APPROACHES TO THE PURIFICATION, ANALYSIS AND CHARACTERIZATION OF ANTIBODY-BASED THERAPEUTICS

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Introduction

Therapeutic antibodies now represent a mainstream therapeutic treatment for many patients' world-wide. As of 2017, some 73 antibody drugs had received marketing approval (EMA&FDA), with many others in clinical and pre-clinical development. Not only are these drugs used for a variety of oncology indications but for numerous other conditions, including cardiovascular and immune diseases. Efforts continue to widen the scope for application of mAbs to new therapeutic areas, including auto-inflammatory conditions and infectious disease. The increasing diversity of mechanisms of actions for these drugs has driven ever increasing molecular complexity, including the design of various conjugates, for both therapeutic and diagnostic applications. Several antibody drug-conjugates have now been successfully developed for cancer treatment, with a robust pipeline of clinical programs under development. Increasing molecular complexity can mean more time and higher risk with regards to process development and scale-up. Specialized in-process or release analytics, characterization assays and formulation data will often be required to support clinical stage development.

While many challenges exist in therapeutic antibody design and production, as well as in implementing appropriate cell-based assays and *in vivo* studies, there have also been many challenges and advancements relating to the purification, analysis and characterization of these molecules. New clarification and filtration technologies have been developed that substantially reduce particulates and residuals entering the capture chromatography step, thereby reducing the burden on downstream processing unit operations. Affinity chromatography, particularly, the use of Protein A, remains a workhorse for antibody capture chromatography in platform, process development and commercial manufacturing environments. Automated solutions to platform purification continue to progress, as the need for higher throughput, lower cost technologies for smaller volume, early-stage protein purification continues. Mass spectrometry is now a well adopted analytical and characterization tool for therapeutic antibodies, providing a rich breadth of information from apparent purity to post-translational modifications of specific amino acid residues. The need to reduce the cost of goods, increase efficiency and reduce timelines is driving the shift for antibody manufacturing towards single-use and continuous downstream processing technologies and processes. These technologies will continue to be developed, will be implemented more broadly and are likely to become dominant in the coming years.

In order to overcome the challenges associated with therapeutic antibody development, a variety of new tools and methodologies have been developed within the purification, analytics and characterization areas over the last several years. The intent of this book is to bring some of these new developments together, under one roof, for the interested reader. Experts from a variety of areas have contributed to this work, with chapters covering areas from protein engineering for affinity chromatography through to chromatography monoliths and applications of mass spectrometry to antibody characterization. It is hoped that this monograph will provide a starting point for readers to delve more deeply into the recent literature relating to antibody purification, analytics and characterization method development.

CHAPTER

1

LC-MS characterization of antibody-based therapeutics: recent highlights and future prospects

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The first monoclonal antibody therapy, Muromonab-CD3 to treat kidney rejection, was approved for use in the US and Europe in 1986 and marked the beginning of a major shift in the pharmaceutical industry away from small molecule drugs toward antibody-based therapies. Today, there are well over 100 approved antibody-based therapies on the market with approximately 60 in late stage clinical review and hundreds more in phase 1 and 2 clinical study [1,2]. Unlike small molecule drugs, antibody therapeutics are large, complex, heterogeneous and dynamic proteins that require sophisticated analytical strategies to thoroughly characterize them and to ensure good quality control and patient safety.

Liquid chromatography coupled with mass spectrometry (LC-MS) is a powerful technology that is at the core of many assays for the characterization of antibody-based therapeutics. With a small amount of material and minimal sample preparation, LC-MS can generate detailed information about multiple antibody features that inform on their purity, heterogeneity, function and stability. Few other technologies can claim to match LC-MS for sensitivity, specificity and throughput. Even fewer have the flexibility of LC-MS that enables it to keep pace with the rapid development of new biotherapeutic modalities. LC-MS characterization of antibody-based therapeutics encompasses a wide range of assays including molecular weight determination, amino acid sequencing, the identification of modifications, host cell protein analysis, antibody-drug conjugate analysis, stability testing, PK/PD studies as well as higher order structure analysis (Fig. 1.1).

Each year, hundreds of research articles are published describing new applications and technological advances relating to the characterization of antibody-based therapeutics by LC-MS. This continual development is driven by the needs of a growing biopharmaceutical sector that is continually developing more effective and safer antibody-based therapeutics.

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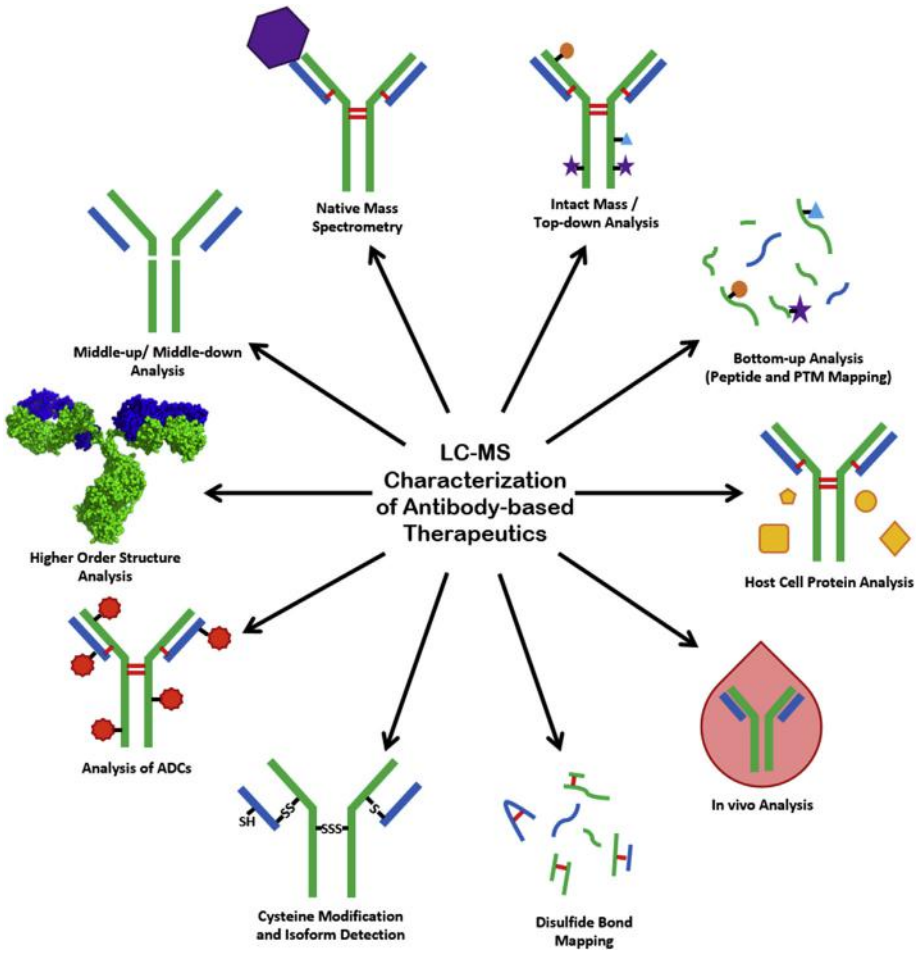

FIG. 1.1 Schematic illustration of some of the many applications of LC-MS for the biophysical characterization of antibody-based therapeutics. "Higher Order Structure Analysis" image is PDB 1IGT [156] rendered using Pymol [157].

This trend is likely to continue for some time as LC-MS gains greater acceptance within the biopharmaceutical community and with regulatory authorities, and as new application areas are explored.

This chapter will cover current applications and recent advances in LC-MS-based biophysical characterization of antibody-based therapeutics. Section 1 will briefly describe some of the more established LC-MS techniques in this field, while Section 2 will focus on the LC-MS analysis of specific attributes, including lesser known post-translational modifications (PTMs) and drug conjugates. Finally, Section 3 will explore recent innovations and trends in LC-MS-based analysis that are likely to significantly impact the manner in which antibody-based therapeutics are analyzed in the future.

Section 1 − LC-MS technologies frequently used for the characterization of antibody-based therapeutics

In this section, a brief overview will be provided of the more established LC-MS technologies for the characterization of antibody-based therapeutics.

Analysis of intact antibodies

Intact mass analysis is a simple, rapid MS technique where the mass of an antibody is determined, with little or no sample preparation. It can be used as a high throughput assay for monitoring the identity, purity and PTMs of recombinant antibody-based therapeutics. Electrospray ionization-MS (ESI-MS) is well-suited for analyzing large proteins, such as antibodies, because it generates multiply charged ions that can be analyzed using mass spectrometers with a relatively limited scanning range but capable of producing high resolution mass spectra, such as Orbitrap and quadrupole time-of-flight (Q-TOF) mass spectrometers [3]. ESI-MS can be connected in-line with separation techniques, such as reverse phase liquid chromatography (RPLC), to remove salts and other buffer components that interfere with ionization and detection, and can be automated to run unattended. Although unit mass resolution of intact antibodies has been achieved [4,5], intact mass analysis is usually done at a lower resolution because ultra-high resolution results in a significant drop in signal intensity with minimal benefit. Even at unit mass resolution, an antibody is so large that the monoisotopic ion is too minor a component to be observed within the dynamic range of current mass spectrometers [6]. As a result, intact mass analysis measures the average mass of an antibody instead of the monoisotopic mass.

The different "proteoforms" of an antibody can be quickly distinguished by intact mass LC-MS based on the molecular weights observed. "Proteoform" is a term coined by the Consortium for Top-Down Proteomics referring to one of "all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications" [7]. The amino acid sequence and modifications of each proteoform can often be inferred from the observed mass. Intact mass analysis has its limitations: it cannot detect modifications that result in zero mass change (e.g., disulfide scrambling, aspartic acid isomerization) and can struggle to distinguish modifications resulting in small mass changes (e.g., deamidation and oxidation), especially when these are present in low abundance. Highly heterogeneous proteins can be challenging to analyze by intact mass LC-MS as the complex spectra are often difficult to deconvolute and interpret. However, it is often possible to remove common sources of heterogeneity such as N-linked glycosylation or C-terminal lysines prior to intact mass analysis so that other more pertinent or low-level features such as glycation, drug conjugation or the heterodimer/homodimer ratio of bispecifics can be more easily detected.

Middle-up analysis

In a typical "middle-up" analysis workflow, an antibody is selectively cleaved and/or reduced into "large" (typically > 7 kDa − refer to Lermyte et al. for a discussion of terminology [8]) polypeptide fragments prior to mass analysis. The antibody is usually enzymatically

cleaved close to the hinge and the inter-chain disulfide bonds are chemically reduced. For example, the most popular preparation for "middle-up" analysis of an antibody involves cleavage below the hinge with the enzyme IdeS followed by reduction of disulfide bonds with dithiothreitol (DTT) treatment, resulting in a heavy chain Fc/2 fragment, a heavy chain Fd fragment and the light chain. The mass of each fragment is measured by intact mass LC-MS analysis, often after chromatographic separation. Since smaller in size, the mass resolution attained for the fragments is greater than the resolution attained for the intact antibody. Furthermore, the molecular weight profiles for the fragments are usually less complex and easier to interpret. "Middle-up" analysis is useful for defining the regional location of a modification or sequence error and for differentiating and characterizing Fc and Fab glycosylation. This technique has seen a large growth in popularity in the last 10 years as more specific enzymes for cleaving at the hinge have become commercially available. A more detailed description of the enzymes available for specific cleavage at the hinge region of an antibody and applications is provided in Section 3.

Bottom-up analysis

"Bottom-up" analysis involves cleaving an antibody into small peptides (typically <3 kDa) with one or more proteases and resolving the peptides chromatographically (typically by RPLC) prior to analysis by MS and MS/MS. Specific proteases such as trypsin (most common), LysC, AspN or GluC (also called *Straphylococcus aureus* Protease V8), or non-specific proteases such as pepsin can be used. Protease cleavage can be performed on antibodies free in solution or immobilized in gels or on stationary phases [9], resulting in release of peptides into solution. "Bottom-up" LC-MS is used in antibody characterization to confirm amino acid sequence, to identify and locate modifications and determine their relative abundance at each site, and to perform detailed comparisons between samples [10]. MS/MS spectra of the peptide ions generated from gas-phase fragmentation techniques, such as collision-induced dissociation (CID), can be matched to the expected antibody amino acid sequence using database searching and sequence match algorithms to confirm identity. Failing this, unidentified MS/MS spectra can be interpreted manually to determine novel amino acid sequences or the presence of a modification. Finally, "bottom-up" analysis of antibody digests prepared without reduction can provide information on the disulfide bond linkages present [11].

The term "extended bottom-up" has been used to describe LC-MS analysis of peptides intermediate in size to those typically generated for "bottom-up" or "middle-up" analysis [12]. In an extended bottom-up assay, antibodies are incubated for a limited period with secreted aspartic protease 9 (Sap9), a non-specific protease, at slightly acidic pH to generate peptides with an average size of approximately 3.5 kDa [13]. Standard "bottom-up" LC-MS conditions are used, though the standard C18 RPLC column is substituted for a C8 column to compensate for the increased hydrophobicity of the larger peptides. The advantages of "extended bottom-up" are improved sequence coverage and more confident identification of IgGs, especially when distinguishing between similar IgGs, because of the increased length of the identified peptides and increased likelihood of identifying unique peptides [13].

Higher order structure analysis by mass spectrometry

Higher order structure mass spectrometry (HOS-MS) analysis is used to study antibody-antigen interactions and to probe changes in the three-dimensional structure of antibody-based therapeutics that result from formulation in different buffers [14], degradation processes [15], post-translational modifications [16] or drug conjugation [17]. HOS-MS can evaluate the structural similarity of lots of the same biotherapeutic or of a biosimilar with an originator molecule [18]. HOS-MS has the advantage of being able to detect antibody structural changes with medium to high resolution. As such, it complements other HOS analysis techniques such as circular dichroism, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [19].

In a typical HOS-MS experiment, an antibody or antibody-antigen pair in the native state is labeled at solvent-exposed residues, digested into peptides and analyzed by LC-MS. Peptides containing the exposed residues are identified by the mass increase due to the label. Labeled peptides are mapped onto the 3-D model (usually a homology model) of the antibody to determine how the structure is being impacted by the experimental conditions. For example, upon antigen binding, labeling will decrease at the binding site and may increase or decrease at peripheral sites due to allosteric effects. The most popular HOS-MS assay is hydrogen/deuterium exchange mass spectrometry (HDX-MS, also called HX-MS) where solvent-exposed backbone amide hydrogens are exchanged with deuterium from the buffer [20]. This technology has the ability to detect and determine the location of dynamic changes in the orientation of the protein backbone. In covalent labeling mass spectrometry (CL-MS), specific amino acid side chains are covalently modified with chemical labels [21]. This approach has the advantages of detecting changes in the orientation of side chains (which in some cases may be more relevant to function than changes in the protein backbone) and producing permanent labels that can be more easily identified and quantified by well established "bottom-up" assays. Finally, cross-linking MS (XL-MS) uses multifunctional reagents to form covalent bonds between spatially proximal amino acid side chains, allowing for the determination of the distance between two interacting proteins or two residues of the same protein [22].

Host cell protein analysis

Host cell proteins (HCPs) are derived from the cell line used to express a therapeutic antibody and are released into the culture media via secretion or cell lysis. LC-MS/MS studies have identified thousands of HCPs present in the spent culture media [23,24]. Downstream purification protocols will remove many HCPs but it can be challenging to completely remove all of them. Many factors influence the composition and abundance of HCP impurities in purified antibody products including HCP initial abundance in the cell media and their tendency to co-elute or associate non-covalently with the therapeutic protein during the purification process [23,25–28]. Even after multiple steps of purification, HCPs can be present in a protein drug substance at concentrations of up to 100 ppm. Therefore, setting and achieving acceptable limits for HCP contamination in the final product is an important requirement for all biotherapeutics.

HCP abundance is usually monitored by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies raised against the host cell proteins. ELISA is a simple, quantitative measure of total HCP content but provides no information about the identity of HCPs present and is dependent on HCPs being reactive to the polyclonal antibody used. LC-MS, on the other hand, can identify and quantify individual HCPs and is not dependent on immunogenicity. The challenge for LC-MS based assays lies in the fact that HCPs are typically many orders of magnitude less abundant than the biotherapeutic product. The majority of LC-MS strategies for HCP analysis exploit the impressive separation capabilities of two-dimensional chromatography (2D-LC) to resolve the antibody/HCP digests prior to MS analysis [24,29–38]. Most employ high pH RPLC in the first LC dimension and low pH RPLC in the second. HCP identification is done using MS/MS or MSe analysis coupled with database searching. Ion mobility mass spectrometry (IM-MS) can add an additional dimension of separation to aid in the detection [33]. HCP quantification can be achieved with MRM using isotopically labeled peptide internal standards, using the Hi3 fragment ion strategy on a Q-TOF instrument or by spectral counting in data-independent acquisition mode [29,39]. Detection and quantification of HCPs in NISTmAb reference material (see Section 3) down to 1 ppm has been reported [33].

MS-based methods for HCP detection and quantification are evolving but currently still suffer from lengthy analysis times and poor inter-lab reproducibility [33]. However, innovative techniques, such as using sample concatenation [38], are being explored to shorten 2D-LC-MS analysis times. ELISA will likely remain the primary assay for HCP monitoring and product release for some time to come but, ideally, a combination of ELISA and LC-MS would be more effective for HCP monitoring and control [40].

Section 2 – LC-MS characterization of antibody attributes

Antibody-based therapeutics may be modified in many ways over the course of production, purification and storage. In this section, we will describe some of these modifications as well as possible ways to detect and quantify them. Some modifications are typically only present on a minority of the antibody proteins but may still be functionally significant. Table 1.1 lists the mass difference and location associated with post-translational modifications known to occur to antibodies and antibody-based therapeutics.

Confirmation of primary sequence and detection of cleavages

LC-MS is routinely used to confirm the identity of antibody-based therapeutics. A comprehensive workflow for amino acid sequence confirmation includes both intact mass LC-MS analysis and "bottom-up" LC-MS/MS sequencing of the peptide digest. The mass observed for an antibody by intact mass LC-MS analysis on a medium to high-resolution mass spectrometer should be within ~ 20 ppm of the theoretical average mass for the predicted amino acid sequence plus known PTMs (such as disulfide bonds or glycosylation) [3]. A discrepancy between the observed and calculated masses may be indicative of an amino acid sequence error, proteolytic cleavage, poor disulfide bond formation or unexpected

TABLE 1.1 Masses of post-translational modifications observed in antibody-based therapeutics.

Modification	Mass difference (monoisotopic)	Location
Loss of Lys	−128.0950	C-terminal Lys
Dehydroalanine formation	−33.9877	Cys
Thioether bond formation	−31.9721	Disulfide bond
Succinimide formation from Asp	−18.0106	Asp
Succinimide formation from Asn	−17.0265	Asn
Pyroglutamic acid formation	−17.0265	N-terminal Gln
Disulfide bond formation	−2.0145	2 Cys residues
Deamidation	+0.9840	Asn (or Gln)
Kynurenine formation (Trp oxidation)	+3.9949	Trp
Methionine sulfoxide formation (Met oxidation)	+15.9949	Met
Hydroxytryptophan/oxindolylalanine formation (Trp oxidation)	+15.9949	Trp
Hydroxyproline formation	+15.9949	Pro
Trisulfide formation	+31.9721	Disulfide bond
N-formylkynurenine formation (Trp oxidation)	+31.9898	Trp
Sulfation	+79.9568	Ser, Thr or Tyr
Phosphorylation	+79.9663	Ser, Thr or Tyr
Cysteinylation	+119.0041	Cys
O-xylosylation	+132.1161	Ser
Citric acid imide modification	+156.0059	N-terminus
Glycation	+162.0528	N-terminus or Lys
Citric acid amide modification	+174.0164	N-terminus
Glutathionylation	+305.0682	Cys
O-linked HexNAcHexNeuAc	+656.2276	Ser or Thr
O-linked $HexNAcHexNeuAc_2$	+947.3230	Ser or Thr
N-linked glycan (example: $HexNAc_4Hex_3Fuc$ (G0F))	+1444.5339	Asn of NXS or NXT (or occasionally NXC)

PTMs. "Bottom-up" LC-MS/MS is the preferred method for confirming amino acid sequence and it is common practice to perform multiple LC-MS/MS experiments using two or three different proteases in order to attain LC-MS/MS coverage of the entire sequence. If a sequence error is suspected, "middle-up" analysis is often used to narrow down the location of the mass difference in order to facilitate the identification of the amino acid change by "bottom-up" LC-MS/MS.

Unexpected cleavages may occur in antibodies, especially near the N- and C-termini, at the solvent-exposed flexible loops connecting β-strands or in the hinge region [41]. Mass separation assays such as CE-SDS or SDS-PAGE can detect extensive cleavage but LC-MS is superior at detecting cleavages resulting in loss of only a few amino acids. Some protein cleavages are only apparent under reducing or denaturing conditions because cleaved portions of the protein are held together by disulfide bonds or non-covalent associations. Minor cleavages near the termini may not affect antibody activity (though loss of tags may cause purification problems) but do increase antibody heterogeneity and can be indicative of longer term stability problems. Partial loss of the C-terminal lysine is a very common cleavage. Other common cleavages include loss of terminal glycine (N- or C-terminal) and loss of one or more histidines from a His-tag. Cleavage is often catalyzed by the side chains of certain amino acids and is influenced by pH (with antibodies typically most stable at pH 5−7), temperature, the presence of metal ions (especially Cu^{II}) as well as protein structure [41]. Peptide bonds especially prone to non-enzymatic fragmentation include Xaa-Ser (where Xaa is any amino acid), Asp-Xaa (at pH < 5, particularly Asp-Pro), Asn-Xaa and Gly-Gly [41]. β-elimination of a disulfide bond can cause cleavage at Xaa-Cys. If the dehydroalanine resulting from β-elimination of a disulfide bond is hydrolyzed (Fig. 1.2C), cleavage will occur N-terminal to the former Cys (an alternative end-product of β-elimination is a thioether bond). In the hinge region of IgG1, the dominant sites of cleavage are Asp-Lys at pH < 5 and at Ser-Cys due to β-elimination at pH > 7 [41].

Cleavage may also be enzymatic and caused by host cell proteases [42]. Co-purification of host cell proteases may only become apparent during a stability stress test. It is often possible to inhibit enzymatic cleavage with protease inhibitors but improved protocols to remove the proteases during production and purification is more desirable.

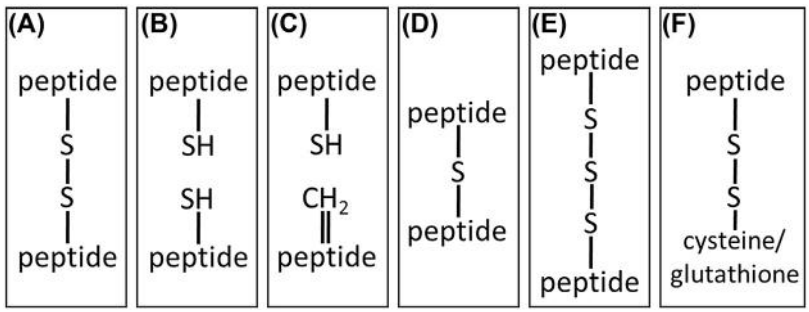

FIG. 1.2 Schematic of different cysteine modifications: (A) a disulfide bond, (B) two free sulfhydryls resulting from reduction of a disulfide bond, (C) a free sulfhydryl **(top)** and dehydroalanine **(bottom)** resulting from β-elimination of a disulfide bond, (D) a thioether bond, (E) a trisulfide and (F) cysteinylation or glutathionylation.

Cysteine modifications

Ensuring that disulfide bonds are formed correctly is an important step in characterizing recombinant antibodies. A typical IgG has 1 intra-chain disulfide bond for each of the 12 domains, 2 disulfide bonds attaching the light chains to the heavy chains and 2 to 11 (subtype-dependent) hinge disulfide bonds connecting the two heavy chains [43]. Scrambling, reduction or modification of these disulfide bonds may negatively affect the biotherapeutic. For example, the reduction of an antibody disulfide bond to free sulfhydryls has been observed to decrease antigen affinity [44], increase propensity for aggregation [45–48], and/or decrease thermal stability [49]. Some common modifications of cysteine residues are shown in Fig. 1.2.

Free sulfhydryls (Fig. 1.2B) have been observed in recombinantly expressed antibodies and in antibodies extracted from serum [48–50]. The free sulfhydryl abundance of a protein can be measured spectroscopically using the Ellman's reagent method [51] or free sulfhydryl-reactive fluorescent labels [52] but these methods can suffer from poor sensitivity and precision and are unable to provide information about the location or distribution of free sulfhydryls. The reduction of a disulfide bond only increases the mass of a protein by 2 Da but free sulfhydryls can be easily and sensitively detected by intact mass LC-MS if they are labeled with a maleimide derivative, such as maleimide-PEG2-biotin, so that the presence of a free sulfhydryl is observed as a significant mass shift [53]. Robotham and Kelly determined the locations of the free sulfhydryls in antibodies by first labeling the free sulfhydryls with d_0-NEM, then labeling the remaining cysteines with d_5-NEM after reduction [53]. Protease digestion was performed under mildly acidic conditions to prevent label loss and scrambling and LC-MS/MS was used to identify peptides containing a free sulfhydryl based on their high d_0/d_5-NEM ratio. In instances where a free sulfhydryl is solvent exposed, it may become cysteinylated and glutathionylated, resulting in characteristic mass shifts of +119 Da and +305 Da, respectively (Fig. 1.2F) [46].

The disulfide bond connecting the light chain to the heavy chain in IgG1 is prone to thioether bond formation (Fig. 1.2D), particularly when exposed to elevated temperature or basic conditions [54,55]. Formation of a thioether bond decreases the mass of an intact antibody by 32 Da (the mass of sulfur). The thioether bond is created when the dehydroalanine and cysteine formed from β-elimination of the disulfide bond react to form a covalent bond by Michael addition [55]. Since this bond is not reducible, the thioether-linked light chain-heavy chain pair is observed under reducing condition during "bottom-up" or "middle-up" LC-MS analysis. Thioether formation reduces Fab flexibility and could affect bivalent binding [55]. The heavy-light chain disulfide bond, as well as the hinge interchain disulfide bonds, are also susceptible to trisulfide formation where a sulfur atom is inserted into the disulfide bond (Fig. 1.2E)[56]. Trisulfides are hypothesized to form during fermentation due to the reaction of disulfide bonds with hydrogen sulfide. Gu et al. determined the location and relative abundance of trisulfides in natural and recombinant antibodies using "bottom-up" LC-MS of non-reduced proteins digested with LysC at pH 6.5 [56]. Trisulfide-linked peptides had a mass 32 Da greater than the predicted disulfide-linked peptides. Although they increase biotherapeutic heterogeneity, there is no evidence that trisulfides affect antibody binding or activity and the majority of trisulfides are rapidly converted to disulfide bonds in vivo [56].

Disulfide bond scrambling may occur with any antibody-based biotherapeutic but is a special concern for IgG2 and IgG4 isotypes. IgG2 can exist as three different isoforms of disulfide bond arrangements: IgG2-A, IgG2-B and IgG2-A/B (Fig. 1.3)[43]. The isoforms differ in terms of higher order structure, which influences thermal stability and binding efficiency [57,58]. IgG4 is also prone to disulfide bond scrambling with the hinge cysteines switching between inter-chain and intra-chain disulfide bonds. As a result, the IgG4 heavy and light chains may be non-covalently associated and Fab arm switching with endogenous IgG4 antibodies in vivo has been observed [59]. The relative abundance of different disulfide bond isoforms can be determined by "bottom-up" LC-MS/MS analysis of non-reduced antibody digests. MS/MS fragmentation with electron transfer dissociation (ETD) or electron capture dissociation (ECD) selectively cleaves the disulfide bond to release the linked peptides whereas collision-induced dissociation (CID) results in preferential fragmentation of the peptide backbone [60]. These techniques can be used in sequence (i.e., ETD followed by CID) to identify disulfide bonded peptides with greater confidence [60].

N-linked glycosylation

N-glycosylation is a common post-translational modification of antibodies. Virtually all antibodies are N-glycosylated at a single asparagine residue located in the Fc region (Asn-Ser-Thr) and a smaller proportion of them are N-glycosylated at additional asparagines on the heavy or light chain (e.g., cetuximab)[61]. An N-glycosylation sequon (Asn-X-Ser, Asn-X-Thr or, occasionally, Asn-X-Cys, where X is any amino acid other than proline) is a pre-requisite, though not a guarantee, for N-linked glycosylation. N-glycosylation can be analyzed by intact mass analysis to determine the overall glycosylation of an antibody, by "middle-up" analysis to distinguish N-glycosylation in the Fc and Fab regions and by "bottom-up" analysis to determine the glycosylation at each N-glycosylation site. Standard RPLC is often used for "bottom-up" analysis but alternatively, hydrophilic interaction chromatography (HILIC) and other glycopeptide-enriching chromatographies can be used to separate glycopeptides from non-glycosylated peptides [62]. N-linked glycans can also be released from the protein by PNGaseF treatment, labeled and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) or by HILIC-fluorescence [63].

Generally speaking, the popular cell lines used for protein expression, such as CHO or HEK293, express antibodies with well-defined N-glycans composed of a restricted set of sugar moieties (i.e., N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), mannose (Man), glucose (Glc), galactose (Gal), fucose (Fuc) and N-acetylneuraminic acid (NeuAc), as well as N-glycolylneuraminic acid (NeuGc) in mouse cell lines). Therefore, it is often possible to infer the number and general composition of the N-glycans present by mass addition alone. Mass spectrometry cannot distinguish between sugar isomers; "HexNAc" is used to indicate that a sugar is GlcNAc or GalNAc and "Hex" to indicate Man, Glc or Gal. Glycans occupying the Fc N-glycosylation site are typically non-sialylated, biantennary complex-type glycans while those occupying the Fab sites tend to be more heterogeneous and sialylated. Typically, each N-linkage site is occupied by a variety of glycan species resulting in a heterogeneous mass profile for glycosylated antibodies. N-glycans

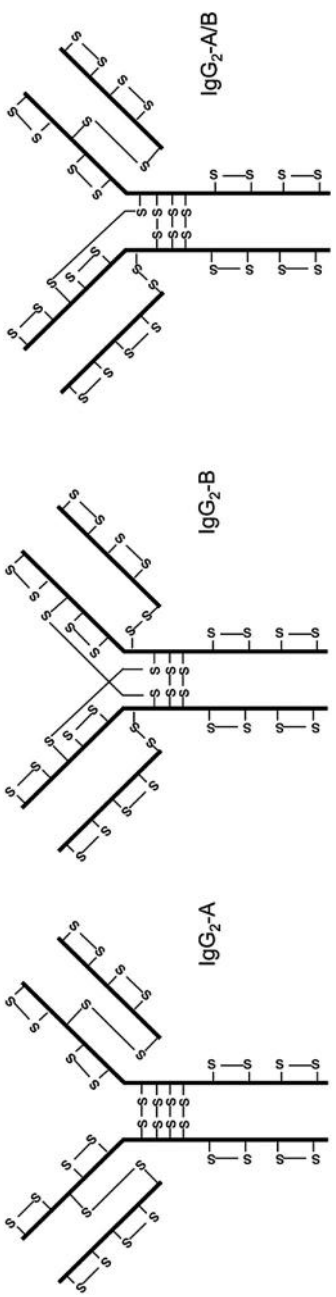

FIG. 1.3 Disulfide bond isoforms observed in IgG2 antibodies. *Adapted from Liu, H. and K. May, Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. mAbs 2012:4(1):17–23 with permission, © 2012 Landes Bioscience.*

are often removed with PNGaseF or trimmed to a common core with exoglycosidases prior to LC-MS in order to facilitate the characterization of other antibody features. Glycosylation analysis by intact mass or "middle-up" LC-MS can be complicated by the presence of O-linked glycosylation or glycation and care must be taken to distinguish these modifications from N-linked glycosylation.

O-linked glycosylation

O-linked glycosylation has been observed on some of the immunoglobulin classes that are not typically used to produce antibody-based therapeutics: IgA1, IgD and IgG3 as well as mouse IgG2b [64–67]. In all of these cases, O-linked glycosylation was located in the hinge region and consisted of mucin-type glycans such as GalNAcGalNeuAc$_2$ (or GalNAcGalNeuGc$_2$ in the case of murine IgG2b). O-linked glycosylation has not been reported on intact IgG1, the most commonly used subclass for recombinant antibodies [68]. However, O-linked glycosylation has been observed on IgG1 Fc fragments expressed alone, on antibody constructs lacking one or both light chains, and on fusion proteins containing an IgG1-type Fc domain (unpublished results). O-linked glycosylation (GalNAcGalNeuAc$_2$) of the Fc fragment of an IgG1 (Rituximab) was observed when the Fc fragment was expressed alone but not when the same fragment was generated from intact Rituximab by papain cleavage [69]. The site of O-linked glycosylation was hypothesized to be located near the hinge because access to this region is greatly improved when the Fab portion of IgG is absent. Other groups have identified unexpected O-linked glycosylation of peptides when they are fused to Fc domains or antibody [70,71] and in some cases, O-glycosylation had a negative effect on the activity of the fusion construct [70].

Intact mass LC-MS analysis can be used to detect mass shifts that are characteristic of O-glycosylation (i.e., +656 Da for HexNAcHexNeuAc and +947 Da for HexNAcHexNeuAc$_2$). O-glycosylation is easier to detect by LC-MS if the N-linked glycans are first removed using PNGaseF. Identifying the site of O-glycosylation is more challenging than for N-glycosylation because there is no consensus sequence for O-glycosylation other than glycosylation occurs at serine and threonine residues. "Top-down" LC-MS analysis has been used to identify O-glycosylation sites [72], however "bottom-up" LC-MS/MS is the preferred method currently. Enrichment using HILIC can aid in the detection of low abundant O-glycopeptides [73,74]. The CID MS/MS spectra of O-glycopeptides are often dominated by glycan oxonium ions (e.g., 204 Da, 366 Da, 292 Da) and by neutral losses of sugar residues from the intact glycopeptide with no evidence of the linkage site. ECD or ETD fragmentation methods are better suited for determining the linking amino acid as these fragmentation methods tend to preserve delicate modifications such as O-glycosylation [75].

Glycation

Non-enzymatic glycation of the N-terminus or the side chain of lysine residues is a common PTM of both recombinant and endogenous mAbs. Glycation of recombinant antibodies typically occurs during fermentation when the expressed protein is exposed to high concentrations of reducing sugars, such as glucose, from the cell feed [76]. Glycation may

also occur during storage if the antibody is exposed to reducing sugars, or to sucrose (a common formulation buffer component) at elevated temperatures and acidic pH [77]. It has even been observed to occur on lyophilized mAbs [78]. Glycation is undesirable because it increases the heterogeneity of a biotherapeutic and may have other negative consequences. A recent study found that glycation in the complementarity-determining region (CDR) interfered with antigen binding [79] and there is mixed evidence as to whether glycation increases mAb aggregation [80,81]. There are also concerns that when exposed to elevated oxidation conditions in vivo, glycated biotherapeutics may further degrade to advanced glycation end products (AGEs) and trigger the expression of AGEs-specific receptors and adverse immune responses [76]. AGEs have also been linked to the discoloration of recombinant antibody product [82]. Given the safety concerns, glycation is an attribute that must be monitored and controlled.

Glycation is detected by intact mass LC-MS analysis as a characteristic mass shift of 162 Da. Glycation is easier to detect if the protein is first deglycosylated, as many N-glycoforms differ from one another by a single hexose (162 Da). Though rare, a 162 Da increase can also be due to O-linked glucosylation and mannosylation where a single glucose or mannose is linked to a serine residue [83,84]. Additionally, two phosphorylations/sulfations also have a similar mass to a glycation (+160 Da). Glycation can be distinguished from these other modifications by determining the site of modification using "bottom-up" LC-MS. Detection can be challenging because glycation abundances tend to be low (<10%) and spread across multiple sites, there is no consensus sequence for glycation other than a solvent accessible primary amine [76], and trypsin and LysC will not cleave at glycated lysine residues. Generally speaking, CID MS/MS spectra of glycated peptides tend to be poor, being dominated by simple losses of water from the hexose residue and with very few informative peptide fragment ions [77]. These challenges can be overcome in a number of ways. For example, boronate-affinity HPLC (BAC-HPLC) can be used to enrich for glycated proteins and BAC-HPLC-UV has been used for determining overall glycation abundance. BAC-HPLC separates glycated and non-glycated forms based on the interaction of the glycated sugar cis-1,2-diol array with the boronate ligands [85]. Treatment of glycated peptide with sodium borohydride or cyanoborohydride stabilizes glycation and generates higher quality CID MS/MS spectra as a result [86]. In addition, alternative MS/MS fragmentation techniques such as electron transfer dissociation (ETD) can yield superior sequence information relative to CID as the modification is not lost during fragmentation, making it possible to confirm the site of glycation [87].

Deamidation and aspartic acid isomerization

Deamidation of asparagine and isomerization of aspartic acid to isoaspartic acid can impact the structure, function and stability of antibody-based therapeutics. These modifications affect antigen-binding if they occur in the CDR region [88,89] and can lead to immunogenicity issues [90]. Asparagine deamidation imparts an additional negative charge to the antibody which can be monitored using charge based assays such as capillary isoelectric focusing (cIEF) or ion exchange chromatography (IEX). "Bottom-up" LC-MS can be used to confirm the site of deamidation. Each asparagine deamidation increases the

mass of a peptide by 1 Da. Conversely, aspartic and isoaspartic acids are isobaric and are more challenging to distinguish by mass spectrometry alone. However, the isomerization of aspartic acid to isoaspartic acid can result in a more hydrophilic peptide and a shorter retention time by RPLC [91]. Using the combination of information provided by both RPLC and MS, sites of deamidation and aspartic acid isomerization can be identified (Fig. 1.4). Special care in sample preparation must be taken during "bottom-up" LC-MS as typical protein digestion conditions can themselves cause deamidation and aspartic acid isomerization. Kori et al. found that asparagine deamidation and isoaspartate artifacts can be reduced significantly by carrying out the digestion procedure in 20 mM Tris buffer, pH 7.8 containing 10% acetonitrile [91]. Aspartic acid isomerization on a model tryptic peptide was 33.8% after an overnight digestion in 20 mM ammonium bicarbonate but only 4.5% in 20 mM Tris 10% acetonitrile. Often, it may be more important to determine which residues are most prone to deamidation rather than the current level of deamidation in a sample as additional deamidation can occur in vivo [92].

Succinimide is a key intermediate in both asparagine deamidation and aspartic acid isomerization and is itself an important modification of antibody-based therapeutics (Fig. 1.5). It is often overlooked as it is unstable at the neutral and basic pH conditions commonly used for "bottom-up" LC-MS sample preparation [93–95]. Cao et al. developed

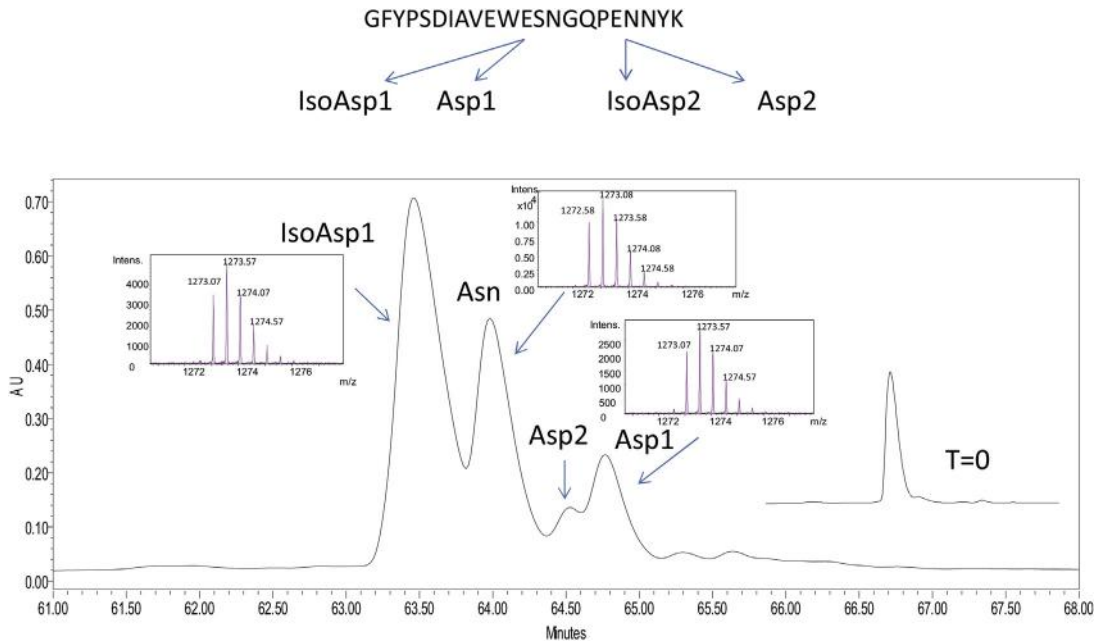

FIG. 1.4 RPLC-UV (214 nm) chromatogram of a synthetic peptide (sequence shown above the chromatogram) after incubation in ammonium bicarbonate, illustrating the separation of multiple deamidation/isoaspartate products. The mass spectra for the major peaks are provided as insets, as is the RPLC-UV chromatogram of the peptide prior to incubation. *Reproduced from Kori, Y., et al., A conventional procedure to reduce Asn deamidation artifacts during trypsin peptide mapping. Journal Of Chromatography B Analytical Technologies In The Biomedical And Life Sciences, 2016;1009–1010:107–113. with permission from Elsevier.*

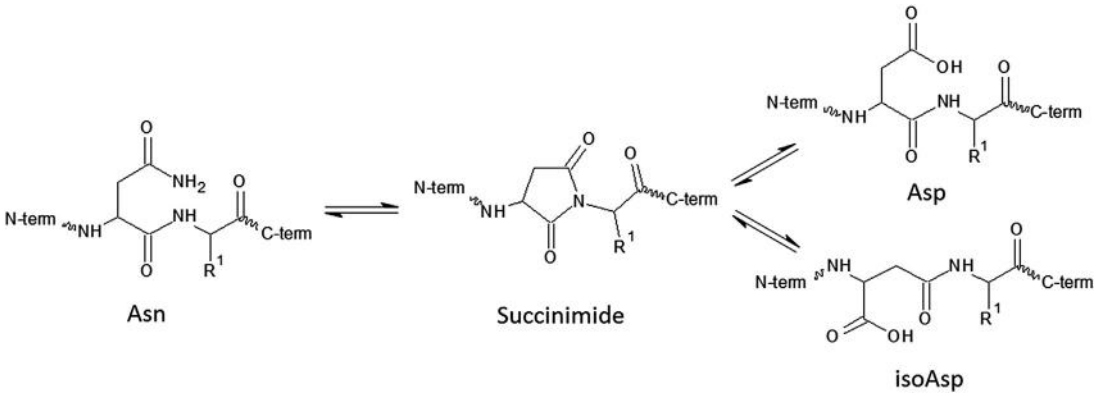

FIG. 1.5 Schematic showing the interconversion of asparagine, aspartic acid, isoaspartic acid and succinimide.

a LC-MS method for detecting and quantifying succinimide that performed reduction, alkylation and proteolytic digestion at pH 5.4–5.8 [95]. The amount of succinimide present on heat stressed antibodies increased with time but was converted almost completely to deamidation products at pH 7.6. Unmodified, deamidated and succinimide forms of an antibody could also be distinguished by hydrophobic interaction chromatography coupled with UV detection (HIC-UV) and the results aligned well with the LC-MS peptide mapping results for the digest prepared under low pH conditions [95].

Oxidation

Oxidation can impact the function and stability of antibody-based therapeutics. Oxidation can be induced by heat, light or the presence of reactive chemical impurities such as reactive oxygen species generated during production and storage [96]. Methionine residues are most susceptible to oxidation [97–99]. Less well appreciated is the fact that other amino acid residues are also prone to oxidation, especially tryptophan. Oxidative degradation products of tryptophan have a chromophore and impart a color to degraded antibody solutions [100]. Oxidation of tryptophan residues in the CDR can have a major impact on antigen affinity [101]. Tryptophans in the CDR may be more susceptible to oxidation given the solvent accessibility and relative flexibility of the CDR loops, though other factors such as side chain orientation and the nature of adjacent residues also contribute to susceptibility to oxidation [101]. Forced oxidation using 2,2-azobis(2-amidinopropane) (AAPH) in the presence of free methionine selectively oxidizes tryptophan residues and allows for the identification of tryptophan residues susceptible to oxidation [96]. Using this technique in combination with "bottom-up" LC-MS, Pavon et al. demonstrated that a single tryptophan in the CDR3 region of two mAbs was susceptible to oxidation [102]. LC-MS identified multiple oxidation products illustrating the complexity of the tryptophan oxidation degradation pathway (Fig. 1.6).

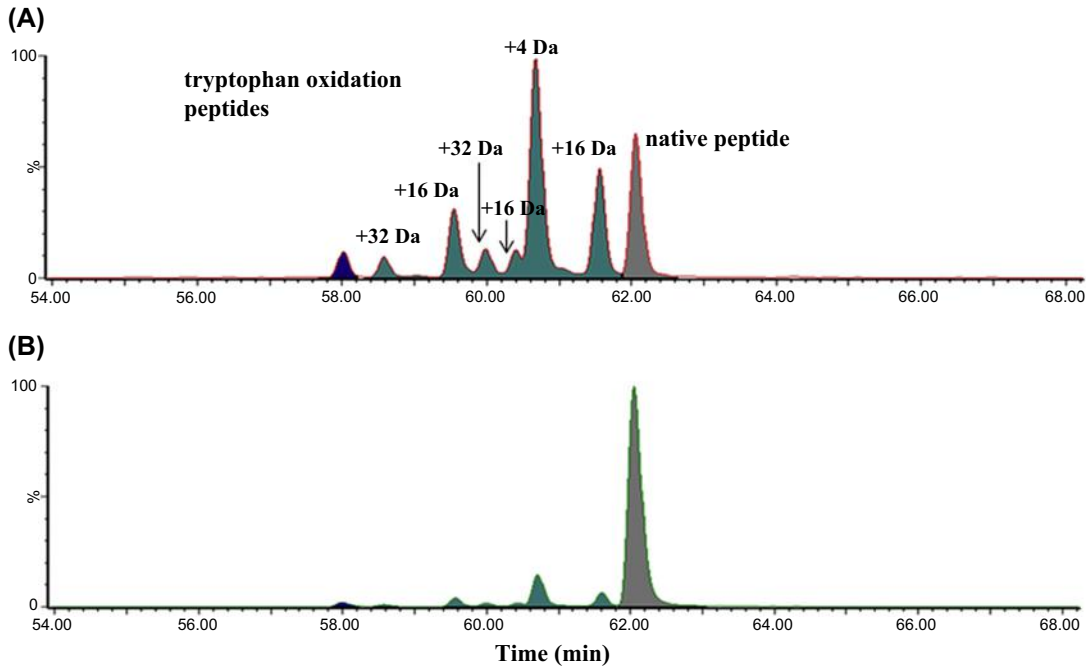

FIG. 1.6 Representative extracted ion chromatogram of the oxidized forms of a tryptophan-containing mAb peptide following forced oxidation with 1.2 mM AAPH. The numbers above each peak indicate the mass difference between the oxidized peptide and the native peptide. The major oxidized peptide peaks correspond to conversion of tryptophan to kynurenine (+4 Da), hydroxytryptophan and/or oxindolylalanine (+16 Da), and N-formylkynurenine (+32 Da). *Adapted from Pavon, J.A., et al., Selective tryptophan oxidation of monoclonal antibodies: oxidative stress and modeling prediction. Anal Chem, 2019:91(3):2192−2200 with permission, © 2019 American Chemical Society.*

Drug conjugates

Antibodies can be intentionally modified with cytotoxic drugs to create antibody drug conjugates (ADCs) - highly specific and potent therapeutics used mainly for cancer treatment. ADCs can be synthesized in many different ways using a variety of linkers and drug payloads. Drug is most commonly attached to the antibody through a linker conjugated to lysine amine groups, free sulfhydryls or modified glycan sugars. Not surprisingly, these unique biotherapeutics can be a challenge to characterize, however, there is a large body of literature demonstrating that LC-MS is well suited for the task [103].

A critical quality attribute of ADCs is their drug to antibody ratio (DAR). Quantitative UV analysis is often used to determine the average DAR of an ADC but can be biased by the presence of free drug in solution. The distribution of drug across the ADC population (i.e. the proportion of antibody molecules having a DAR of 0, 1, 2, 3 … etc.) is more challenging to measure. Hydrophobic interaction chromatography with UV detection (HIC-UV) is frequently used to determine the DAR distribution of ADCs with defined conjugation sites but is challenged by ADCs prepared using random conjugation strategies (i.e. lysine conjugation). On other the hand, intact mass LC-MS analysis can be used to measure the DAR

distribution of almost all ADCs, irrespective of their nature and method of manufacture. Furthermore, LC-MS can identify unusual ADC species (such as antibody with linker but no drug attached) that are not generally detected by other methods.

"Bottom-up" LC-MS is the method of choice for identifying drug conjugation sites; however determining the degree of occupancy at each location can be challenging as the unconjugated and drug-conjugated versions of peptides in ADC digests behave differently by LC-MS. Hill et al. were able to measure site occupancy at 98% of available primary amines on a surrogate ADC prepared by derivatizing the NISTmAb reference material with tandem mass tags [104]. They demonstrated that while many lysines were modified to some extent, the N-terminus as well as Lys187 on the light chain were particularly amenable to conjugation. LC-MS can also be used to measure the amount of free (unconjugated) drug present in ADC preparations [105–107]. For example, Birdsall et al. developed a 2D-LC-MS method using a solid phase extraction (SPE) trap in the first dimension and RPLC in the second [106]. The unconjugated drug-linker products accumulated on the SPE trap whereas the ADC passed through. In this way they were able to measure free drug in ADC preps down to single digit ppm levels - two orders of magnitude more sensitive than equivalent UV methods [106].

In-vivo stability of ADCs is especially important as premature release of drug prior to target engagement can produce undesirable toxic side effects and narrow the therapeutic window. Immuno-enrichment of ADCs from serum followed by intact mass LC-MS analysis has been used to measure DAR and monitor ADC stability in vivo [108–113]. For example, Xu et al. immobilized biotinylated target antigen on paramagnetic streptavidin beads and used it to capture both lysine- and cysteine-conjugated ADCs from the plasma of dosed monkeys [108,109]. Intact mass LC-MS analysis of the immuno-isolated ADCs revealed a gradual reduction of DAR over time, indicating loss of drug during circulation in the blood (Fig. 1.7). Excoffier et al. developed a more universal immune-capture protocol that targeted the Fc portion of ADCs and demonstrated that it could be used to enrich ADCs with both cleavable and non-cleavable linkers from serum [110]. Enriching ADCs from serum at sufficient purity and concentration that the DAR distribution can be determined remains a challenge for ADC analysis.

Other modifications of antibodies and antibody fusion proteins

Tyrosine sulfation is a rare modification of antibodies. Sulfation and phosphorylation both add a mass of approximately 80 Da (79.9568 Da and 79.9663 Da, respectively) and can be distinguished using a mass spectrometer with very high mass accuracy and resolution. Alternatively, sulfation and phosphorylation can be distinguished by their CID fragmentation pattern in MS/MS; sulfation exhibit a 80 Da neutral loss while phosphorylation has a characteristic neutral loss of 98 Da. Zhao et al. observed that a fraction of their CHO-produced human IgG bound to anion exchange resin and had a +80 Da mass difference [114]. Using "bottom-up" analysis, they confirmed that the modification was sulfation. ETD fragmentation was then used to locate the sulfation to a single tyrosine residue. The effect of tyrosine sulfation on antibodies is not known but for many other proteins, tyrosine sulfation recognition is an important driver of protein-protein interactions [115].

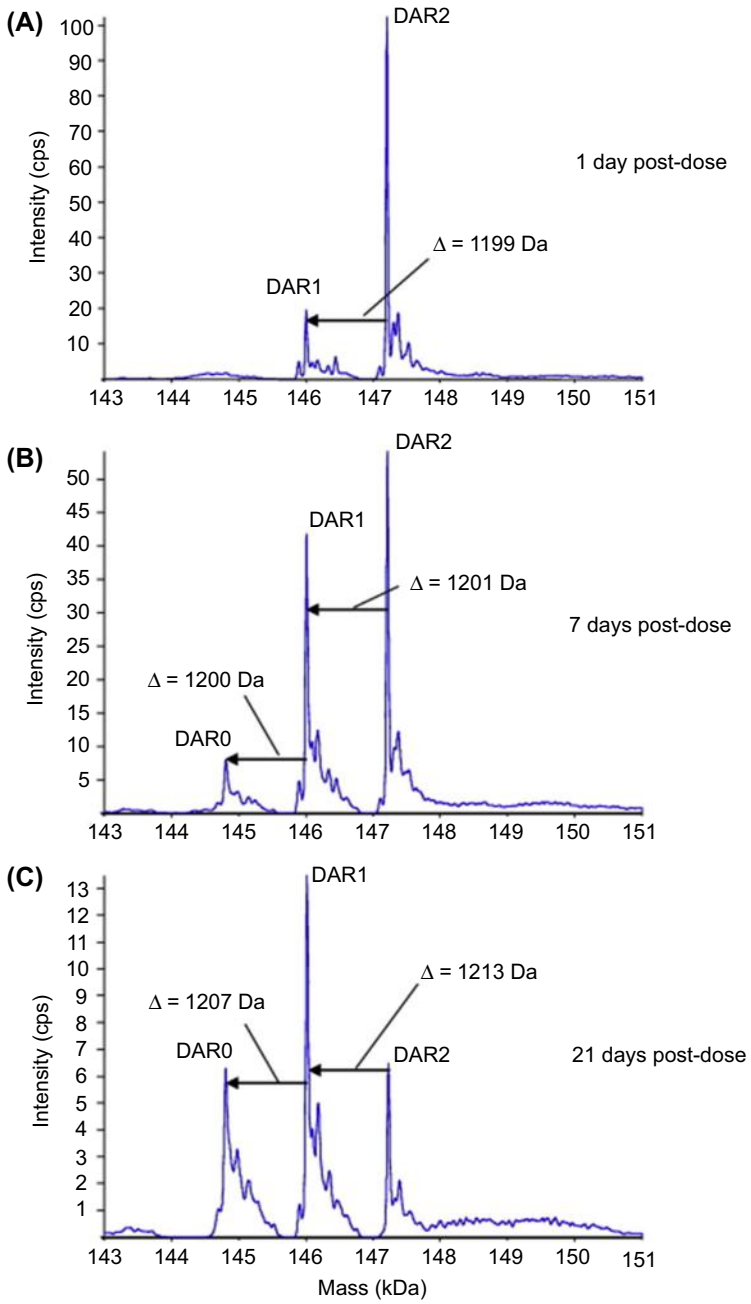

FIG. 1.7 LC-MS spectra showing in vivo drug release from a cysteine-conjugated ADC (anti-MUC16 TDC) in the circulation of cynomolgus monkeys. The ADC had an initial DAR of 2. Using immuno-capture of the ADC from serum, LC-MS monitored the amount of ADC in vivo with a DAR of 0, 1 or 2 at (A) 1 day, (B) 7 days and (C) 21 days post-dose. *Reproduced from Xu, K., et al., Characterization of intact antibody–drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography–mass spectrometry. Anal Biochem 2011:412:56–66 with permission from Elsevier.*

Chumsae et al. have reported covalent modifications of the N-terminus of several antibodies by citric acid resulting in a mass increase of 174 Da or 156 Da [116]. Citric acid is a common buffering agent used in biotherapeutic formulations to maintain the pH between 3 and 6 and is frequently used to elute antibodies from affinity resins during downstream processing. The modifications are hypothesized to be from reaction of citric anhydride with the N-terminal amine, resulting in a +174 Da amide modification and two different +156 Da cyclic imide modifications. Several different N-terminal residues were observed modified but lysine side chain amines were not observed similarly modified. Chumsae et al. hypothesized that other buffering agents with 2 or more juxtaposed carboxylic acids such as adipic acid, malic acid, succinic acid or tartaric acid may also form analogous N-terminal modifications.

The glycine-rich linkers such as $(GGGGS)_n$ or $(GGGGP)_n$ commonly used in antibody-based fusion proteins are prone to modifications such as phosphorylation [117], O-xylosylation [118], xylose-based glycans such as Xyl-Gal-Neu5Ac and Xyl-Gal-Gal-GlcA [119], and hydroxyproline formation [120]. These modifications can be detected during intact mass analysis or "bottom-up" analysis by their characteristic mass changes (see Table 1.1). MS2 or MS3 with CID, HCD and/or ETD may be necessary to confirm the exact site of modification [120]. Since these linker modifications are usually far from the binding region, these modifications are unlikely to interfere with antibody binding but raise potential immunogenicity concerns.

Section 3 — Enabling technologies and future directions for LC-MS-based antibody characterization

New reference materials

In 2016, the NISTmAb reference material 8671 (a monoclonal IgG1κ produced by the National Institute of Standards and Technology, USA) became publicly available to researchers - making available for the first time a highly characterized recombinant monoclonal antibody for method development, system suitability tests and inter-laboratory comparability studies. Prior to 2016, a few recombinant monoclonal antibodies were commercially available (such as Waters' Intact mAb Mass Check Standard and Sigma's SILu™Lite SigmaMAb Universal Antibody Standard) but these antibodies were not well characterized and use was not widespread. Many labs developed their own in-house reference antibodies for method development, which precluded easy comparison of methods developed in different labs. The NISTmAb was thoroughly characterized by a multi-lab, international effort which used multiple methods to assess the identity, purity, aggregation, stability, glycosylation, post-translational modifications and higher order structure. The results of these studies were published in a 3 volume book series "State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization" and follow-up papers [19,121–124]. NISTmAb does not replace the need for product-specific standards when developing a new antibody-based therapeutic or biosimilar but it serves as a useful external standard when comparing methods developed in different labs [125].

Enabling enzymes

Over the past 10 years, new antibody proteases commercialized by companies such as Genovis have opened up new options for specific antibody cleavage and "middle-up"/"middle-down" analysis. Previously, partial cleavage of antibodies was done using the proteases papain and pepsin. Papain cleaves above the hinge of IgGs to generate Fc and Fab fragments. Pepsin cleaves below the hinge to generate a $F(ab')_2$ fragment while the Fc fragment undergoes extensive proteolysis. Pepsin and papain protocols need to be carefully optimized for each monoclonal IgG to avoid over-digestion and yields of the desired fragments are typically 45–55% [126]. The introduction of IdeS (by Genovis under the brand name "FabRICATOR"), an IgG-specific cysteine protease isolated from *Streptococcus pyogenes*, made it possible to specifically cleave human IgG1 into $F(ab')_2$ and Fc/2 fragments in an easy, complete and reproducible manner [127]. IdeS cleaves human IgG at one specific location below the hinge (Fig. 1.8) with much greater specificity and efficiency than pepsin and the protocol does not require optimization for individual antibodies. IdeS is now the most commonly used enzyme in "middle-up"/"middle-down" characterization of IgG subunits.

More recently, Genovis has introduced enzymes for cleavage of IgG at one specific site above the hinge to form Fab and Fc fragments. SpeB (FabULOUS, launched in 2014) cleaves a wide range of IgGs but only under reducing conditions, Kgp (GingisKHAN, launched in 2015) cleaves human IgG1 under very mild reducing conditions (2 mM cysteine) and IgdE (FabALACTICA, launched in 2017) cleaves human IgG1 above the hinge under native conditions. Generation of the Fab fragment is of particular interest because this antibody fragment retains heavy-light chain pairing information. Kgp has been used to determine heavy-light chain pairing and to detect light chain swapping during the generation of bispecific antibodies [128,129]. Heterodimer antibodies with swapped light chains are isobaric to those with correct pairing, but at the Fab fragment level, incorrect pairings are easily identified. In addition, a combination of enzymes that cleave above and below the hinge has been used to isolate the hinge region of an antibody and identify reduced disulfide bonds [130].

FIG. 1.8 Sites of cleavage in the hinge region of IgG1 for enzymes typically used to generate antibody fragments for "middle-up"/"middle-down" LC-MS analysis.

Top-down and middle-down analysis

Interest has been growing in the potential of "top-down" mass spectrometry for the characterization of antibody-based therapeutics. In a "top-down" experiment, intact antibody ions are selected and fragmented within the mass spectrometer to obtain amino acid sequence and PTM information. A variety of fragmentation techniques can be used in "top-down" assays including ultraviolet photodissociation (UVPD), collision-induced dissociation (CID), electron transfer dissociation (ETD), electron capture dissociation (ECD), higher energy collisional dissociation (HCD) or ETD followed by HCD (EThcD). Like intact mass analysis, "top-down" mass spectrometry requires minimal sample preparation (primarily desalting) so processing artifacts such as deamidation, oxidation and S-thiolation (i.e., cysteinylation) are avoided [3,131]. Also, modifications prone to loss or scrambling during "bottom-up" LC-MS/MS tend to remain in place in a "top-down" analysis. The "top-down" approach provides a more holistic view of an antibody compared with "bottom-up" analysis. For example, "top-down" analysis can detect correlations between modifications, such as any correlations between glycosylation and susceptibility to oxidation; this information is lost in "bottom-up" experiments as the different proteoforms are scrambled during digestion [3,129]. Depending on the fragmentation method used, disulfide bonds may still be intact and branched fragments may indicate chain pairing.

In theory, information about the entire protein (including all modifications) is present in a "top-down" spectrum. In practice, however, "top-down" mass spectrometry has several technical challenges. During MS/MS, the precursor ion signal is split into a large number of fragment ion signals. As the size of the precursor protein increases, the number of fragment ions increases and the signal intensity for individual ions decreases. To attain a sufficient signal-to-noise ratio to detect low abundant fragment ions, many microscans must be averaged, typically by performing multiple replicate runs [6,129]. Additionally, fragmentation methods preferentially cleave the antibody in certain regions, usually mediated by the presence of disulfide bonds [129,132]. Better sequence coverage can be attained by using a combination of fragmentation methods, though this results in multiple datasets to analyze and align [133]. Top-down spectra are very complex due to the presence of canonical N- and C-terminus containing fragments as well as internal fragments and branched disulfide bond-containing fragments, all of which may exist at multiple charge states [129]. Sophisticated software is necessary in order to compile microscans, deconvolute data, match fragments and distinguish false positives [133]. As a consequences, sequence coverage for "top-down" analysis of antibodies lags significantly behind that which can be attained by "bottom-up" methods [6,133].

"Middle-down" analysis of antibodies is showing greater promise than "top-down". "Middle-down" LC-MS/MS is carried out in the same way as "top-down" except the antibody is first cleaved into large fragments (usually Fc/2, Fd and light chain, or Fab and Fc) which are analyzed separately by LC-MS/MS (Fig. 1.9) [6]. Since the cleaved fragments are significantly smaller than the full-sized antibody, the MS/MS spectra are simpler and fragment ion signal is improved. However, the fragmentation data is still complex and complete amino acid sequence coverage continues to be an issue. It remains to be seen whether the technical challenges associated with "top-down" and "middle-down" MS will

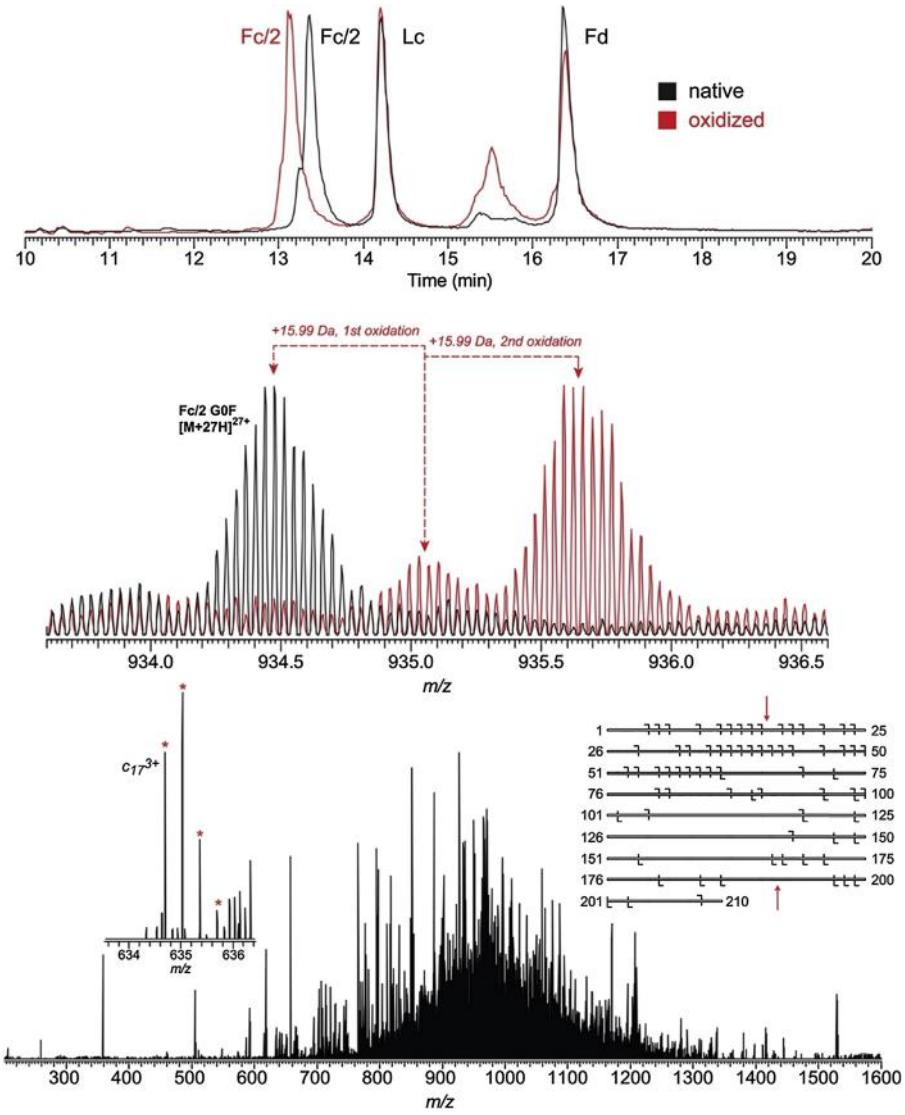

FIG. 1.9 Application of "middle-down" LC-MS for determining the locations of oxidized Met residues in the mAb, Adalimumab. (Top) After IdeS digestion and reduction, the resulting Fd, Fc/2 and light chain fragments were resolved and analyzed by LC-MS on a Orbitrap Fourier Transform mass spectrometer. The oxidized **(***red line (gray in print version)***)** Fc/2 eluted earlier than the control **(***black line***)** Fc/2. (Middle) Expanded view of the overlapped mass spectra for control (*black*) and oxidized Fc/2 (*red* (*gray in print version*)). At isotopic resolution (120,000 resolution at m/z 400), the oxidized Fc/2 is observed with a mass +16 Da and +32 Da greater than control Fc/2, indicating both singly and doubly oxidized form. (Bottom) The "middle-down" ETD fragment ion spectrum for oxidized Fc/2 (single LC run, 3 ms ETD, isolation window 120 Th). The left inset shows a product ion confirming that Met16 is oxidized. The right inset is a scheme of identified product ions, with the positions of oxidized Met indicated by arrows. *Reproduced from Fornelli, L., et al., Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Anal Chem, 2014;86(6):3005−3012 with permission, © 2014 American Chemical Society.*

be surmounted in the coming years and use of these promising technologies becomes more widespread.

Native mass spectrometry

One of the more exciting developments in LC-MS in recent years has been the emergence of native mass spectrometry (native MS) for the characterization of antibody-based therapeutics under non-denaturing conditions. Native MS preserves non-covalent interactions and higher order structure normally lost under the denaturing conditions of RPLC-MS and can be used to study antibody-antigen and antibody-antibody interactions [134,135]. Native MS is similar to intact mass LC-MS analysis except that samples are electrosprayed under neutral, near physiological pH conditions in simple solutions consisting of volatile buffers such as ammonium acetate. The resultant protein ions are not highly charged and their charge state envelopes are shifted to a higher m/z range than protein ions generated under denaturing MS conditions. Therefore, a mass spectrometer with a higher mass range than typical for traditional instruments (up to m/z 8,000−20,000) is necessary for native MS. Native MS is particularly useful for studying antibodies, ADCs and bispecifics that are held together non-covalently, such as cysteine-based ADCs where the cysteines that would normally have formed inter-chain disulfide bonds are instead used for drug conjugation [136,137] (Fig. 1.10). Native MS provides a better picture of the true state of an antibody than does denaturing MS.

Different proteoforms are better resolved with native MS than denaturing LC-MS because the separation between ions is greater at the lower charge states typical of native MS. Wohlschlager et al. took advantage of this improved separation and used intact and "middle-up" native MS on an Exactive Plus EMR Orbitrap to characterize the highly complex glycoforms of Etanercept, a 130 kDa Fc fusion protein with 6 N-linked and up to 26 O-linked glycans [138]. Native MS sufficiently resolved the numerous, complex glycoforms of intact Enbrel and enabled lot to lot comparison.

Ion mobility mass spectrometry

In conventional mass spectrometry, gas-phase ions are resolved solely on the basis of their mass to charge ratio (m/z). In ion mobility mass spectrometry (IM-MS), both the m/z of an ion and the time taken by the ion to pass through a drift tube placed in-line with the mass spectrometer (the "drift time") is recorded. The drift time of an ion is dependent on both its charge and shape. IM-MS provides another dimension of separation that can add greater selectivity to an LC-MS assay and offer a means to probe the structure and conformational stability of ions [139]. IM enhances "bottom-up" LC-MS by resolving overlapping peptide ions with different charge states, improving the signal to noise for weaker ions and distinguishing structural isomers of isobaric ions. For example, ion mobility has been coupled with 1D-RPLC-MS to profile lot to lot N-glycosylation compositional heterogeneity in batches of trastuzumab mAb [140] and with 2D-RPLC-MS to identify trace-level host cell proteins in originator and biosimilar mAbs [36].

Ion mobility is a logical match with native MS which maintains the native shape of protein ions and there are numerous publications demonstrating the use of native-IM-MS for

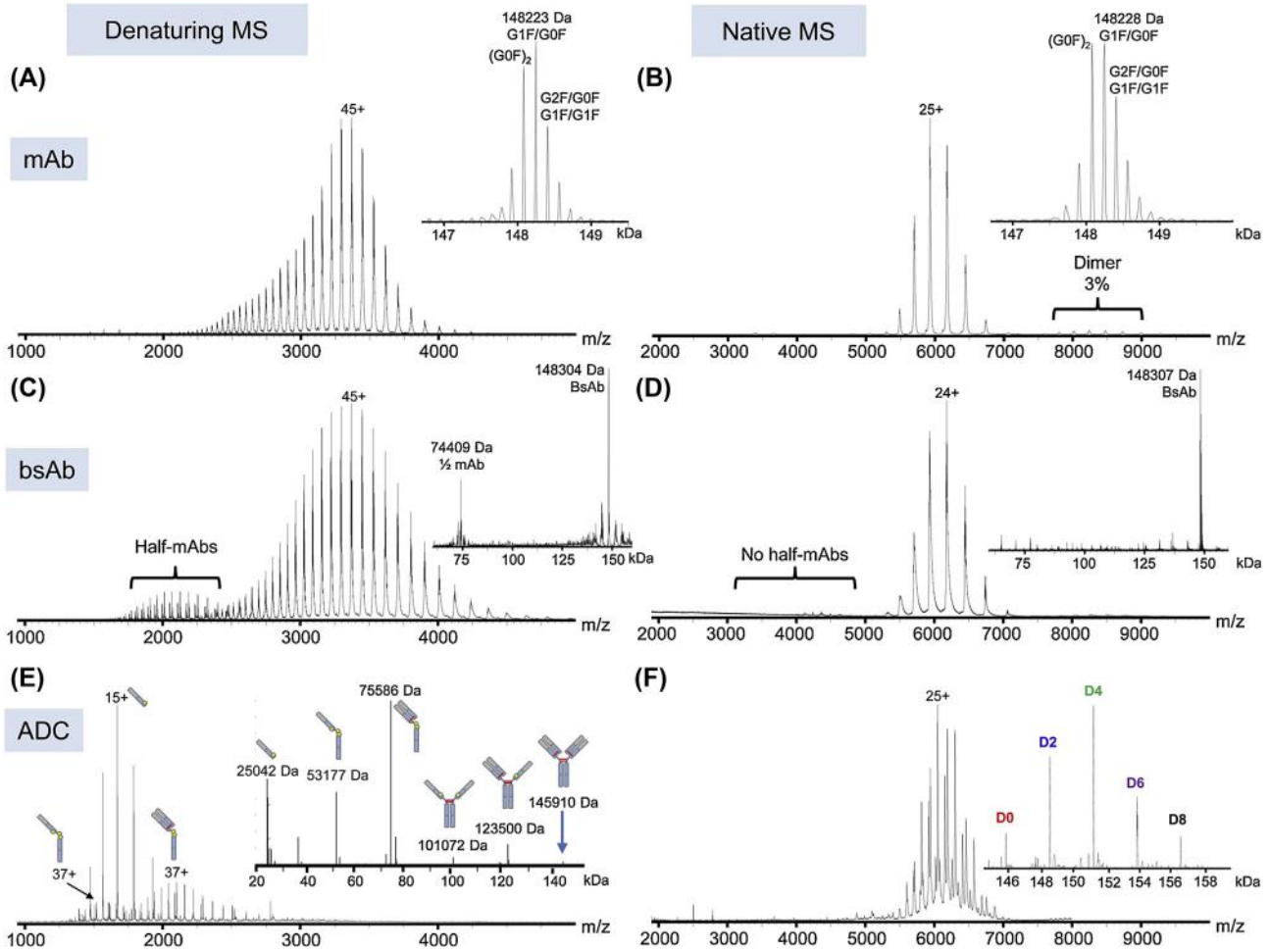

FIG. 1.10 Comparison of denaturing and native LC-MS of antibody-based therapeutics: (A and B) intact trastuzumab, (C and D) a bispecific mAb, and (E and F) the cysteine-conjugated ADC, brentuximab vedotin. The insets show the annotated deconvoluted mass spectra. The intermolecular disulfide bonds in brentuximab vedotin were partially reduced during drug conjugation. The ADC dissociated into fragments under the denaturing conditions but remained intact during native MS analysis. *Reproduced from Terral, G., A. Beck, and S. Cianférani, Insights from native mass spectrometry and ion mobility-mass spectrometry for antibody and antibody-based product characterization. Journal of Chromatography B Analyt Technol Biomed Life Sci 2016;1032:79—90 with permission from Elsevier.*

investigating the structure and non-covalent interactions of antibody-based therapeutics. In one of the first publications, Atmanene et al. used native-IM-MS to study interactions between human JAM-A, a protein expressed in tumors, and anti-JAM-A mAbs [135]. IM-MS resolved on the basis of drift times populations of ions that had alternative disulfide bonding in the JAM-A ectodomain. However, analysis of the antibody/JAM-A interactions by native-IM-MS indicated that ectodomain disulfide heterogeneity did not have a significant effect on antibody affinity or binding stoichiometry. Bagal et al. demonstrated that native-IM-MS could distinguish between the A and B disulfide isoforms of intact IgG2 mAbs. The B isoform had a slightly longer drift time suggesting that it has a larger cross-sectional area relative to the A isoform. More recently native-IM-MS has been incorporated into multi-pronged analytical strategies to compare biosimilars with originator mAbs [141,142].

Native-IM-MS can be coupled with collision induced unfolding (CIU) to study protein conformational stability [139]. In a CIU experiment, the protein ions undergo collisional activation in the gas phase just prior to IM. Unfolding events are detected as changes in the IM drift time. Native-IM-MS CIU was used to compare CBW-03-06, an ADC with engineered sites for drug conjugation, with its parental mAb [143]. The ADC was less prone to unfolding than the parent mAb, suggesting that drug conjugation actually stabilized the conformational structure of the mAb. Native-IM-MS with CIU was also used to study Fab arm exchange in IgG4 antibodies, a process that can be exploited to create bispecifics [144]. Native-IM-MS alone could not distinguish the different IgG4 species but the addition of CIU generated unique signatures for each antibody species present, both native and bispecific. Finally, IM-MS with CIU has been used recently in accelerated stability studies [145]. IM-MS with CIU detected changes in the stressed antibodies indicative of unfolding.

Alternative chromatographies for antibody and ADC analysis

Different chromatographies can be interfaced with native MS to facilitate the separation of proteoforms based on size, charge or affinity prior to MS. For example, Hengel et al. coupled a microscale size exclusion chromatography (SEC) column with native MS for the separation of cysteine-conjugated ADCs immune-isolated from the plasma of dosed rats prior to DAR assessment [146]. The improved separation provided by SEC allowed them to monitor the gradual decrease in DAR occurring over time due to loss of drug-linker and to detect cysteinylation of the deconjugated cysteines. Similarly, Ehkirch et al. demonstrated the potential of coupling SEC with native MS and ion mobility-MS (SEC-native-MS/IM-MS) for the enhanced characterization of antibody-based therapeutics, including ADCs [147]. The same group also developed a four dimensional HIC-SEC-native-IM-MS platform and applied this to the comparison of cysteine-conjugated ADCs in native form and after forced degradation [148]. The addition of HIC separation in the first dimension enabled a greater resolution of all ADC species present, especially those in the degraded samples.

Proteoforms can vary in charge due to the presence of charge-modifying modifications, such as C-terminal lysine, sialic acid and deamidation. Füssl et al. described the development and application of a pH gradient cation exchange chromatography-native mass spectrometry platform for the charge-based separation and identification of antibody species [149,150]. Volatile, low ionic strength buffers were used for the separation so as to be compatible

with native MS. This novel LC-MS platform could distinguish charge isoforms of stressed-tested Adalimumab due to the presence of C-terminal lysine, glycation, deamidation, isoaspartate and possibly succinimide formation [150]. Finally, Chen et al. demonstrated that it is possible to directly interface HIC with electrospray mass spectrometry [151,152]. Normally this would not be possible as the mobile phase uses relatively high concentrations of non-volatile salts which are incompatible with mass spectrometry, however, by replacing the non-volatile salts with ammonium acetate and utilizing more hydrophobic stationary phases, they achieved good HIC separations of antibodies and performed "top-down" HIC-ECD-MS sequencing on the multiply charged antibody ions [152].

Multi-Attribute Method

LC-MS is at the core of a recent technology development called Multi-Attribute Method (MAM) that has generated significant interest within the biopharmaceutical industry. MAM utilizes "bottom-up" LC-MS protocols to simultaneously detect, quantify and monitor multiple features of a protein biologic such as N- and O-glycosylation, oxidation, deamidation, isoaspartate formation and protease cleavage [153,154]. In a conventional biomanufacturing environment, a separate assay is used to assess and monitor each important feature, especially those deemed to be critical quality attributes (CQAs). These assays are based on conventional and well understood analytical technologies such as CE-SDS, CEX-HPLC, cIEF and HILIC, typically coupled with UV and fluorescence detectors that generate a straightforward and quantifiable output. MAM has the potential to consolidate many of these conventional assays into a single LC-MS assay. Moreover, MAM is capable of providing additional information about CQAs such as the location and abundance of individual modifications.

Recently, a white paper was published by a working group with representation from across the biopharmaceutical industry advocating for increased adoption of MAM for identifying and monitoring CQAs, improving control strategies for biomanufacturing and simplifying submissions to regulatory authorities [155]. The potential implications for the industry are enormous. Instrument manufacturers have taken note and are developing LC-MS platforms that are better tailored for MAM and are creating software for the automated control of the entire MAM workflow, from instrument control to data analysis, in a manner that is compliant with regulatory expectations. New peak detection (NPD), the ability to flag new and unexpected features in the LC-MS/MS data, is a critical component of MAM control and analysis software [155]. NPD reduces the risk of false negatives and, as a result, has greatly increased user confidence in the technology. Broad application of MAM in the highly regulated environment of QA/QC labs and GMP manufacturing facilities is still some way off in the future and will require continual improvements in instrument design, improved quality control in the manufacture of HPLC columns, enzymes and other reagents as well as the development of new reference materials suitable for MAM. However, it does appear that MAM, and by implication "bottom-up" LC-MS, will have a major impact on how biotherapeutics are manufactured and characterized in the future.

Conclusion

In this chapter, we have provided an overview of how LC-MS is used for the biophysical characterization of antibody-based therapeutics. LC-MS protein characterization is a broad topic area spanning an enormous body of literature and it is impossible to cover all applications in a single chapter. Instead we have highlighted some current LC-MS applications, particularly for the characterization of different PTMs, and recent developments in the field that illustrate both the scope and future potential of LC-MS for biotherapeutics analysis. In this, we have omitted some other applications of LC-MS for the study of antibody-based therapeutics such as quantitative analysis for PK/PD studies which, though outside the scope of this chapter, also relies heavily on LC-MS.

It is acknowledged that LC-MS capabilities represent a large investment for any organization in terms of upfront capital cost, maintenance and specialized staff. However, LC-MS is unparalleled in terms of specificity, sensitivity and flexibility, and is better able than other analytical technologies to keep pace with the innovative R&D developments happening within the biopharmaceutical sector today. The unprecedented level of detail provided by LC-MS-based assays can lead to improvements in biotherapeutic design and production, resulting in more stable, effective and homogeneous products and, ultimately, better patient outcomes. Already LC-MS-based assays are increasingly being adopted into the biopharmaceutical and biomanufacturing domain and are being used to set more precise specifications for critical quality attributes and to monitor production processes. Multi-attribute monitoring with LC-MS is gaining acceptance as a viable, cost-effective alternative to conventional analytical assays. We expect in the near future to see increasing use of LC-MS at all stages of the product development life cycle of antibody-based therapeutics.

References

[1] FDA, *Purple Book*. In: Research CfBEa, editor. Lists of licensed biological products with reference product exclusivitiy and biosimilarity or interchangeable evaluations; 2019.
[2] Kaplon H, Reichert JM. Antibodies to watch in 2019. mAbs 2019;11(2):219–38.
[3] Donnelly DP, et al. Best practices and benchmarks for intact protein analysis for top-down mass spectrometry. Nat Methods 2019;16(7):587–94.
[4] Jin Y, et al. Comprehensive characterization of monoclonal antibody by Fourier transform ion cyclotron resonance mass spectrometry. mAbs 2019;11(1):106–15.
[5] Valeja SG, et al. Unit mass baseline resolution for an intact 148 kDa therapeutic monoclonal antibody by Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 2011;83(22):8391–5.
[6] Fornelli L, et al. Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Anal Chem 2014;86(6):3005–12.
[7] Smith LM, Kelleher NL, Consortium for Top Down P. Proteoform: a single term describing protein complexity. Nat Methods 2013;10(3):186–7.
[8] Lermyte F, et al. Top or middle? Up or down? Toward a standard lexicon for protein top-down and allied mass spectrometry approaches. J Am Soc Mass Spectrom 2019;30(7):1149–57.
[9] Shen M, et al. Restricted proteolysis and LC-MS/MS to evaluate the orientation of surface-immobilized antibodies. Anal Chem 2019;91(7):4913–9.
[10] Mouchahoir T, Schiel JE. Development of an LC-MS/MS peptide mapping protocol for the NISTmAb. Anal Bioanal Chem 2018;410(8):2111–26.

[11] Baker JJ, et al. Rapid identification of disulfide bonds and cysteine-related variants in an IgG1 knob-into-hole bispecific antibody enhanced by machine learning. Anal Chem 2019;91(1):965–76.
[12] Laskay UA, et al. Proteome digestion specificity analysis for rational design of extended bottom-up and middle-down proteomics experiments. J Proteome Res 2013;12(12):5558–69.
[13] Srzentic K, et al. Advantages of extended bottom-up proteomics using Sap9 for analysis of monoclonal antibodies. Anal Chem 2014;86(19):9945–53.
[14] Manikwar P, et al. Correlating excipient effects on conformational and storage stability of an IgG1 monoclonal antibody with local dynamics as measured by hydrogen/deuterium-exchange mass spectrometry. J Pharmacol Sci 2013;102(7):2136–51.
[15] Zhang A, et al. Distinct aggregation mechanisms of monoclonal antibody under thermal and freeze-thaw stresses revealed by hydrogen exchange. Pharm Res (N Y) 2012;29(1):236–50.
[16] Houde D, et al. Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics 2010;9(8):1716–28.
[17] Pan LY, Salas-Solano O, Valliere-Douglass JF. Antibody structural integrity of site-specific antibody-drug conjugates investigated by hydrogen/deuterium exchange mass spectrometry. Anal Chem 2015;87(11):5669–76.
[18] Pan J, Zhang S, Borchers CH. Comparative higher-order structure analysis of antibody biosimilars using combined bottom-up and top-down hydrogen-deuterium exchange mass spectrometry. Biochim Biophys Acta 2016;1864(12):1801–8.
[19] State-of-the-Art and emerging technologies for therapeutic monoclonal antibody characterization - defining the next generation of analytical and biophysical techniques. In: Schiel JB, O Davis D, editors. ACS symposium series, vol. 3. Washington, DC: American Chemical Society; 2014.
[20] Weis DD. Hydrogen exchange mass spectrometry of proteins : fundamentals, methods, and applications. Chichester, West Sussex: John Wiley & Sons, Inc. xxiii; 2016. p. 350. 46 unnumbered pages of plates.
[21] Limpikirati P, Liu T, Vachet RW. Covalent labeling-mass spectrometry with non-specific reagents for studying protein structure and interactions. Methods 2018;144:79–93.
[22] Sinz A. Cross-linking/mass spectrometry for studying protein structures and protein-protein interactions: where are we now and where should we go from here? Angew Chem Int Ed Engl 2018;57(22):6390–6.
[23] Henry SM, et al. ELISA reagent coverage evaluation by affinity purification tandem mass spectrometry. mAbs 2017;9(7):1065–75.
[24] Kumar A, et al. Elucidation of the CHO super-ome (CHO-SO) by proteoinformatics. Journal of Proteomics Research 2015;14(11):4687–703.
[25] Ranjan S, et al. Investigation of cathepsin D-mAb interactions using a combined experimental and computational tool set. Biotechnol Bioeng 2019;116(7):1684–97.
[26] Luo H, et al. Cathepsin L causes proteolytic cleavage of Chinese-Hamster-Ovary cell expressed proteins during processing and storage: identification, characterization, and mitigation. Biotechnol Prog 2019;35(1):e2732.
[27] Lintern K, et al. Residual on column host cell protein analysis during lifetime studies of protein A chromatography. J Chromatogr 2016;1461:70–7.
[28] Tran B, et al. Investigating interactions between phospholipase B-Like 2 and antibodies during Protein A chromatography. J Chromatogr 2016;1438:31–8.
[29] Doneanu CE, et al. Analysis of host-cell proteins in biotherapeutic proteins by comprehensive online two-dimensional liquid chromatography/mass spectrometry. mAbs 2012;4(1):24–44.
[30] Zhang Q, et al. Comprehensive tracking of host cell proteins during monoclonal antibody purifications using mass spectrometry. mAbs 2014;6(3):659–70.
[31] Doneanu CE, Chen W. Analysis of host-cell proteins in biotherapeutic proteins by LC/MS approaches. Methods Mol Biol 2014;1129:341–50.
[32] Soderquist RG, et al. Development of advanced host cell protein enrichment and detection strategies to enable process relevant spike challenge studies. Biotechnol Prog 2015;31(4):983–9.
[33] Doneanu CE, et al. Enhanced detection of low-abundance host cell protein impurities in high-purity monoclonal antibodies down to 1 ppm using ion mobility mass spectrometry coupled with multidimensional liquid chromatography. Anal Chem 2015;87(20):10283–91.
[34] Farrell A, et al. Quantitative host cell protein analysis using two dimensional data independent LC-MS(E). Anal Chem 2015;87(18):9186–93.

[35] Zhang Q, et al. Characterization of the co-elution of host cell proteins with monoclonal antibodies during protein A purification. Biotechnol Prog 2016;31(3):708−17.
[36] Fang J, et al. Advanced assessment of the physicochemical characteristics of Remicade® and Inflectra® by sensitive LC/MS techniques. mAbs 2016;8(6):1021−34.
[37] Stoll D, et al. Characterization of therapeutic antibodies and related products by two-dimensional liquid chromatography coupled with UV absorbance and mass spectrometric detection. Journal of Chromatography B Analyt Technol Biomed Life Sci 2016;1032:51−60.
[38] Yang F, et al. A 2D LC-MS/MS strategy for reliable detection of 10-ppm level residual host cell proteins in therapeutic antibodies. Anal Chem 2018;90(22):13365−72.
[39] Madsen JA, et al. Toward the complete characterization of host cell proteins in biotherapeutics via affinity depletions, LC-MS/MS, and multivariate analysis. mAbs 2015;7(6):1128−37.
[40] Falkenberg H, et al. Mass spectrometric evaluation of upstream and downstream process influences on host cell protein patterns in biopharmaceutical products. Biotechnol Prog 2019;35(3):e2788.
[41] Vlasak J, Ionescu R. Fragmentation of monoclonal antibodies. mAbs 2011;3(3):253−63.
[42] Yang B, et al. Discovery and characterization of CHO host cell protease-induced fragmentation of a recombinant monoclonal antibody during production process development. J Chromatogr B Analyt Technol Biomed Life Sci 2019;1112:1−10.
[43] Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. mAbs 2012;4(1):17−23.
[44] Harris RJ. Heterogeneity of recombinant antibodies: linking structure to function. In: Mire-Sluis AR, editor. State of the art analytical methods for the characterization of biological products and assessment of comparability. Basel (Switzerland: Karger; 2005. p. 117−27.
[45] Chung WK, et al. Effects of antibody disulfide bond reduction on purification process performance and final drug substance stability. Biotechnol Bioeng 2017;114(6):1264−74.
[46] Buchanan A, et al. Engineering a therapeutic IgG molecule to address cysteinylation, aggregation and enhance thermal stability and expression. mAbs 2013;5(2):255−62.
[47] Trivedi MV, Laurence JS, Siahaan TJ. The role of thiols and disulfides on protein stability. Curr Protein Pept Sci 2009;10(6):614−25.
[48] Huh JH, et al. The identification of free cysteine residues within antibodies and a potential role for free cysteine residues in covalent aggregation because of agitation stress. J Pharmacol Sci 2013;102(6):1701−11.
[49] Lacy ER, Baker M, Brigham-Burke M. Free sulfhydryl measurement as an indicator of antibody stability. Anal Biochem 2008;382(1):66−8.
[50] Zhang W, Czupryn MJ. Free sulfhydryl in recombinant monoclonal antibodies. Biotechnol Prog 2002;18(3):509−13.
[51] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82(1):70−7.
[52] Wu CW, Yarbrough LR. N-(1-pyrene)maleimide: a fluorescent cross-linking reagent. Biochemistry 1976;15(13):2863−8.
[53] Robotham AC, Kelly JF. Detection and quantification of free sulfhydryls in monoclonal antibodies using maleimide labeling and mass spectrometry. mAbs 2019;11(4):757−66.
[54] Tous GI, et al. Characterization of a novel modification to monoclonal antibodies: thioether cross-link of heavy and light chains. Anal Chem 2005;77(9):2675−82.
[55] Zhang Q, et al. IgG1 thioether bond formation in vivo. J Biol Chem 2013;288(23):16371−82.
[56] Gu S, et al. Characterization of trisulfide modification in antibodies. Anal Biochem 2010;400(1):89−98.
[57] Dillon TM, et al. Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. J Biol Chem 2008;283(23):16206−15.
[58] Resemann A, et al. Rapid, automated characterization of disulfide bond scrambling and IgG2 isoform determination. mAbs 2018;10(8):1200−13.
[59] Labrijn AF, et al. Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. Nat Biotechnol 2009;27(8):767−71.
[60] Guan X, Zhang L, Wypych J. Direct mass spectrometric characterization of disulfide linkages. mAbs 2018;10(4):572−82.
[61] Zauner G, et al. Glycoproteomic analysis of antibodies. Mol Cell Proteomics 2013;12(4):856−65.

[62] Chen CC, et al. Interaction modes and approaches to glycopeptide and glycoprotein enrichment. Analyst 2014;139(4):688–704.
[63] Huhn C, et al. IgG glycosylation analysis. Proteomics 2009;9(4):882–913.
[64] Plomp R, et al. Hinge-region O-glycosylation of human immunoglobulin G3 (IgG3). Mol Cell Proteomics 2015;14(5):1373–84.
[65] Kim H, et al. O-glycosylation in hinge region of mouse immunoglobulin G2b. J Biol Chem 1994;269(16):12345–50.
[66] Mattu TS, et al. The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fcalpha receptor interactions. J Biol Chem 1998;273(4):2260–72.
[67] Takahashi N, et al. Complete amino acid sequence of the delta heavy chain of human immunoglobulin D. Proc Natl Acad Sci U S A 1982;79(9):2850–4.
[68] Darula Z, Medzihradszky KF. Analysis of mammalian O-Glycopeptides-We have made a good start, but there is a long way to go. Mol Cell Proteomics 2018;17(1):2–17.
[69] Ahmed AA, et al. Structural characterization of anti-inflammatory immunoglobulin G Fc proteins. J Mol Biol 2014;426(18):3166–79.
[70] Zhong X, et al. Pyroglutamate and O-linked glycan determine functional production of anti-IL17A and anti-IL22 peptide-antibody bispecific genetic fusions. J Biol Chem 2013;288(2):1409–19.
[71] Hashii N, et al. In-depth site-specific O-Glycosylation analysis of therapeutic Fc-fusion protein by electron-transfer/higher-energy collisional dissociation mass spectrometry. Biologicals 2019;58:35–43.
[72] Hanisch FG. Top-down sequencing of O-glycoproteins by in-source decay matrix-assisted laser desorption ionization mass spectrometry for glycosylation site analysis. Anal Chem 2011;83(12):4829–37.
[73] Largy E, et al. Orthogonal liquid chromatography-mass spectrometry methods for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level. J Chromatogr A 2017;1498:128–46.
[74] Jiang J, et al. Site-specific qualitative and quantitative analysis of the N- and O-glycoforms in recombinant human erythropoietin. Anal Bioanal Chem 2014;406(25):6265–74.
[75] Mulagapati S, Koppolu V, Raju TS. Decoding of O-linked glycosylation by mass spectrometry. Biochemistry 2017;56(9):1218–26.
[76] Wei B, et al. Glycation of antibodies: modification, methods and potential effects on biological functions. mAbs 2017;9(4):586–94.
[77] Gadgil HS, et al. The LC/MS analysis of glycation of IgG molecules in sucrose containing formulations. J Pharmacol Sci 2007;96(10):2607–21.
[78] Quan CP, et al. Susceptibility of rhDNase I to glycation in the dry-powder state. Anal Chem 1999;71(20):4445–54.
[79] Mo J, et al. Quantitative analysis of glycation and its impact on antigen binding. mAbs 2018;10(3):406–15.
[80] Banks DD, et al. The effect of sucrose hydrolysis on the stability of protein therapeutics during accelerated formulation studies. J Pharmacol Sci 2009;98(12):4501–10.
[81] Fischer S, Hoernschemeyer J, Mahler HC. Glycation during storage and administration of monoclonal antibody formulations. Eur J Pharm Biopharm 2008;70(1):42–50.
[82] Butko M, et al. Recombinant antibody color resulting from advanced glycation end product modifications. Anal Chem 2014;86(19):9816–23.
[83] Tanaka M, et al. O-linked glucosylation of a therapeutic recombinant humanised monoclonal antibody produced in CHO cells. Eur J Pharm Biopharm 2013;83(2):123–30.
[84] Martinez T, et al. Characterization of a novel modification on IgG2 light chain. Evidence for the presence of O-linked mannosylation. J Chromatogr A 2007;1156(1–2):183–7.
[85] Viski K, et al. Parallel development of chromatographic and mass-spectrometric methods for quantitative analysis of glycation on an IgG1 monoclonal antibody. J Chromatogr B Analyt Technol Biomed Life Sci 2016;1032:198–204.
[86] Brady LJ, Martinez T, Balland A. Characterization of nonenzymatic glycation on a monoclonal antibody. Anal Chem 2007;79(24):9403–13.
[87] Zhang Q, et al. Application of electron transfer dissociation mass spectrometry in analyses of non-enzymatically glycated peptides. Rapid Commun Mass Spectrom 2007;21(5):661–6.

[88] Dick LWJ, Qiu D, Cheng KC. Identification and measurement of isoaspartic acid formation in the complementarity determining region of a fully human monoclonal antibody. Journal of Chromatography B Analyt Technol Biomed Life Sci 2009;877(30):3841−9.
[89] Yan B, et al. Succinimide formation at Asn 55 in the complementarity determining region of a recombinant monoclonal antibody IgG1 heavy chain. J Pharmaceut Sci 2009;98(10):3509−21.
[90] Mamula MJ, et al. Isoaspartyl post-translational modification triggers autoimmune responses to self-proteins. J Biol Chem 1999;274(32):22321−7.
[91] Kori Y, et al. A conventional procedure to reduce Asn deamidation artifacts duringtrypsin peptide mapping. Journal Of Chromatography B Analytical Technologies In The Biomedical And Life Sciences 2016;1009−1010:107−13.
[92] Liu H, Nowak C, Patel R. Modifications of recombinant monoclonal antibodies in vivo. Biologicals; 2019.
[93] Patel K, Borchardt RT. Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. Pharmaceut Res 1990;7(7):703−11.
[94] Bhatt NP, Patel K, Borchardt RT. Chemical pathways of peptide degradation. I. Deamidation of adrenocorticotropic hormone. Pharmaceut Res 1990;7(6):593−9.
[95] Cao M, et al. Characterization and quantification of succinimide using peptide mapping under low-pH conditions and hydrophobic interaction chromatography. Anal Biochem 2019;566:151−9.
[96] Folzer E, et al. Selective oxidation of methionine and tryptophan residues in a therapeutic IgG1 molecule. J Pharmaceut Sci 2015;104(9):2824−31.
[97] Bertolotti-Ciarlet A, et al. Impact of methionine oxidation on the binding of human IgG1 to Fc Rn and Fc gamma receptors. Mol Immunol 2009;46(8−9):1878−82.
[98] Wang W, et al. Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. Mol Immunol 2011;48(6−7):860−6.
[99] Gao X, et al. Effect of individual Fc methionine oxidation on FcRn binding: Met252 oxidation impairs FcRn binding more profoundly than Met428 oxidation. J Pharmaceut Sci 2015;104(2):368−77.
[100] Li Y, et al. Characterization of the degradation products of a color-changed monoclonal antibody: tryptophan-derived chromophores. Anal Chem 2014;86(14):6850−7.
[101] Hageman T, et al. Impact of tryptophan oxidation in complementarity-determining regions of two monoclonal antibodies on structure-function characterized by hydrogen-deuterium exchange mass spectrometry and surface plasmon resonance. Pharmaceut Res 2018;36(1):24.
[102] Pavon JA, et al. Selective tryptophan oxidation of monoclonal antibodies: oxidative stress and modeling prediction. Anal Chem 2019;91(3):2192−200.
[103] Beck A, et al. Cutting-edge multi-level analytical and structural characterization of antibody-drug conjugates: present and future. Expet Rev Proteonomics 2019;16(4):337−62.
[104] Hill JJ, et al. An accurate TMT-based approach to quantify and model lysine susceptibility to conjugation via N-hydroxysuccinimide esters in a monoclonal antibody. Sci Rep 2018;8(1):17680.
[105] Abecassis PY, Amara C. In vivo testing of drug-linker stability. Methods Mol Biol 2013;1045:101−16.
[106] Birdsall RE, et al. A sensitive multidimensional method for the detection, characterization, and quantification of trace free drug species in antibody-drug conjugate samples using mass spectral detection. mAbs 2016;8(2):306−17.
[107] Zmolek W, et al. A simple LC/MRM-MS-based method to quantify free linker-payload in antibody-drug conjugate preparations. Journal of Chromatography B Analyt Technol Biomed Life Sci 2016;1032:144−8.
[108] Xu K, et al. Characterization of intact antibody−drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography−mass spectrometry. Anal Biochem 2011;412:56−66.
[109] Xu K, et al. Characterization of the drug-to-antibody ratio distribution for antibody−drug conjugates in plasma/serum. Bioanalysis 2013;5(9):1057−71.
[110] Excoffier M, et al. A new anti-human Fc method to capture and analyze ADCs for characterization of drug distribution and the drug-to-antibody ratio in serum from pre-clinical species. Journal of Chromatography B Analyt Technol Biomed Life Sci 2016;1032:149−54.
[111] He J, et al. Characterization of in vivo biotransformations for trastuzumab emtansine by high-resolution accurate-mass mass spectrometry. mAbs 2018;10(7):960−7.

[112] Jin W, Burton L, Moore I. LC-HRMS quantitation of intact antibody drug conjugate trastuzumab emtansine from rat plasma. Bioanalysis 2019;10(11):851–62.
[113] Li KS, et al. Automated on-tip affinity capture coupled with mass spectrometry to characterize intact antibody-drug conjugates from blood. J Am Soc Mass Spectrom 2018;29(7):1532–7.
[114] Zhao J, et al. Characterization of a novel modification of a CHO-produced mAb: evidence for the presence of tyrosine sulfation. mAbs 2017;9(6):985–95.
[115] Yang YS, et al. Tyrosine sulfation as a protein post-translational modification. Molecules 2015;20(2):2138–64.
[116] Chumsae C, et al. Discovery of a chemical modification by citric acid in a recombinant monoclonal antibody. Anal Chem 2014;86(18):8932–6.
[117] Tyshchuk O, et al. Detection of a phosphorylated glycine-serine linker in an IgG-based fusion protein. mAbs 2017;9(1):94–103.
[118] Wen D, et al. Discovery and investigation of O-xylosylation in engineered proteins containing a (GGGGS)n linker. Anal Chem 2013;85(9):4805–12.
[119] Spahr C, Shi SD, Lu HS. O-glycosylation of glycine-serine linkers in recombinant Fc-fusion proteins: attachment of glycosaminoglycans and other intermediates with phosphorylation at the xylose sugar subunit. mAbs 2014;6(4):904–14.
[120] Spahr C, et al. High-resolution mass spectrometry confirms the presence of a hydroxyproline (Hyp) post-translational modification in the GGGGP linker of an Fc-fusion protein. mAbs 2017;9(5):812–9.
[121] State-of-the-Art and emerging technologies for therapeutic monoclonal antibody characterization - monoclonal antibody therapeutics: structure, function, and regulatory space. In: Schiel JB, O Davis D, editors. ACS symposium series, vol. 1. American Chemical Society; 2014.
[122] State-of-the-Art and emerging technologies for therapeutic monoclonal antibody characterization - biopharmaceutical characterization: the NISTmAb case study. In: Schiel JB, O Davis D, editors. ACS symposium series, vol. 2. American Chemical Society; 2014.
[123] Brinson RG, et al. Enabling adoption of 2D-NMR for the higher order structure assessment of monoclonal antibody therapeutics. mAbs 2019;11(1):94–105.
[124] Hudgens JW, et al. Interlaboratory comparison of hydrogen-deuterium exchange mass spectrometry measurements of the Fab fragment of NISTmAb. Anal Chem 2019;91(11):7336–45.
[125] Nupur N, Rathore AS. Usability of NISTmAb reference material for biosimilar analytical development. Anal Bioanal Chem 2019;411(13):2867–83.
[126] Andrew SM, Titus JA. Fragmentation of immunoglobulin G. Curr Protoc Cell Biol 2003;17(1):16.4.1–16.4.10.
[127] Sjogren J, Olsson F, Beck A. Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. Analyst 2016;141(11):3114–25.
[128] van den Bremer ETJ, et al. Cysteine-SILAC mass spectrometry enabling the identification and quantitation of scrambled interchain disulfide bonds: preservation of native heavy-light chain pairing in bispecific IgGs generated by controlled fab-arm exchange. Anal Chem 2017;89(20):10873–82.
[129] Srzentic K, et al. Multiplexed middle-down mass spectrometry as a method for revealing light and heavy chain connectivity in a monoclonal antibody. Anal Chem 2018;90(21):12527–35.
[130] Faid V, et al. Middle-up analysis of monoclonal antibodies after combined IgdE and IdeS hinge proteolysis: investigation of free sulfhydryls. J Pharmaceut Biomed Anal 2018;149:541–6.
[131] Auclair JR, et al. Artifacts to avoid while taking advantage of top-down mass spectrometry based detection of protein S-thiolation. Proteomics 2014;14(10):1152–7.
[132] Fornelli L, et al. Top-down analysis of immunoglobulin G isotypes 1 and 2 with electron transfer dissociation on a high-field Orbitrap mass spectrometer. Journal of Protemics 2017;159:159–67.
[133] Fornelli L, et al. Accurate sequence analysis of a monoclonal antibody by top-down and middle-down orbitrap mass spectrometry applying multiple ion activation techniques. Anal Chem 2018;90(14):8421–9.
[134] Dyachenko A, et al. Tandem native mass-spectrometry on antibody-drug conjugates and submillion da antibody-antigen protein assemblies on an orbitrap EMR equipped with a high-mass quadrupole mass selector. Anal Chem 2015;87(12):6095–102.
[135] Atmanene C, et al. Extending mass spectrometry contribution to therapeutic monoclonal antibody lead optimization: characterization of immune complexes using noncovalent ESI-MS. Anal Chem 2009;81(15):6364–73.
[136] Valliere-Douglass JF, McFee WA, Salas-Solano O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 2012;84(6):2843–9.

[137] Terral G, Beck A, Cianférani S. Insights from native mass spectrometry and ion mobility-mass spectrometry for antibody and antibody-based product characterization. Journal of Chromatography B Analyt Technol Biomed Life Sci 2016;1032:79−90.
[138] Wohlschlager T, et al. Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals. Nat Commun 2019;9(1):1713.
[139] Dixit SM, Polasky DA, Ruotolo BT. Collision induced unfolding of isolated proteins in the gas phase: past, present, and future. Curr Opin Chem Biol 2018;42:93.
[140] Damen CW, et al. Electrospray ionization quadrupole ion-mobility time-of-flight mass spectrometry as a tool to distinguish the lot-to-lot heterogeneity in N-glycosylation profile of the therapeutic monoclonal antibody trastuzumab. J Am Soc Mass Spectrom 2009;20(11):2021−33.
[141] Pisupati K, et al. A multidimensional analytical comparison of remicade and the biosimilar remsima. Anal Chem 2017;89(9):4838−46.
[142] Brown KA, et al. Rapid characterization of structural and functional similarity for a candidate bevacizumab (Avastin) biosimilar using a multipronged mass spectrometry-based approach. Drug Testing and Analysis; 2019.
[143] Botzanowski T, et al. Insights from native mass spectrometry approaches for top- and middle- level characterization of site-specific antibody-drug conjugates. mAbs 2017;9(5):801−11.
[144] Hernandez-Alba O, et al. Native mass spectrometry, ion mobility, and collision-induced unfolding for conformational characterization of IgG4 monoclonal antibodies. Anal Chem 2018;90(15):8865−72.
[145] Kerr RA, Keire DA, Ye H. The impact of standard accelerated stability conditions on antibody higher order structure as assessed by mass spectrometry. mAbs 2019;11(5):930−41.
[146] Hengel SM, et al. Measurement of in vivo drug load distribution of cysteine-linked antibody-drug conjugates using microscale liquid chromatography mass spectrometry. Anal Chem 2014;86(7):3420−5.
[147] Ehkirch A, et al. Hyphenation of size exclusion chromatography to native ion mobility mass spectrometry for the analytical characterization of therapeutic antibodies and related products. J Chromatogr B Analyt Technol Biomed Life Sci 2018;1086:176−83.
[148] Ehkirch A, et al. A novel online four-dimensional SEC×SEC-IM×MS methodology for characterization of monoclonal antibody size variants. Anal Chem 2018;90(23):13929−37.
[149] Füssl F, et al. Charge variant analysis of monoclonal antibodies using direct coupled pH gradient cation exchange chromatography to high-resolution native mass spectrometry. Anal Chem 2018;90(7):4669−76.
[150] Füssl F, et al. Comprehensive characterisation of the heterogeneity of adalimumab via charge variant analysis hyphenated on-line to native high resolution Orbitrap mass spectrometry. mAbs 2019;11(1):116−28.
[151] Chen B, et al. Online hydrophobic interaction chromatography-mass spectrometry for top-down proteomics. Anal Chem 2016;88(3):1885−91.
[152] Chen B, et al. Online hydrophobic interaction chromatography-mass spectrometry for the analysis of intact monoclonal antibodies. Anal Chem 2018;90(12):7135−8.
[153] Rogers RS, et al. Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. mAbs 2015;7(5):881−90.
[154] Wang Y, et al. Simultaneous monitoring of oxidation, deamidation, isomerization, and glycosylation of monoclonal antibodies by liquid chromatography-mass spectrometry method with ultrafast tryptic digestion. mAbs 2016;8(8):1477−86.
[155] Rogers RS, et al. A view on the importance of "Multi-Attribute method" for measuring purity of biopharmaceuticals and improving overall control strategy. AAPS J 2018;20:1−8.
[156] Harris LJ, et al. Refined structure of an intact IgG2a monoclonal antibody. Biochemistry 1997;36(7):1581−97.
[157] Schrodinger LLC. The PyMOL molecular graphics system. 2015. Version 2.2.3.

CHAPTER

2

Engineering of Protein A for improved purification of antibodies and Fc-fused proteins

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List of abbreviations

CIP Cleaning In Place
DBC Dynamic Binding Capacity
Fc Fragment crystallizable
Fab Fragment antigen binding
HCP Host Cell Protein
IgG Immunoglobulin G
mAb monoclonal Antibody

Antibody purification

Monoclonal antibodies (mAbs) make up the largest category of biopharmaceuticals today and the number of clinically approved therapeutic mAbs increases continuously [1]. *In vivo* administration of monoclonal antibodies and fragment crystallizible (Fc)-fusion proteins for the treatment of diseases, such as cancer and inflammatory diseases, requires production and purification of large amounts of proteins with exceptional purity [2]. With the upstream production of mAbs, a complex mixture of impurities like host cell proteins (HCPs), DNA and viruses is obtained [3]. A variety of separation techniques have been developed for the downstream isolation of immunoglobulin G (IgG) antibodies and Fc-fusion proteins [2]. The methods used can be divided into chromatographic and non-chromatographic. Historically, the non-chromatographic methods, with examples such as crystallization [4], precipitation [5] and aqueous two-phase partitioning [6], are less common. Chromatographic methods, including ion exchange chromatography and affinity chromatography, often based

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on bacterial surface proteins binding to conserved regions of antibodies, are widely used at lab-scale as well as for industrial-scale separation. Ion exchange chromatography is economically more justified, but suffers from lower selectivity than the more widely used affinity-based strategies which have consequently taken the lead. Instead, ion exchange is frequently used downstream of the affinity-based unit operation as a polishing step [2,7]. The most well-established affinity chromatography method is based on staphylococcal Protein A, an affinity ligand which offers excellent recovery and purity [8]. Despite the successful use of Protein A affinity media, due to the high costs associated with proteinaceous chromatographic material, efforts are still ongoing toward the development of non-chromatographic methods, although with limited success [7,8]. Hence, Protein A still remains the gold standard for mAb purification and new improved Protein A resins are continuously being launched to match the increasing demands [9].

Antibody binding proteins

There are several antibody binding proteins that are naturally displayed on bacterial surfaces and can be utilized for antibody purification [10]. Of these, Protein A is the most commonly used and will be further discussed in detail in the following section. Besides Protein A, there is streptococcal Protein G, which has the advantage of binding all IgG subclasses, including e.g. mouse IgG1, human IgG3 and rat IgG2a and b, to which Protein A binds weakly or not at all [11]. Furthermore, Protein G binds to both the Fc and the fragment antigen binding (Fab) of IgG, in both cases to the heavy chain's constant domains [12,13]. Protein G can thus be used for purification of particular subclasses/isotypes of IgG, to which Protein A has no or low affinity, as well as for certain antibody fragments where Protein A is not an alternative. Peptostreptococcal Protein L binds to the variable part of some kappa light chains [14] and can therefore also be used in purification of antibodies, both full-length and fragments such as Fab and single chain variable fragments containing a compatible kappa light chain. Additionally there are recombinant fusion protein products such as Protein A/G [15], Protein LG [16] and Protein LA [17] that combine binding domains from Protein A, G and L, with the intent to broaden the binding specificity and allow for purification of more subclasses than each protein on its own, as well as of samples where the subclass of antibody may not be known.

Staphylococcal Protein A

Protein A is found on the cell wall of *Staphylococcus aureus* where it is thought, through various mechanisms, to increase the virulence of the bacterium. One of these mechanisms is initiated by binding to the constant part of IgG, between the C_H2 and C_H3 domain of the Fc [18]. By doing so the bacteria can avoid opsonization, evade phagocytosis and thereby remain longer in the body. Protein A has also been shown to bind the V_H domain of the Fab fragment of V_H3 family antibodies on the surface of certain B cells and by doing so triggering apoptosis of said cells, which has a weakening effect on the immune system [19,20].

Staphylococcal Protein A contains five highly homologous domains, E, D, A, B and C, each consisting of 56–58 amino acids [21,22] (Fig. 2.1A). All five domains fold into tightly packed three helical bundles with strong hydrophobic cores, where the surface formed by helices

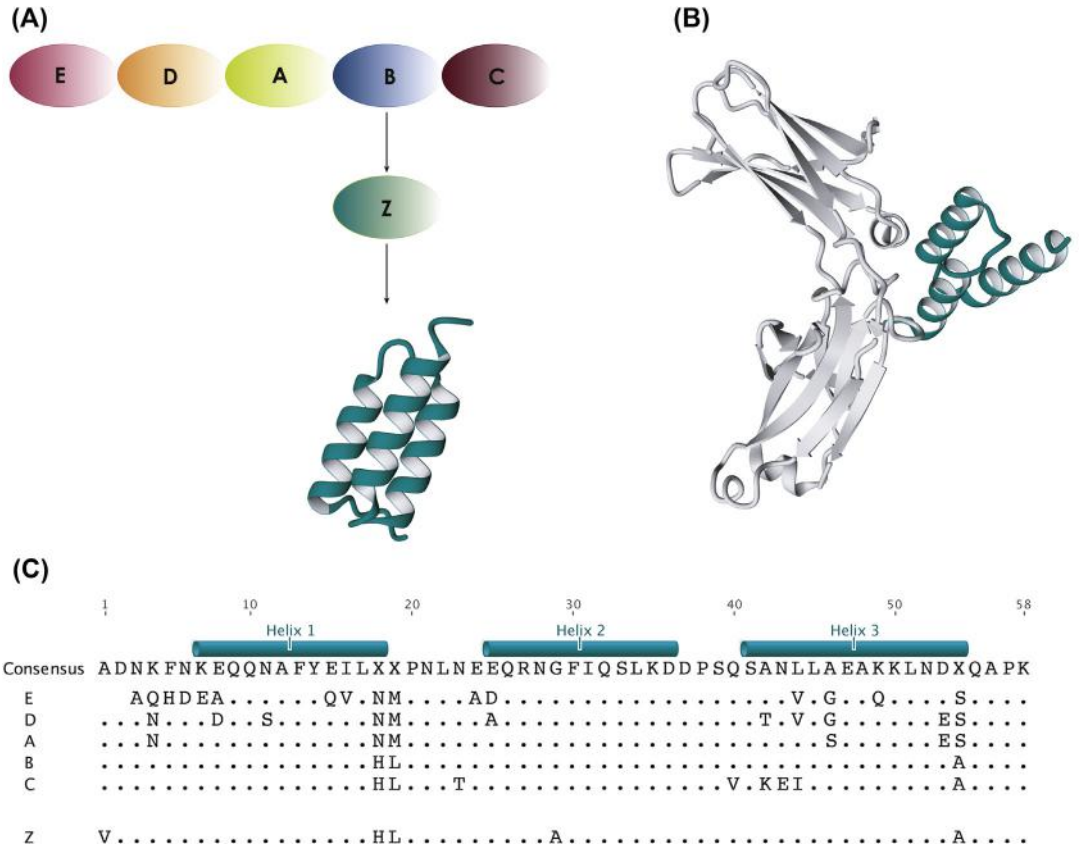

FIG. 2.1 An overview of staphylococcal Protein A. (A) Schematic illustration of the IgG binding domain structure of staphylococcal Protein A. The Z domain is derived from the B domain of Protein A, and its ribbon structure is shown in teal (2spz.pdb [24]). (B) Ribbon representation of the interaction of the B domain with the IgG heavy chain, binding in the wedge between C_H2 and C_H3 (5u4y.pdb [25]). (C) Sequence alignment of the five Protein A IgG-binding domains as well as the engineered Z domain, which differs from the other domains in that it has lost its affinity to the Fab fragment.

one and two is responsible for Fc-binding [18,22] (Fig. 2.1B). The B domain of Protein A has been engineered into the Z domain by two mutations: Ala1Val for cloning purposes and Gly29Ala to make the protein less sensitive to hydroxylamine treatment. The purpose of the latter was to make the domain compatible with site-specific cleavage when used as purification tag [23]. One consequence of this mutation was the loss of binding to the variable region of V_H3-containing IgGs, and thus to smaller antigen binding fragments. Since then, a plethora of changes have been made to the different Protein A domains in order to improve their use in antibody purification, which in the coming sections is illustrated by selected examples. An amino acid sequence alignment of the five natural Protein A domains as well as the Z domain is shown in Fig. 2.1C.

Protein A-based purification

Protein A-based affinity chromatography is the most commonly used capture step when purifying antibodies and Fc-fusion proteins. During 2016 it was reported to be used in 86% of FDA submissions of monoclonal antibodies [9]. As the initial capture in a purification setup it provides a convenient purification step, resulting in highly pure and concentrated product with excellent recoveries of up to 99%, effective removal of HCPs and DNA, as well as virus depletion, only requiring some polishing steps thereafter in order to achieve an antibody pure enough for clinical purposes. Moreover, the inherent high tolerance for acidic pH, denaturants such as urea or guanidine hydrochloride and lower concentrations of sodium hydroxide makes Protein A-based chromatography resins amenable to milder CIP procedures [26,27]. Since the initial launch, Protein A resins have had a steady increase in both capacity, productivity and sales [9]. Most Protein A resins on the market are based on either the full-length recombinant Protein A or on multimeric proteins constructed from four to six repeats of the B or C domain or modified versions thereof [28]. The numerous Protein A-based products on the market are mostly chromatographic matrices where the protein is immobilized on e.g. agarose, porous glass or polymethacrylate beads [28–30]. Though well-functioning and established, these matrices have the drawback of limited flow rates due to back pressure caused by diffusion into the pores of the solid support, as well as requiring filtration of the sample before application to avoid fouling and clogging of the matrix. There are alternatives that are not based on bead supports, such as monolithic affinity chromatography [8,31] and membrane adsorbers [32] which allow for higher flow rates and lower back pressure, circumventing some of the issues with bead-based chromatography media. To date, their low capacity and availability only in small scale has yet to improve in order to meet the demands for large scale purification [9,33,34].

Despite the unprecedented specificity and efficiency of Protein A affinity chromatography, extensive engineering efforts have demonstrated that the domains of Protein A can be tailored for specific requirements. A major focus has been put on the loss of performance over time of Protein A-based resins, which has been attributed to fouling of the resin by proteinaceous material or to degradation of the ligand under the alkaline conditions used for cleaning [28]. Other efforts have addressed the requirement for low pH elution that might have a negative impact on the purified protein and investigated how e.g. altered binding specificity or stability of the ligand can allow milder elution conditions. Strategies based on site-directed mutagenesis, terminal truncations or insertions have successfully been employed to generate improved Protein A-derived domains that can be used in the form of multimeric chromatography ligands in antibody purification. An engineered, improved affinity resin should ideally have a higher binding capacity, prolonged lifetime, improved alkaline stability or better elution behavior.

Engineering of Protein A domains for enhanced alkaline stability

Early engineering efforts of the Protein A primary sequence focused on improvements in chemical stability linked to its initial use as an affinity fusion tag. These efforts used the B domain, which can be considered as a consensus sequence of the five domains in

Protein A, as a template to design the synthetic Z domain (Fig. 2.1C) [23]. Both this engineering effort and the comprehensive structural investigation of Protein A-derived domains [18,35,36] have been instrumental for the further development of novel Protein A-based purification matrices.

Improved tolerance to harsh cleaning conditions makes it possible to reuse the expensive resin in additional cycles in industrial applications, which has been a driving force for engineering efforts to improve ligand performance. The highly optimized resins available today allow them to be used in 50−200 product cycles [30]. A recent comprehensive evaluation of twelve commercially available Protein A stationary phases show that several robust options exist where the ligands have been engineered for improved performance relative the wild-type protein [28]. Alkali stabilization is commonly approached via rational, site-directed mutagenesis. Due to the triple helix conformation and hydrophobic core of its domains [35], the wild-type Protein A is already relatively stable at extreme pH conditions and toward exposure to denaturing agents such as urea [26,27]. Tolerance to sodium hydroxide is of particular relevance since it is a preferred cleaning agent for CIP of chromatographic resins. It effectively solubilizes lipids, proteins and nucleic acids and desorbs bound species from chromatographic matrices. Sodium hydroxide also inactivates microorganisms, destroys endotoxins, is inexpensive and can easily be monitored and removed [26]. Early studies demonstrated that wild-type Protein A retains ca. 99% binding capacity when treated with 0.5 M NaOH for 15 min [26,37], which is remarkable for a protein-based agent but still insufficient for repeated use in robust and demanding industrial applications. The most susceptible residue to high pH conditions is asparagine due to its tendency to undergo deamidation or backbone cleavage reactions that are accelerated at high pH [38−40]. Glutamines are also known to be somewhat sensitive to high pH, but to a much lower extent than asparagines [38].

When designing the Z domain to be stable to hydroxylamine cleavage, a particularly alkali-sensitive Asn28-Gly29 motif [41] was simultaneously removed via the Gly29Ala substitution. However, the Z domain contains eight asparagine residues and major protein engineering efforts have focused on substituting these to other residues to obtain more alkali-stable variants. The susceptibility to modification of a given residue is sequence and conformation dependent [41−43] and thus difficult both to predict and to measure in the context of the already relatively stable Protein A sequence. Most engineering efforts have been based on empirical relative comparisons with existing Protein A-based domains or multimeric affinity ligands. The importance of asparagine residues for alkaline sensitivity is illustrated by position 23 in the Protein A domains. All domains have an asparagine residue in this position except for the C domain that has a threonine. This contributes to the C domain having the highest alkali resistance of all wild-type Protein A domains [9]. The inherent alkaline stability of the C domain has inspired its use in a multimeric form in antibody purification [44,45].

A systematic evaluation of the roles of seven of the eight asparagines in the Z domain has indicated that the substitution of Asn23 was most beneficial to increase alkaline tolerance [46]. Asparagine 3 was not included in the study due to its location in an unstructured region close to the N-terminus, and that region can easily be redesigned in multimeric ligands. This study was based on a bypass mutagenesis strategy where a structurally destabilized variant of the Z domain (Phe30Ala [47]) was used to more facilely detect minor differences in stability

that would be challenging to measure using a more stable template domain. In general, only minor effects on affinity and no clear effects on secondary structure content were observed. The binding capacities of a panel of mutants were monitored over 15 consecutive IgG-purification cycles with the Z domain variants coupled to the resin and a 30 min 0.5 M NaOH cleaning regimen used between runs. The data indicated that residues Asn6, Asn11, Asn43 and Asn52 were not important for alkali-dependent loss of binding capacity since their performance was similar to the Phe30Ala mutant alone [46]. A possible explanation for this result is their location in, or in close proximity to, helical regions that are less flexible, and therefore less accessible to modification [41,48,49]. However, Asn43Glu has been linked to somewhat reduced IgG-binding in a later report [50], which illustrates that a complicated interplay between different parameters needs to be considered. The Asn23Thr mutant showed the most significant improvement over Phe30Ala alone and this particular substitution has also been shown to increase the stability of the Z domain, without the destabilizing Phe30Ala mutation, although at the cost of a minor decrease in the binding to IgG [50]. In the context of an optimized domain scaffold for engineered binding proteins based on the Z domain that are referred to as affibody molecules, Asn23Thr is commonly combined with Asn3Ala and Asn6Ala (Fig. 2.2A), where the latter two are mainly related to increased storage stability and minimized risk related to side-chain modifications [50]. In contrast to Asn23Thr, the Asn21Ala substitution led to a clear loss of binding capacity following alkaline exposure versus the control Phe30Ala [46]. The poor performance of the Asn21-variant indicates that this position deserves special attention and its sensitivity might be attributed to its location in an exposed loop region rather than the chemical composition of its side chain. Taken together, these studies illustrate that there are several structurally compatible substitutions that can be considered and that consensus design approaches are well suited for rational engineering purposes. Similar work focusing on histidine, serine, aspartic acid and threonine, which all represent asparagine substitutions represented in wild-type sequences, have for example been used to construct an alkali-stabilized tetrameric variant of the Z domain carrying Asn3His, Asn6Asp and Asn23Ser substitutions [51]. Interestingly, there are also examples where substitutions of non-asparagine residues have been shown to further enhance alkali resistance, which is exemplified by the superior resistance of a domain carrying a Gly29Trp modification over Gly29Ala in the C domain [52].

Milder elution conditions in Protein A-based chromatography

Even though Protein A purification has excellent qualities and is extensively used for initial capture in IgG and Fc-fusion protein purification processes, the acidic pH needed to break the strong interaction between Protein A and antibodies for elution does pose a major drawback. IgG is generally eluted from a Protein A column using citrate, glycine or acetate buffers at a pH around 3.5. The low pH can cause aggregation of the antibody, which is problematic since aggregation can lead to inactivation of the protein or to immunogenic responses [53], and therefore needs to be kept at a minimum. Multiple reports have been published on the subject, showing that low pH exposure is a key determinant for aggregation of antibodies and Fc-fusion proteins [54–57]. The extent of the problem varies from antibody to antibody, and certain subclasses such as IgG4 and IgG2 have been reported to be more

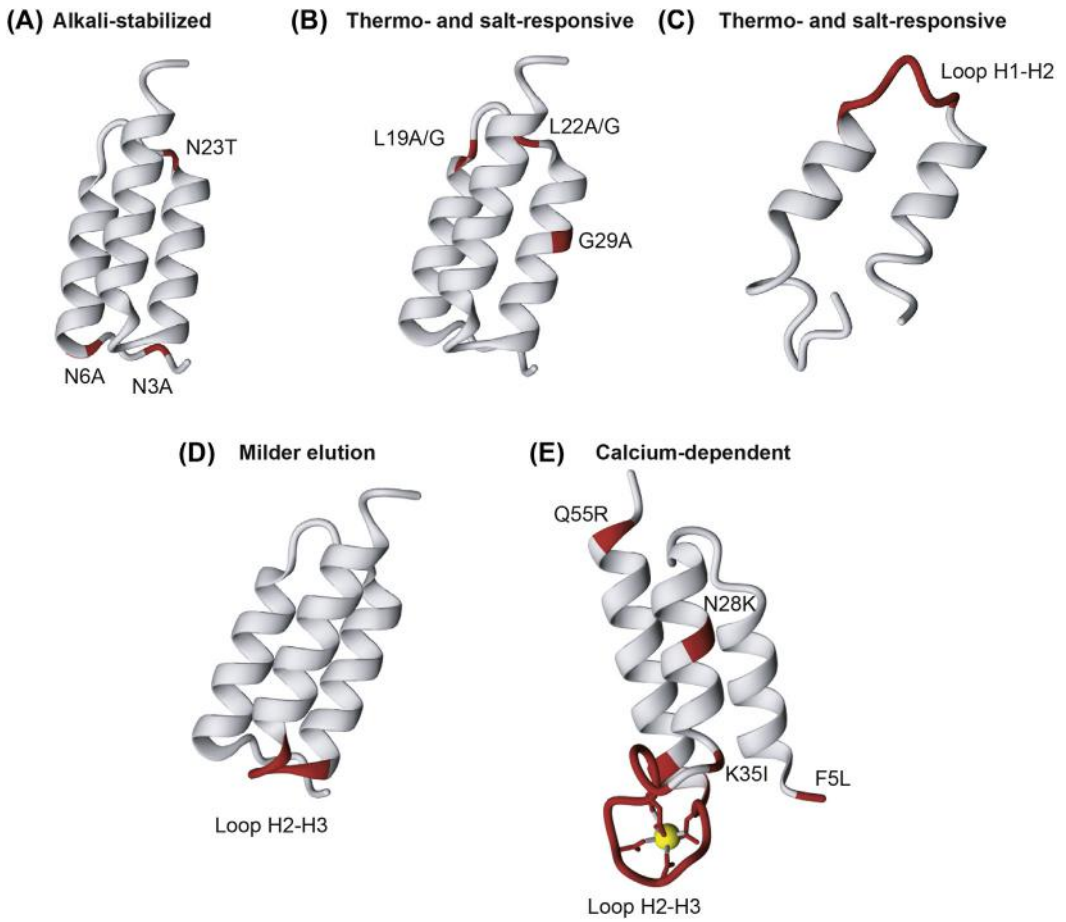

FIG. 2.2 Examples of engineering efforts of Protein A domains in order to improve their properties in regards to alkali stability or elution. Ribbon diagrams are based on 2spz.pdb [24] (A, B and D), 1zda.pdb [83] (C) and 6fgo.pdb [90] (E). (A) Mutation of asparagines at positions 3, 6 and 23 of the Z domain were used to increase the alkali stability of the protein domain. (B) Mutations of the B domain at positions 19, 22 and 29 have rendered the domain thermo- or salt-responsive in its elution of Fc-containing molecules. (C) Exchange of the loop between helix 1 and 2 of a mini-domain based on the Z domain to an elastin turn resulted in a thermo- or salt-responsive binding to IgG. (D) Exchange to or extension with glycines in the loop between helix 2 and 3 of the Z domain resulted in an increased elution pH. (E) Introduction of an EF-hand loop in exchange for the loop between helix 2 and 3 together with mutations at positions 5, 28, 35 and 55 (Z domain numbering) resulted in a calcium-dependent domain able to elute antibodies at pH 5.5 or higher by calcium removal with EDTA. In order to visualize the mutations in Z_{Ca} the domain has been rotated compared to the domains in (A)-(D) .

aggregation prone than others, e.g. IgG1, which appears to be more susceptible to degradation [57–59]. Even though IgG1 is the most commonly used subclass for therapeutic antibodies to date, there are approved therapies based on IgG2 and IgG4 [60]. Research has shown that the purification using Protein A with acidic elution accelerates the aggregation of antibodies compared to only subjecting them to a low pH environment [54,56,61].

Moreover, increasing the salt concentration was reported to increase aggregation further [58,62]. Varying data have been presented regarding the role of buffering agents, where in one case citrate was reported to result in a higher aggregation rate than acetate and glycine [62], while another study indicated that it is the concentration of the buffering agent rather than the buffering agent itself that is the major contributor to protein aggregation [58]. Further, it has been reported that the identity of the base used in the neutralizing step is important for the extent of aggregation [58,63]. Additionally, exposure to elevated temperatures after elution might also influence aggregation, explaining why it is important to limit the hold time of the elution pool at elevated temperatures [54,55,62]. For some antibodies, and especially for acid-labile Fc-fusion proteins, these issues may rule out Protein A chromatography as an option for purification. Therefore, extensive research efforts have been put into engineering of Protein A domains, as well as finding alternative elution buffer compositions in order to raise the pH required for elution from a Protein A-based resin.

It is worth mentioning that the low pH is not only negative but is often included in purification processes as a virus inactivation step. Virus removal is indeed a very important part of the purification process of a biological therapeutic and as previously stated there are reports claiming that the actual chromatography step, more than the low pH itself, has an impact on the rate of aggregation. In that case, a low pH hold after mild elution may be an alternative for virus inactivation, still avoiding protein aggregation. If low pH is to be avoided during the entire purification process, virus clearance may be done by alternative methods such as virus filtration, anion exchange chromatography or by using detergents [3,64].

Several approaches have been described to improve the elution conditions in Protein A chromatography, which can hopefully also expand the scope of Protein A purification for more acid-sensitive antibodies as well as for Fc-containing proteins.

Elution buffer additives for milder elution

One way to increase the pH or decrease the aggregation propensity of the target protein in the elution buffer is to use additives. A common protein refolding promotor is arginine, and addition of 0.5−2 M arginine, or its derivatives acetyl-arginine and agmatine respectively, to the elution buffer has been proposed to allow an increase of the elution pH to 4−4.3 [65,66]. Other suggested additives are 1 M urea, which is said to decrease aggregates, but in the reported case without effect on the pH necessary for elution of the captured antibodies [54]. Ejima et al. could show that the addition of 1−2 M of guanidine hydrochloride could increase the pH needed for elution to 4.3. However, the eluted product showed aggregates, in amounts similar to those after low pH elution. It was also evaluated whether other amino acids as additives such as lysine, proline and glycine could improve elution pH but these amino acids had no effect on the pH necessary for elution of the antibodies [66]. Also addition of propylene glycol, that was supposed to positively affect the aggregation rate, was reported to instead increase the aggregate content for one particular Fc-fusion protein [54]. As can be seen from the examples above, changing the buffer composition can have an effect on elution pH and/or aggregation rate during purification, but the effects may be difficult to predict. While these different additives may be beneficial in the purification of certain aggregation-prone antibodies or Fc-fusion proteins, the effects may differ depending on the protein to

be purified and need to be assessed for each purification process. Although arginine addition has been shown to increase the pH needed for elution, concentrations as high as 2 M would be very costly in a large-scale purification process.

Structural engineering for improved elution

Due to the high affinity of the interaction between Protein A and IgG, the low pH required for elution of antibodies from Protein A resins could possibly be altered by lowering this affinity. Position Gly29 has been shown to be involved in the interaction with V_H3-containing antibody variable domains [67] and upon substitution to alanine a significant loss of Fab-binding occurs [68]. This effect is the basis for a common argument to use ligands based on the Z domain rather than wild-type Protein A since the lack of binding to the variable region enables more homogenous elution at marginally higher pH [52,69,70]. Therefore, several substitutions of Gly29 have been explored to further decrease the Fab-binding; particular focus has been on Gly29Lys, Gly29Arg and Gly29Leu [71]. In an effort to expand on this advantage of Z domain-derived ligands over wild-type Protein A, rational mutants supposed to further decrease V_H3-binding have also been computationally designed by combining Gly29Ala, Ser33Glu and Asp36Arg substitutions in an inherently alkali-stable C domain template [72]. Such subtle modifications of the binding interface can be useful for modification of the column elution conditions. Moreover, two variants of the alkali-stabilized tetrameric ligand used in MabSelect Sure™ matrices have been shown to allow elution at about 0.5 pH units higher than when using the original domain. In the first version His18Ser was evaluated and in the second Asn28Ala was also included, both mutations targeting positions involved in the binding of the B/Z domain to Fc [18,73]. This example again illustrates that minor modifications of the ligand can result in process improvements while retaining the yield [74].

More extensive engineering efforts have been made to allow for milder elution in Protein A purification. The use of truncated variants of Protein A domains represents an early approach to enable elution at a higher pH [75]. A rational method to decrease the required pH for elution is to incorporate histidine residues in the binding site to electrostatically interfere with residues within the Fc when the pH is reduced [76,77]. Tsukamoto et al. reported an additional promising example where a variant of the B domain carrying a Asp36His substitution displayed a 100-fold decrease in affinity to IgG at pH 5.0 compared to the wild-type B domain and thereby allows for elution at higher pH [78].

Koguma et al. have presented a variant of the B domain of Protein A, in which they have introduced mutations in order to decrease the hydrophobicity of the amino acid side chains buried inside the domain, and by doing so decreasing the overall stability [79]. This was done by mutating the leucines at positions 19 and 22 to alanines or glycines. Additionally, the molecule included the Gly29Ala mutation also present in the Z domain (Fig. 2.2B) [80]. These mutations have rendered the protein domain temperature-dependent, in such a way that IgG can be bound at temperatures below 10 °C and released upon raising the temperature to 40 °C [79,81]. While this invention is promising in order to be able to elute antibodies at neutral pH, the need to raise the temperature to 40 °C can be suboptimal, both due to possible product instabilities at elevated temperatures and the inconvenience to perform such a

temperature increase in a large-scale process. As an alternative, elution is also possible using a 1 M NaCl pulse, which may be a better route for elution in a large-scale process rather than raising the temperature [81].

Loop engineering

A few endeavors have focused on engineering of the loops of the Protein A domains. Reiersen and Rees [82] used a minidomain [83] based on the B domain, only containing helices 1 and 2, stabilized by a disulfide bond. The connecting loop between the helices (HDPNLN, residues 19–24 in 1zda.pdb [83]) was switched to a sequence derived from a typical elastin turn (GVPGVG) (Fig. 2.2C). This resulted in a protein variant that could bind IgG at room temperature, and enabled elution at cold temperatures, or by removing salt from the column by pure water, as the engineered mini-domain showed a salt-induced folding in the presence of sodium sulfate [82,84]. More recent applications have demonstrated affinity precipitation of antibodies using various designs of stimuli-responsive elastin-like polypeptides fused to, for example, the Z domain or dimer of the Z domain [85,86]. This approach shows an alternative to circumvent the traditional chromatographic approach to enable less expensive antibody purification. Although promising, these approaches are currently not broadly used in industry.

Other work have instead focused on engineering of the other loop in the Z domain, the four amino acid loop (DPSQ, residues 37–40 in consensus sequence (Fig. 2.1C)) between helix two and three (Fig. 2.2D). By extending the loop with six glycine residues, or by exchanging the four residues for glycines, Gülich et al. could destabilize the domain resulting in a lower affinity to IgG. This made it possible to elute IgG at a pH of 4.5 from columns coupled to the engineered domains, i.e. ten times less acidic than what is traditionally used in Protein A chromatography [87].

Kanje et al. have used another engineering approach, utilizing the calcium coordinating properties of the EF-hand loop in order to make the Z domain dependent on calcium for its binding to IgG. A library of calcium-binding loops based on sequences from naturally occurring EF-hand motifs was introduced in exchange for the DPSQ loop between helices two and three, together with randomizations of positions known to be important for the hydrophobic core. Through phage display selection and error-prone PCR, a variant of the Z domain displaying a calcium-dependent binding to IgG was developed, named Z_{Ca}. This domain has a loop, consisting of 12 amino acids, that coordinates calcium in the same way as the canonical EF-hand loops [88,89] (Fig. 2.2E) and binds to IgG in a similar manner as its parent domain. Furthermore, in addition to the incorporated loop, Z_{Ca} has several substitutions (Phe5Leu, Asn28Lys and Lys35Ile) in positions known to interact with Fc [18,73], as well as the substitution Gln55Arg, a position proposed to form an intramolecular hydrogen bond [50]. This protein variant has been shown to release polyclonal human IgG at pH 5.5 in a calcium-dependent manner by depletion of the bound calcium ion using EDTA. The novel Z_{Ca} matrix was shown to have a similar binding capacity at saturation as a comparable parental Z domain matrix [90]. Recent data demonstrate that Z_{Ca} can be multimerized and that the dynamic binding capacity of the molecule, at 5 min residence time, increases upon multimerization. Remarkably, a tetrameric Z_{Ca} allows elution of IgG2

and IgG4 at neutral pH [91]. However, a possible improvement of the domain for CIP treatment needs to be investigated as the molecule is currently not as alkali resistant as other optimized Protein A variants. Still this domain is very promising for acid-sensitive Fc-containing molecules that can now be purified using higher, or even neutral, elution pH while maintaining the great properties of the parent Z domain when it comes to specificity and binding capacity.

Improving the binding capacity of Protein A resins

Since therapeutic mAbs are used to treat a variety of diseases with large patient populations requiring repeated doses of the sought-after drug, there are large product quantity goals to be met [92]. Continuous advances in upstream processing, such as optimization of growth media, cell feeding strategies and most importantly selection of a suitable cell line, have substantially increased mAb expression levels over the years [93]. As a result, ways to increase the productivity downstream to handle the higher cell culture titers have also been developed. To optimize the expensive but crucial Protein A chromatography step, focus has been on improving the Protein A resin binding capacity. Great progress has been made since Protein A media was first used in the 1970s, and in recent years dynamic binding capacities (DBCs) have increased markedly as more advanced Protein A resins have been made commercially available by a variety of manufacturers [94,95]. The higher capacity resins have the potential to reduce buffer consumption, minimize column sizes and require smaller amounts of valuable Protein A media which could have a large impact on process economics. As a consequence, the current demand for vast amounts of mAbs can be realized more easily at a more affordable price.

Approaches to improve the binding capacity include the choice of coupling chemistry, ligand multimerization and other spacer technologies as well as adjusting the ligand density and particle/pore size. There are different types of binding capacities and the mentioned factors can have effects on either the static or the dynamic binding capacity of a chromatography resin, or ultimately both [28]. The static binding capacity is a measure of the maximum amount of protein that can bind to the resin disregarding any excess protein loss. DBC is measured under operating conditions before a certain protein breakthrough and takes into account the flow rate and thus mass transfer properties related to the chromatographic support [96]. The static binding capacity is therefore never lower than the DBC but in most cases significantly higher because of mass transfer limitations [95,97].

Base matrices and ligand density

Modification of base matrix properties such as the particle size is often employed by manufacturers, but will only be briefly mentioned here. Decreasing the particle size has been shown to have a significant influence on the DBC by providing faster mass transfer, though the generation of a larger pressure drop has to be factored in as well [28,98]. Another general material property that is often altered to achieve higher DBC values is the pore size. By increasing the pore size, the space available for antibody binding to take place is increased

and steric hindrances can be minimized. This is especially important in the case of antibody purification since antibodies are large molecules with low pore diffusion rates. However, larger pore sizes reduce total resin surface area and result in a lower static binding capacity [97]. In the case of GE Healthcare's resin MabSelect Xtra™, which has an increased pore size compared to the earlier MabSelect™, the loss in static binding capacity was counteracted by increasing the ligand density. Naturally, a higher density of Protein A ligands coupled to the resin causes an increase in the static binding capacity, although only to a certain point after which intermolecular steric effects impede further capacity gains. Ghose et al. showed that significantly lower ligand densities than those normally used for commercial matrices can be used to achieve the same static binding capacity because of steric effects between the adjacent ligands at densities close to commercial [92]. MabSelect SuRe™ LX, a high ligand density resin based on the same alkali-stable ligand used in MabSelect SuRe™, is also suggested to give rise to serious inter-ligand steric effects affecting the binding capacity [30]. Ligand utilization is drastically reduced for these resins and may negatively impact process economics [92].

Ligand multimerization

Beyond modifications of the base matrix to which the ligand is attached and the number of ligands attached to the surface, engineering of the Protein A ligand itself has had substantial effects on the binding capacity. To provide more antibody binding sites, connecting a number of Protein A domains and forming longer ligands has become a common tool for increasing resin binding capacities [95,99–102]. The additional binding sites have been shown to increase the static binding capacity, at least up to an octamer of B domains. In solution, a nonamer of B domains showed the most promising results when Freiherr von Roman and Berensmeier [96] investigated the relationship between the number of antibody-binding domains of individual ligands and their binding performance. Ranging from a dimer up to a nonamer, a fairly linear dependency was observed when the ligands were not coupled to a solid support. However, measurements in solution do not reflect the true conditions of Protein A chromatography. The ligand density on an acrylamido-based stationary phase reached a maximum for a certain number of repeated domains of each site-specifically coupled ligand and subsequently dropped for higher order multimers, most likely as a consequence of the increased steric hindrance. In this case, the substantially reduced ligand density for a nonameric ligand compared to an octamer resulted in a decrease of both the static binding capacity and DBC, rendering the octamer the most promising candidate for antibody purification on this chromatographic support [97]. Even if ligand densities would have been optimized for these specific resins, no major capacity increases would likely have been observed since the nonamer was the largest ligand that they could successfully produce and purify.

Positive effects seen on DBCs as a result of ligand multimerization can also be caused by the extended length of the ligand, which can function as a spacer from the chromatographic support. Besides providing more binding sites, the ligand becomes more accessible for antibody binding, which could facilitate mass transport during purification. However, longer residence times may be required in order to observe the maximum differences in DBC for long multimeric ligands [95]. Recently, increased accessibility alone was suggested to affect binding capacities for some ligands when multimeric variants of the previously described

Z domain equipped with a calcium-binding loop were compared using surface plasmon resonance. A large difference in binding capacity between a dimer and a trimer could be explained by the additional functional site for binding, whereas smaller capacity differences between a mono- and dimer and a tri- and tetramer, respectively, indicated that the added domains were not able to bind to another antibody molecule but might instead be more accessible and reduce mass transfer resistance which allows antibody binding to more ligands [91]. This reasoning is supported by several studies showing that each of the five domains of Protein A cannot simultaneously bind to one antibody each even in solution [92,103]. Intramolecular steric effects appear to block some domains from simultaneous binding and is consequently also a factor that needs to be taken into account when developing a Protein A chromatography resin [92].

Optimization of the linker regions

To prevent intraligand steric effects and achieve DBC values closer to the static binding capacities, the flexibility and accessibility of the binding sites of the ligands can be optimized by introducing linkers between the domains of the multimeric proteins [97,104]. Linker lengths ranging from zero to 15 amino acids have been suggested by Hober and Johansson among others. The N− and C-terminal ends of two domains can thus be linked directly or spaced apart by a number of amino acids which should be carefully chosen to avoid destabilization of the protein [101,104]. Due to its unique structure and ability to form turns in a protein, prolines should not be included for maintaining flexibility. For example, Björkman and Rodrigo showed the effect of substituting the C-terminal proline (Pro57) present in the Z domain and Protein A to an isoleucine. Higher observed DBCs were attained as a consequence of increased flexibility of the individual domains. It is possible that the same proline is responsible for the intraligand steric hindrance that prevents the domains from simultaneous binding to one antibody each and that the substitution of this amino acid can increase domain usage in all multimeric ligands [105]. Freiherr von Roman and Berensmeier kept the proline but applied an isoleucine as an amino acid linker between the domains when studying a spectrum of multimeric Protein A ligands [97]. In general, long amino acid sequences introduced into the ligand should be assessed for sensitivity to proteases that could be present in the sample [106]. When choosing a linker, one should also consider the influence on resin stability to alkaline conditions by e.g. excluding alkali-susceptible residues such as asparagine and glutamine [105].

Multimeric ligands may not only come with advantages but can also be accompanied by some problems. Other critical aspects of Protein A chromatography beyond DBC, for instance purity and recovery, could be jeopardized by the increased surface area of longer multimeric ligands which could also create more sites for non-specific interactions. The reduction of impurities like HCPs has been observed to be less efficient for some resins with higher binding capacities. However, no significant differences were observed by Müller and Vajda in HCP removal and recovery when comparing two Protein A resins with a tetrameric or hexameric ligand from Tosoh in a recent study [95]. Pabst et al. also observed fairly consistent HCP levels for a number of Protein A stationary phases with varying binding capacities. However, depending on the mAb that was purified, differences in HCP levels within each stationary phase were observed [28].

Ligand coupling

To further enhance the accessibility of chromatography ligands and improve DBCs, the ligand coupling to the matrix can be optimized. There are a variety of ways of coupling a recombinant Protein A ligand to a matrix. For early Protein A stationary phases, the ligand was commonly immobilized onto the matrix covalently via amine coupling [28]. The utilization of primary amino groups to attach the ligand to the matrix leads to a heterogeneous immobilization with the risk of reduced binding site accessibility at the coupled positions [103]. Since then, directed ligand immobilization rather than random coupling has been shown to improve binding capacities. Site-specific coupling chemistry by utilizing a terminal cysteine residue has been shown to be an efficient and highly reproducible strategy that is commonly used by manufacturers today [95,97,99,101,107,108]. Cysteine is a unique amino acid with its side chain thiol group that can form a stable thioether bond or disulfide bridge. The cysteine can either be placed N-terminally or C-terminally in the protein, alone or combined with a short linker to act as a spacer from the resin backbone to facilitate the coupling reaction and possibly also increase ligand mobility and accessibility. The linker should preferably have similar alkali-resistance as the ligand [99]. Already in 1989, directed immobilization via a cysteine as part of a 19-amino-acid linker C-terminally of the Z domain was investigated and revealed a similar binding capacity to commercial wild-type Protein A resins at the time, even though the ligand density in this study was tenfold lower. Inserting the cysteine at the C-terminus is especially useful to obtain functionally active ligands since successfully immobilized ligands will be full-length [103]. Regarding the selection of amino acids for the linker, lysine and histidine residues were suggested by Ander et al. [99] A Lys-Cys-Lys tag was incorporated C-terminally by Freiherr von Roman and Berensmeier when coupling multimeric versions of the B domain of Protein A to a solid support via thiol chemistry and compared to coupling of the same ligands without the cysteine. For the hexamer, the static binding capacity was increased by as much as 41% through site-directed coupling. For an octamer a smaller increase was observed, which was presumably due to the fact that binding capacities were already near the theoretical maximum values [97]. Methods of coupling cysteine-equipped ligands to resin surfaces have exploited a number of different sulfhydryl-reactive chemical groups, such as maleimide or epoxide groups [97,107]. Further approaches to increase the distance of the Protein A ligand from the stationary phase and improve ligand availability have been explored. Recently, a dextran-grafted agarose-based matrix combined with site-specific thiol immobilization showed improved binding capacities over non-grafted media [108].

All things considered, a combination of ligand polymerization and site-directed coupling chemistry is an established approach among manufacturers to increase the binding capacity of Protein A ligands. However, it is important to keep in mind that the binding capacity varies depending on the protein to be purified and the conditions used for purification. For example, Fc-fusion proteins of lower molecular weight than mAbs but with the same Fc domain have consistently shown lower binding capacities on the same Protein A resin [92]. Within biopharmaceutical production, it is fairly common that a range of therapeutic mAbs and Fc-fusion proteins are purified using the same Protein A stationary phase to simplify process development. This approach poses a risk of product loss since purification processes using Protein A media often are run close to the conditions for the specified binding

capacity to minimize resin costs [92]. Independent of stated binding capacity, a Protein A resin should be selected and evaluated for its specific application. Further, high DBC is not the sole factor of an efficient purification process since varying residence times also play a role in the process productivity [109].

Summary

Protein A-based affinity chromatography has, for a long time, been by far the most used method for industrial antibody and Fc-fusion protein purification. Thus, there has been a large interest in understanding and improving Protein A as well as its matrices for better performance and longer life span. In this chapter we have described some of the efforts that have been put into the development and understanding of Protein A and some of its IgG-binding domains. One of the most important features of industrially used affinity matrices is high stability in the range of environments where it is utilized. Regarding Protein A, this includes proteolytic resistance, alkaline stability and endurance to low pH. Due to the high conformational stability of Protein A, its inherent tolerance to different, fairly harsh conditions, is rather high. Despite this, there has been a demand for an even more alkali-stable protein ligand, to allow for safe CIP while maintaining a long lifetime of the matrix. This has led to further, and very successful, development of a number of new variants that show even higher alkaline stability than the original protein. Furthermore, the low pH used for elution of the bound antibodies or Fc-fused proteins from Protein A matrices can be detrimental to the target protein due to loss of activity and/or formation of aggregates. Hence, a number of different approaches have been taken to permit milder, higher pH elution. Different additives in the elution buffer have been evaluated with variable success. Moreover, various engineering efforts of the Protein A domains have focused on reducing the harshness of the elution conditions in Protein A chromatography. This has been achieved by introducing mutations in the binding site of the affinity ligand or its hydrophobic core, or by destabilization of the domain by loop engineering to enable elution at higher pH.

Another important feature of a protein purification matrix is the required residence time, tolerated flowrates and binding capacity of the matrix under operational conditions. In order to achieve optimal resin performance, a lot of effort has been put into the development of the matrix and its properties as well as the methods for covalent coupling of the protein ligands to the matrix. Furthermore, the protein density at the surface of the matrix has been investigated in order to reach the highest binding capacity possible without inducing precipitation during the elution step. Also, the number of interconnected IgG-binding domains as well as the length of the spacer between them have been investigated. Here it has been important to achieve enough flexibility for optimal binding without introducing vulnerability to proteases or extreme pH.

In summary, many innovative approaches have been devised to engineer novel variants of Protein A with refined properties for improved antibody and Fc-fused protein purification. Several of these approaches have been successful and have led to a number of new marketed products. Despite this, we foresee that the development of novel Protein A alternatives to further enhance its properties will continue and new innovative approaches will be investigated in the future.

References

[1] Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. mAbs 2015;7(1):9–14.
[2] Roque AC, Silva CS, Taipa MA. Affinity-based methodologies and ligands for antibody purification: advances and perspectives. J Chromatogr A 2007;1160(1–2):44–55.
[3] Liu HF, Ma J, Winter C, Bayer R. Recovery and purification process development for monoclonal antibody production. mAbs 2010;2(5):480–99.
[4] Smejkal B, Agrawal NJ, Helk B, et al. Fast and scalable purification of a therapeutic full-length antibody based on process crystallization. Biotechnol Bioeng 2013;110(9):2452–61.
[5] Tscheliessnig A, Satzer P, Hammerschmidt N, Schulz H, Helk B, Jungbauer A. Ethanol precipitation for purification of recombinant antibodies. J Biotechnol 2014;188:17–28.
[6] Mao LN, Rogers JK, Westoby M, Conley L, Pieracci J. Downstream antibody purification using aqueous two-phase extraction. Biotechnol Prog 2010;26(6):1662–70.
[7] Murphy C, Devine T, O'Kennedy R. Technology advancements in antibody purification. Antib Technol J 2016;6:17–32.
[8] Gagnon P. Technology trends in antibody purification. J Chromatogr A 2012;1221:57–70.
[9] Bolton GR, Mehta KK. The role of more than 40 years of improvement in protein A chromatography in the growth of the therapeutic antibody industry. Biotechnol Prog 2016;32(5):1193–202.
[10] Boström T, Nilvebrant J, Hober S. Purification systems based of bacterial surface proteins. In: Protein purification. IntechOpen; 2012.
[11] Bjorck L, Kronvall G. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J Immunol 1984;133(2):969–74.
[12] Sauer-Eriksson AE, Kleywegt GJ, Uhlen M, Jones TA. Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. Structure 1995;3(3):265–78.
[13] Derrick JP, Wigley DB. Crystal structure of a streptococcal protein G domain bound to an Fab fragment. Nature 1992;359(6397):752–4.
[14] Nilson BH, Solomon A, Bjorck L, Akerstrom B. Protein L from Peptostreptococcus magnus binds to the kappa light chain variable domain. J Biol Chem 1992;267(4):2234–9.
[15] Eliasson M, Olsson A, Palmcrantz E, et al. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. J Biol Chem 1988;263(9):4323–7.
[16] Kihlberg BM, Sjobring U, Kastern W, Bjorck L. Protein LG: a hybrid molecule with unique immunoglobulin binding properties. J Biol Chem 1992;267(35):25583–8.
[17] Svensson HG, Hoogenboom HR, Sjobring U. Protein LA, a novel hybrid protein with unique single-chain Fv antibody- and Fab-binding properties. Eur J Biochem 1998;258(2):890–6.
[18] Deisenhofer J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-A resolution. Biochemistry 1981;20(9):2361–70.
[19] Hillson JL, Karr NS, Oppliger IR, Mannik M, Sasso EH. The structural basis of germline-encoded VH3 immunoglobulin binding to staphylococcal protein A. J Exp Med 1993;178(1):331–6.
[20] Goodyear CS, Silverman GJ. Death by a B cell superantigen: in vivo VH-targeted apoptotic supraclonal B cell deletion by a Staphylococcal Toxin. J Exp Med 2003;197(9):1125–39.
[21] Lofdahl S, Guss B, Uhlen M, Philipson L, Lindberg M. Gene for staphylococcal protein A. Proc Natl Acad Sci U S A 1983;80(3):697–701.
[22] Tashiro M, Montelione GT. Structures of bacterial immunoglobulin-binding domains and their complexes with immunoglobulins. Curr Opin Struct Biol 1995;5(4):471–81.
[23] Nilsson B, Moks T, Jansson B, et al. A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng 1987;1(2):107–13.
[24] Tashiro M, Tejero R, Zimmerman DE, Celda B, Nilsson B, Montelione GT. High-resolution solution NMR structure of the Z domain of staphylococcal protein A. J Mol Biol 1997;272(4):573–90.
[25] Ultsch M, Braisted A, Maun HR, Eigenbrot C. 3-2-1: structural insights from stepwise shrinkage of a three-helix Fc-binding domain to a single helix. Protein Eng Des Sel 2017;30(9):619–25.
[26] Hale G, Drumm A, Harrison P, Phillips J. Repeated cleaning of protein A affinity column with sodium hydroxide. J Immunol Methods 1994;171(1):15–21.

[27] Shukla AA, Hinckley P. Host cell protein clearance during protein A chromatography: development of an improved column wash step. Biotechnol Prog 2008;24(5):1115−21.
[28] Pabst TM, Thai J, Hunter AK. Evaluation of recent Protein A stationary phase innovations for capture of biotherapeutics. J Chromatogr A 2018;1554:45−60.
[29] Hahn R, Schlegel R, Jungbauer A. Comparison of protein A affinity sorbents. J Chromatogr B Analyt Technol Biomed Life Sci 2003;790(1−2):35−51.
[30] Liu Z, Mostafa SS, Shukla AA. A comparison of protein A chromatographic stationary phases: performance characteristics for monoclonal antibody purification. Biotechnol Appl Biochem 2015;62(1):37−47.
[31] Pfaunmiller EL, Paulemond ML, Dupper CM, Hage DS. Affinity monolith chromatography: a review of principles and recent analytical applications. Anal Bioanal Chem 2013;405(7):2133−45.
[32] Boi C. Membrane adsorbers as purification tools for monoclonal antibody purification. J Chromatogr B Analyt Technol Biomed Life Sci 2007;848(1):19−27.
[33] Farid SS. Process economics of industrial monoclonal antibody manufacture. J Chromatogr B Analyt Technol Biomed Life Sci 2007;848(1):8−18.
[34] Love JC, Love KR, Barone PW. Enabling global access to high-quality biopharmaceuticals. Current Opinion in Chemical Engineering 2013;2(4):383−90.
[35] Gouda H, Torigoe H, Saito A, Sato M, Arata Y, Shimada I. Three-dimensional solution structure of the B domain of staphylococcal protein A: comparisons of the solution and crystal structures. Biochemistry 1992;31(40):9665−72.
[36] Deis LN, Wu Q, Wang Y, et al. Suppression of conformational heterogeneity at a protein-protein interface. Proc Natl Acad Sci U S A 2015;112(29):9028−33.
[37] Girot P, Moroux Y, Duteil XP, Nguyen C, Boschetti E. Composite affinity sorbents and their cleaning in place. J Chromatogr 1990;510:213−23.
[38] Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. J Biol Chem 1987;262(2):785−94.
[39] Palmer B, Angus K, Taylor L, Warwicker J, Derrick JP. Design of stability at extreme alkaline pH in streptococcal protein G. J Biotechnol 2008;134(3−4):222−30.
[40] Gulich S, Linhult M, Stahl S, Hober S. Engineering streptococcal protein G for increased alkaline stability. Protein Eng 2002;15(10):835−42.
[41] Kosky AA, Razzaq UO, Treuheit MJ, Brems DN. The effects of alpha-helix on the stability of Asn residues: deamidation rates in peptides of varying helicity. Protein Sci 1999;8(11):2519−23.
[42] Kossiakoff AA. Tertiary structure is a principal determinant to protein deamidation. Science 1988;240(4849):191−4.
[43] Lura R, Schirch V. Role of peptide conformation in the rate and mechanism of deamidation of asparaginyl residues. Biochemistry 1988;27(20):7671−7.
[44] Zhao L, Wei W, Huang Y, et al. A novel rProtein A chromatographic media for enhancing cleaning-in-place performance. J Immunol Methods 2018;460:45−50.
[45] Hall M, Larsson S, Muranyi A, Rodrigo G, Zou J, Aberg P-M, Inventors, GE H, Bioprocess R&D, AB, assignee. Chromatography ligand comprising domain C from *Staphylococcus aureus* protein A for antibody isolation. Patent US9290549B2. 2016.
[46] Linhult M, Gulich S, Graslund T, et al. Improving the tolerance of a protein a analogue to repeated alkaline exposures using a bypass mutagenesis approach. Proteins 2004;55(2):407−16.
[47] Cedergren L, Andersson R, Jansson B, Uhlen M, Nilsson B. Mutational analysis of the interaction between staphylococcal protein A and human IgG1. Protein Engineering 1993;6(4):441−8.
[48] Patel K, Borchardt RT. Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. Pharm Res (N Y) 1990;7(7):703−11.
[49] Robinson NE. Protein deamidation. Proc Natl Acad Sci U S A 2002;99(8):5283−8.
[50] Feldwisch J, Tolmachev V, Lendel C, et al. Design of an optimized scaffold for affibody molecules. J Mol Biol 2010;398(2):232−47.
[51] Li SC, Li X, Voss C, et al. Inventors; University of Western Ontario JSR Corp, assignee. Alkali-resistant variants of protein A and their use in affinity chromatography. Patent US9051375B2. 2015.
[52] Minakuchi K, Murata D, Okubo Y, Nakano Y, Yoshida S. Remarkable alkaline stability of an engineered protein A as immunoglobulin affinity ligand: C domain having only one amino acid substitution. Protein Sci 2013;22(9):1230−8.

[53] Arosio P, Barolo G, Muller-Spath T, Wu H, Morbidelli M. Aggregation stability of a monoclonal antibody during downstream processing. Pharm Res (N Y) 2011;28(8):1884–94.
[54] Shukla AA, Gupta P, Han X. Protein aggregation kinetics during Protein A chromatography. Case study for an Fc fusion protein. J Chromatogr A 2007;1171(1–2):22–8.
[55] Ejima D, Tsumoto K, Fukada H, et al. Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. Proteins 2007;66(4):954–62.
[56] Mazzer AR, Perraud X, Halley J, O'Hara J, Bracewell DG. Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold. J Chromatogr A 2015;1415:83–90.
[57] Liu B, Guo H, Xu J, et al. Acid-induced aggregation propensity of nivolumab is dependent on the Fc. mAbs 2016;8(6):1107–17.
[58] Hari SB, Lau H, Razinkov VI, Chen S, Latypov RF. Acid-induced aggregation of human monoclonal IgG1 and IgG2: molecular mechanism and the effect of solution composition. Biochemistry 2010;49(43):9328–38.
[59] Ito T, Tsumoto K. Effects of subclass change on the structural stability of chimeric, humanized, and human antibodies under thermal stress. Protein Sci 2013;22(11):1542–51.
[60] Kretschmer A, Schwanbeck R, Valerius T, Rosner T. Antibody isotypes for tumor immunotherapy. Transfus Med Hemotherapy 2017;44(5):320–6.
[61] Gagnon P, Nian R, Leong D, Hoi A. Transient conformational modification of immunoglobulin G during purification by protein A affinity chromatography. J Chromatogr A 2015;1395:136–42.
[62] Joshi V, Shivach T, Kumar V, Yadav N, Rathore A. Avoiding antibody aggregation during processing: establishing hold times. Biotechnol J 2014;9(9):1195–205.
[63] Potty A, Xenopoulos A. Stress-induced antibody aggregates. BioProcess Int 2013;11(3):44–52.
[64] Roberts PL. Virus elimination during the purification of monoclonal antibodies by column chromatography and additional steps. Biotechnol Prog 2014;30(6):1341–7.
[65] Arakawa T, Philo JS, Tsumoto K, Yumioka R, Ejima D. Elution of antibodies from a Protein-A column by aqueous arginine solutions. Protein Expression and Purification 2004;36(2):244–8.
[66] Ejima D, Yumioka R, Tsumoto K, Arakawa T. Effective elution of antibodies by arginine and arginine derivatives in affinity column chromatography. Anal Biochem 2005;345(2):250–7.
[67] Graille M, Stura EA, Corper AL, et al. Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. Proc Natl Acad Sci U S A 2000;97(10):5399–404.
[68] Ljungberg UK, Jansson B, Niss U, Nilsson R, Sandberg BE, Nilsson B. The interaction between different domains of staphylococcal protein A and human polyclonal IgG, IgA, IgM and F(ab')2: separation of affinity from specificity. Mol Immunol 1993;30(14):1279–85.
[69] Hakoda M, Kamatani N, Hayashimoto-Kurumada S, et al. Differential binding avidities of human IgM for staphylococcal protein A derive from specific germ-line VH3 gene usage. J Immunol 1996;157(7):2976–81.
[70] Ghose S, Allen M, Hubbard B, Brooks C, Cramer SM. Antibody variable region interactions with Protein A: implications for the development of generic purification processes. Biotechnol Bioeng 2005;92(6):665–73.
[71] Spector S, Inventor; EMD Millipore Corp, assignee. Immunoglobulin-binding proteins with improved specificity. Patent US8592555B2. 2013.
[72] Yoshida S, Murata D, Taira S, et al. Rational design and engineering of protein A to obtain the controlled elution profile in monoclonal antibody purification. Chem Bio Inf J 2012;12:1–13.
[73] Jendeberg L, Persson B, Andersson R, Karlsson R, Uhlen M, Nilsson B. Kinetic analysis of the interaction between protein A domain variants and human Fc using plasmon resonance detection. J Mol Recogn 1995;8(4):270–8.
[74] Pabst TM, Palmgren R, Forss A, et al. Engineering of novel Staphylococcal Protein A ligands to enable milder elution pH and high dynamic binding capacity. J Chromatogr A 2014;1362:180–5.
[75] Bottomley SP, Sutton BJ, Gore MG. Elution of human IgG from affinity columns containing immobilised variants of protein A. J Immunol Methods 1995;182(2):185–92.
[76] Watanabe H, Matsumaru H, Ooishi A, et al. Optimizing pH response of affinity between protein G and IgG Fc: how electrostatic modulations affect protein-protein interactions. J Biol Chem 2009;284(18):12373–83.
[77] Watanabe H, Matsumaru H, Ooishi A, Honda S. Structure-based histidine substitution for optimizing pH-sensitive Staphylococcus protein A. J Chromatogr B Analyt Technol Biomed Life Sci 2013;929:155–60.

[78] Tsukamoto M, Watanabe H, Ooishi A, Honda S. Engineered protein A ligands, derived from a histidine-scanning library, facilitate the affinity purification of IgG under mild acidic conditions. J Biol Eng 2014;8:15.
[79] Koguma I, Yamashita S, Sato S, Okuyama K, Katakura Y. Novel purification method of human immunoglobulin by using a thermo-responsive protein A. J Chromatogr A 2013;1305:149–53.
[80] Sato S. Inventor; Nomadic Bioscience Co Ltd, assignee. Polypeptide, an affinity chromatography material, and a method for separating and/or purifying immunoglobulin. Patent US8198409B2. 2012.
[81] Krepper W, Satzer P, Beyer BM, Jungbauer A. Temperature dependence of antibody adsorption in protein A affinity chromatography. J Chromatogr A 2018;1551:59–68.
[82] Reiersen H, Rees AR. An engineered minidomain containing an elastin turn exhibits a reversible temperature-induced IgG binding. Biochemistry 1999;38(45):14897–905.
[83] Starovasnik MA, Braisted AC, Wells JA. Structural mimicry of a native protein by a minimized binding domain. Proc Natl Acad Sci U S A 1997;94(19):10080–5.
[84] Reiersen H, Rees AR. Sodium sulphate reactivates a protein A minidomain with a short elastin beta-turn. Biochem Biophys Res Commun 2000;276(3):899–904.
[85] Madan B, Chaudhary G, Cramer SM, Chen W. ELP-z and ELP-zz capturing scaffolds for the purification of immunoglobulins by affinity precipitation. J Biotechnol 2013;163(1):10–6.
[86] Swartz AR, Xu X, Traylor SJ, Li ZJ, Chen W. One-step affinity capture and precipitation for improved purification of an industrial monoclonal antibody using Z-ELP functionalized nanocages. Biotechnol Bioeng 2018;115(2):423–32.
[87] Gulich S, Uhlen M, Hober S. Protein engineering of an IgG-binding domain allows milder elution conditions during affinity chromatography. J Biotechnol 2000;76(2–3):233–44.
[88] Gifford JL, Walsh MP, Vogel HJ. Structures and metal-ion-binding properties of the Ca2+-binding helix-loop-helix EF-hand motifs. Biochem J 2007;405(2):199–221.
[89] Marsden BJ, Shaw GS, Sykes BD. Calcium binding proteins. Elucidating the contributions to calcium affinity from an analysis of species variants and peptide fragments. Biochem Cell Biol 1990;68(3):587–601.
[90] Kanje S, Venskutonyte R, Scheffel J, Nilvebrant J, Lindkvist-Petersson K, Hober S. Protein engineering allows for mild affinity-based elution of therapeutic antibodies. J Mol Biol 2018;430(18 Pt B):3427–38.
[91] Scheffel J, Kanje S, Borin J, Hober S. Optimization of a calcium-dependent Protein A-derived domain for mild antibody purification. mAbs 2019:1–10.
[92] Ghose S, Hubbard B, Cramer SM. Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials. Biotechnol Bioeng 2007;96(4):768–79.
[93] Huang YM, Hu W, Rustandi E, Chang K, Yusuf-Makagiansar H, Ryll T. Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. Biotechnol Prog 2010;26(5):1400–10.
[94] Ey P, Prowse SJ, Jenkin C. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry 1978;15(7):429–36.
[95] Muller E, Vajda J. Routes to improve binding capacities of affinity resins demonstrated for Protein A chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2016;1021:159–68.
[96] Carlsson M. How to interperet protein binding capacity: chromatography resins. 2018. https://www.gelifesciences.com/en/us/news-center/protein-binding-chromatography-resins-10001. [Accessed 9 April 2019]. Published.
[97] Freiherr von Roman M, Berensmeier S. Improving the binding capacities of protein A chromatographic materials by means of ligand polymerization. J Chromatogr A 2014;1347:80–6.
[98] Baur D, Angelo JM, Chollangi S, et al. Model assisted comparison of Protein A resins and multi-column chromatography for capture processes. J Biotechnol 2018;285:64–73.
[99] Ander M, Bauren G, Bjorkman T, Rodrigo G, Inventors; GE H. Bioprocess R&D AB, assignee. Mutated immunoglobulin-binding polypeptides. Patent US9663558B2. 2017.
[100] Hall M, Larsson S, Muranyi A, Rodrigo G, Zou J, Aberg P-M, Inventors; GE H. Bioprocess R&D AB, assignee. Chromatography ligand comprising domain C from *Staphylococcus aureus* protein A for antibody isolation. Patent US8329860B2. 2012.
[101] Hober S, Johansson HJ, Inventors; GE H. Bioprocess R&D AB, assignee. Mutant protein. Patent US9296791B2. 2016.

[102] Johansson HJ, Palmgren R, Inventors; GE H. Bioprocess R&D AB, assignee. Affinity chromatography matrix. Patent WO2013081540A1. 2013.
[103] Ljungquist C, Jansson B, Moks T, Uhlen M. Thiol-directed immobilization of recombinant IgG-binding receptors. Eur J Biochem 1989;186(3):557–61.
[104] Hober S, Lindbo S, Nilvebrant J. Bispecific applications of non-immunoglobulin scaffold binders. Methods 2019;154:143–52.
[105] Bjorkman T, Rodrigo G, Inventors; GE H. Bioprocess R&D AB, assignee. Affinity chromatography matrix. Patent WO2012074463A1. 2012.
[106] Chen X, Zaro JL, Shen WC. Fusion protein linkers: property, design and functionality. Adv Drug Deliv Rev 2013;65(10):1357–69.
[107] Zhang X, Duan Y, Zeng X. Improved performance of recombinant protein A immobilized on agarose beads by site-specific conjugation. ACS Omega 2017;2(4):1731–7.
[108] Zhao L, Zhu K, Huang Y, et al. Enhanced binding by dextran-grafting to Protein A affinity chromatographic media. J Separ Sci 2017;40(7):1493–9.
[109] Tugcu N, Roush DJ, Goklen KE. Maximizing productivity of chromatography steps for purification of monoclonal antibodies. Biotechnol Bioeng 2008;99(3):599–613.

CHAPTER

3

High fidelity affinity purification of Fc-fusion molecules from product related impurities

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Glossary

DBC Dynamic binding capacity
DNA Deoxyribonucleic acid
DS Drug substance
Fab Fragment antigen binding
CD3 Cluster of differentiation 3
HFAP High fidelity affinity purification
HTPD High throughput process development
mAb Monoclonal antibody
NaPi Sodium phosphate
Fc Fragment crystallizable
PRI Product related impurity
RNA Ribonucleic acid
LRV Log_{10} reduction value

Introduction

The increasing complexity of biologics is straining traditional manufacturing technologies. The number and diversity of antibody-like molecules and Fc constructs, such as bispecifics, multispecifics and fusion proteins is increasing. Purification processes for clinical manufacturing of these molecules are anticipated to be heavily reliant on the protein A chromatography-based purification platform, which is firmly established as the industry

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standard for production of traditional monoclonal antibodies. Protein A offers a very efficient and economical means to achieve high purity after a single purification step. Significantly, the product related impurities (e.g., aggregates, oxidized and/or deamidated forms) typically only occur at low levels and their removal can be addressed by subsequent conventional chromatography steps. However, modern antibody like constructs are more complex and contain many more possible product related variants arising from heavy and light chain mispairings, as well as misfolding. Furthermore, the complexity can be exacerbated by process generated variants (e.g., proteolytic cleavages and inactive molecules). Contrary to standard antibodies, the levels of these impurities are significantly higher and, in some cases, can constitute most of the product containing mixture. Unfortunately, the very feature of protein A that has enabled it to be so ubiquitous, *i.e.*, being a universal ligand for Fc, means that it copurifies not only the desired drug molecule, but all the product related impurities as well. Removal of these impurities is particularly challenging and has placed a strain on the subsequent conventional chromatography steps.

We describe a new solution to this purification challenge and one that is engineered to ensure high product quality in a single purification step. Affinity resins can be developed that exhibit exquisite selectivity, binding just the correctly formed drug substance and effectively purifying it away from the multitude of product related impurities. We refer to this as high fidelity affinity purification (HFAP). In HFAP, the affinity resins do not bind a common structural element of target variants but are tailored to bind the molecular feature that engenders critical product quality attributes and high potency. Each HFAP resin can be developed within 3 months and subsequent scaling up of production of large quantities for implementation in cGMP manufacturing facilities can be completed in timeframes that match clinical development timelines. We demonstrate 2 case studies of HFAP resins developed for Fc fusion proteins in which the affinity ligand on the resin specifically recognizes the correct conformation of the fusion partner. Originally envisaged as polish resins to be applied after protein A chromatography, the affinity resins work just as effectively as capture resins from the crude feed stream. Thus, in a single step HFAP resins capture drug substance, provide robust HCP clearance and removal of product related impurities. Moreover, negating the need for a separate capture step with protein A, HFAP resins simplify the purification process, dramatically improving the economics. In addition, we describe 2 other case studies directed toward the solving of the product related impurity (PRI) problems that beset the production of bispecific antibody formats.

The 4 case studies describe not only the performance of new HFAC resins and how they afforded a solution to our collaborating partner, but are also selected to highlight the many different aspects of the discovery and development as well as the motivations and design behind each of the resins. By encompassing each of these diverse aspects, we seek to offer each reader the ability to explore those insights that are most relevant to them. Thus, one study details the scale-up and implementation of the resin, while another seeks to show the power and diversity enabled by the speed that this can be done. In each case study the motivation is different, but all of them share the common objective of efficient removal of product related impurities. Prior to the case studies an overview of the process of affinity ligand discovery and affinity resin development is provided.

It is notable that the development model being expounded is very much “one resin-one company”, i.e., the affinity resin is exclusive to the sponsoring partner company and not made widely available. This requires some elaboration. There is clearly a need and demand

for catalog resins that target particular domains, e.g. KappaSelect (GE Healthcare) that serves a wider market. However, Avitide has eschewed this strategy because there also exist many unique problems that seek unique solutions and hence created an unmet need. We believe both strategies are necessary to address the plethora of purification problems. Many companies appreciate the advantages of an exclusive resin. First, there are intellectual property aspects that might extend well beyond the composition of matter patent of the actual drug substance. Second, there is also the conceptual appeal that the affinity resin is tailored to the molecule and not shoehorning the molecule to the affinity resin. From Avitide's perspective it is important to recognize that each resin is an entirely new product designed from the ground up, but its development is based on a standardized process/approach Again, we have taken our inspiration from the bioprocessing industry. Just as the production of mAbs has been platformed, so too we have platformed the resin discovery and development process, such that each program need only be fine-tuned to a particular molecule and associated objective.

How affinity resins are discovered, developed and manufactured

The array of biologic modalities entering clinical development and the correspondingly large diversity of structures brings with it a significant challenge for downstream purification. The modalities encompass proteins of varying shapes and sizes, vaccines, viruses, virus like particles, gene therapies, DNA, RNA, whole cells, cell related products (e.g. exosomes) and the range is expanding. To meet the challenge presented by this extraordinary structural variety, Avitide has developed a corresponding diversity in affinity ligand architectures based upon peptides and proteins. Each of these ligand architectures contains a high sequence diversity exceeding 1×10^{10} molecules. Currently, Avitide employs 48 distinct ligand architectures in each discovery effort. Our experience indicates only a select few ligand formats will be successful in generating bioprocess-ready ligands for any specific therapeutic modality hence, to ensure success for every purification project, a diverse repertoire of ligand architectures is required. A high quality affinity ligand can be envisaged by 4 major criteria: affinity, specificity, stability, and manufacturability. Our large ligand diversity ensures each resin delivered meets these standards. To be reliant upon a single ligand architecture is extremely risky. Indeed, even with five different ligand architectures we estimate that we would fail to meet the high demands required of a modern affinity resin 50% of the time!

The first step to developing a high-performance affinity resin is discovery of a high affinity ligand, thus all candidate ligands are screened on a fully automated Octet HTX (ForteBio/Molecular Devices) biolayer interferometry (BLI) platform for determination of binding kinetics and affinity [1]. Prior to this, initial ligand hits are filtered first with Luminex technology in a highly multiplexed approach [2]. For a ligand to be considered suitable for bioprocessing it must also demonstrate the requisite stability to sodium hydroxide, the preferred industry standard sanitization agent for chromatographic columns, as well as other cleaning agents and denaturants. In order for a ligand to discriminate between product related impurities, it must display different affinities for the desired product and the impurities. In the ideal case, the difference is almost binary with little or no binding to the impurities. However, this is not mandatory as we have prepared an affinity resin that achieves the desired purification from ligands with just a 3-fold difference in affinity between the drug substance and a

product related impurity. This concept is expanded further in each of the case studies. Only affinity ligands with nanomolar affinities that pass this rigorous testing are progressed to evaluation on resin.

During resin development, the affinity ligands are coupled with industry standard and proven base beads available from major chromatography vendors. Parameters for resin preparation and resin screening approaches are described in detail in each of the case studies that follow.

Modern pharmaceutical development programs emphasize speed to market. For example, it is now standard in the industry to quote programs from DNA to IND filing measured in months for mAbs, although this requires more time for other modalities. This time pressure is particularly intense for biotech startups, which have to balance burn rate and valuations for raising venture capital. Hence, to be of practical value, affinity resin development programs must be matched with clinical, and especially process development timelines. To meet this demand, Avitide develops affinity resins from concept to demonstrated working prototype bioprocess suitable resins in 12 weeks.

For an affinity resin to be bioprocess compatible/ready, it must meet the requirements of cGMP manufacturing in a modern biopharmaceutical facility to support current and future production. Without this critical last commitment, the resin would be merely an academic curiosity. Avitide manufactures affinity resins for cGMP use in an ISO9001 accredited quality environment. In addition, to further address continuity of supply, a supply risk mitigation strategy to meet the requirement of the sponsor companies is established to ensure the timely production of the sponsor's drug.

Case study 1: Fc-fusion protein #1

This case study demonstrates that although a PRI removing-resin can be used as a polish resin following protein A, process economics can sometimes demand that the resin replace protein A and also serve as the primary capture for the desired drug molecule (free of PRIs) directly from the clarified feed stream.

The molecule to be purified was an Fc-fusion protein. Since the fusion partner was a native homodimer, the design used a knob-in-hole Fc domain, with the fusion partner domain fused to just one of the heavy chains. Thus, the nature of the PRIs is similar to that observed for knob-in-hole bispecifics, plus a significant amount of aggregated species (Fig. 3.1).

Affinity ligand discovery focused on the fusion partner (in its native homodimer state) and not the Fc region. In discovering affinity ligands that discriminate between the drug molecule and PRIs, it is beneficial to screen with each of the impurities as well as the drug substance. In this instance, the product related impurities were too numerous and were difficult to separate using traditional technologies. Although >60 ligands with high affinity to the drug substance were discovered, it was not possible to demonstrate differential affinities toward the product related impurities using the Octet. Thus, determination of those ligands that were able to discriminate the product related impurities was performed on resin using high throughput process development (HTPD) approaches.

When moving beyond static binding experiments in filter plates and screening resins in column format it is not practical for us to employ common HTPD devices such as

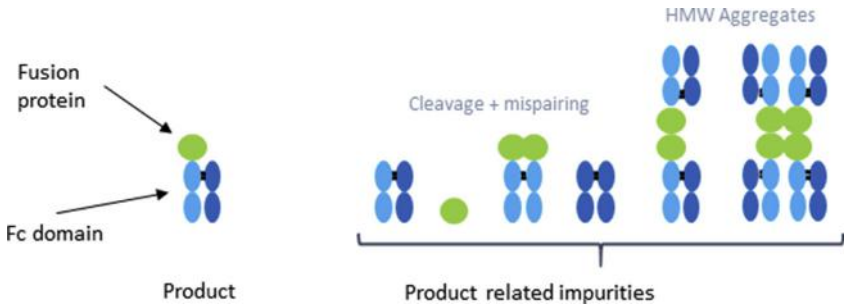


FIG. 3.1 Cartoon representation product related impurities of the Fc-fusion protein.

RoboColumns™ or PhyTip™. Instead, we self-pack 0.18 mL (3 × 25 mm) and 0.7 mL (3 × 100 mm) columns on demand and operate these in highly parallelized format using the ProteinMaker™ (Protein BioSolutions, Gaithersburg, MD, Fig. 3.2). This instrument can run 12 columns in parallel under complete computer control that can be pre-programmed to run unattended. It offers very fine flow rate control for achieving desired residence times in the range from 30 s to 6 min and, critically for this application, it collects fractions directly into microplates enabling subsequent product quality analysis. The microplate fraction collection enables direct placement into HPLC autosamplers for ease of sample transfer on liquid handler platforms for product quality analysis. Building on the experience of the first-generation instrument, a second generation instrument has even greater capabilities − up to 24 columns in parallel, each with inline UV detection to complement the fraction collection. This technology has enabled high throughput affinity resin column testing within the time-frame of a 6-week resin development program. We have yet to learn of another technology

FIG. 3.2 High throughput parallel mini-column chromatography is enabled by ProteinMaker™ instruments.

that makes it possible to screen so many columns from so many different resins available only in small quantities, so quickly and efficiently.

The result of this screening effort was the selection of AVI-297 resin that met all the strict performance requirements encompassing selectivity between PRIs, binding capacity, purity and CIP robustness. Most importantly, the selectivity criteria were met, with a 10-fold reduction in product related impurities ensuring enrichment of the drug substance in protein A purified material from 69% to 97% (Table 3.1). To improve overall process economics, the focus shifted from use of the resin as a polishing step following protein A capture to the primary capture resin with simultaneous removal of the product related impurities.

The resin also achieved a high degree of product selectivity, achieving 95% purity of the drug substance. Moreover, the overall purification performance of resin AVI-297 compared very well with protein A. A complete list of performance parameters is listed in Table 3.2. It is worth noting the robust HCP clearance that was further improved by introduction of a wash step by our partner to achieve approximately 700 ppm residual HCPs. Other performance parameters also befit the resin status as a bioprocess suitable resin. The ability to clean and sanitize the AVI-297 resin with 0.1 M NaOH exceeded the minimum specifications, enabling 35 contact cycles of 15 min each before a 10% reduction in binding capacity was observed. Sanitization was planned for every fifth cycle, so the anticipated lifetime exceeded

TABLE 3.1 Purity profile after affinity purification of Fc fusion protein with protein A or AVI-297 resin.

Purification process	% Drug Molecule	% HMW	% LMW
Protein A purified	69	20	11
AVI-297 affinity resin used as polish after protein A capture	> 97	< 3	0
AVI-297 affinity resin (capture from clarified feedstream)	> 95	< 3	< 2

TABLE 3.2 Performance metrics for AVI-297 resin used as primary capture from HCCF.

Performance criteria	Results
DBC	25 g/L
Yield	93 %
Elution	pH 3.5
Elution volume	2.5 CV
Residual HCP	
PBS wash only	1210 ppm
With proprietary wash	700 ppm
HCP reduction	2.4 log
DNA reduction	3.2 log
DNA (ppm)	1
0.1 M NaOH CIP	35 cycles

TABLE 3.3 Effective binding capacity comparison between protein A and AVI-297 resin.

MabSelect Sure™ DBC	22 g/L
Proportion of DS	70 %
Effective DBC	16 g/L
AVI-297 resin DBC	25 g/L
Proportion of DS	96 %
Effective DBC	24 g/L
Fold increase	1.5

100 cycles. Column lifetime is a very big determinant of overall process economics, particularly for programs with high mass production requirements, thus this was an important metric in being able to supplant the use of protein A capture.

The binding capacity merits some discussion. The 25 g/L binding capacity was slightly higher than that achieved with MabSelect SuRe™ for this fusion protein (Table 3.3). However, further consideration shows the difference is much greater than the absolute amounts suggest. This arises from the presence of the product related impurities. Since only 70% of the protein bound to protein A is the desired drug molecule, the effective capacity is reduced to 16 g/L. In contrast, since the AVI-297 resin binds ostensibly only drug molecules there is little impact on effective binding capacity by the product related impurities. The net effect is that the selective Avitide resin translates to a 50% higher effective capacity. There is also an add-on effect from removing the product related impurities in the first step. Since the bulk of the product related impurities have been removed, the total protein loaded on the first polish column is reduced by 30%, hence columns can be resized accordingly, and buffer consumption reduced.

Having established the merits of the affinity resin, preparation for larger scale manufacturing was necessary. Ligand density was a balance between capacity and yield (Fig. 3.3). Binding capacity reached a plateau as ligand density was increased. As ligand density was increased further, yield was compromised. This placed an upper limit on ligand density and hence defined this process specification for the resin. Subsequently, batches of resin were prepared at a scale of several liters to demonstrate robust process control. Additionally, batches were deliberately prepared at the limit of the specifications to demonstrate that the resulting resins would still provide the required performance.

The selectivity of the resin, both in terms of product related impurities and HCP were important features of the resin. Thus, it was natural that the release criteria for the resin was couched in those terms. This is a defining feature of purpose built affinity resins —quality control of the resin demonstrates qualification for that purpose using authentic materials from the intended application. This contrasts with generic resins where the specifications are set for surrogate proteins, e.g. BSA or lysozyme, that are not process relevant, particularly for high resolution separations. Consequently, the onus is upon the user to demonstrate that each lot of the generic resin works as intended in their application. Lot-to-lot variation in generic resin purification performance has given rise to the practice of “lot picking”, an often cited drawback of adapting generic resins for high resolution separations [3–5].

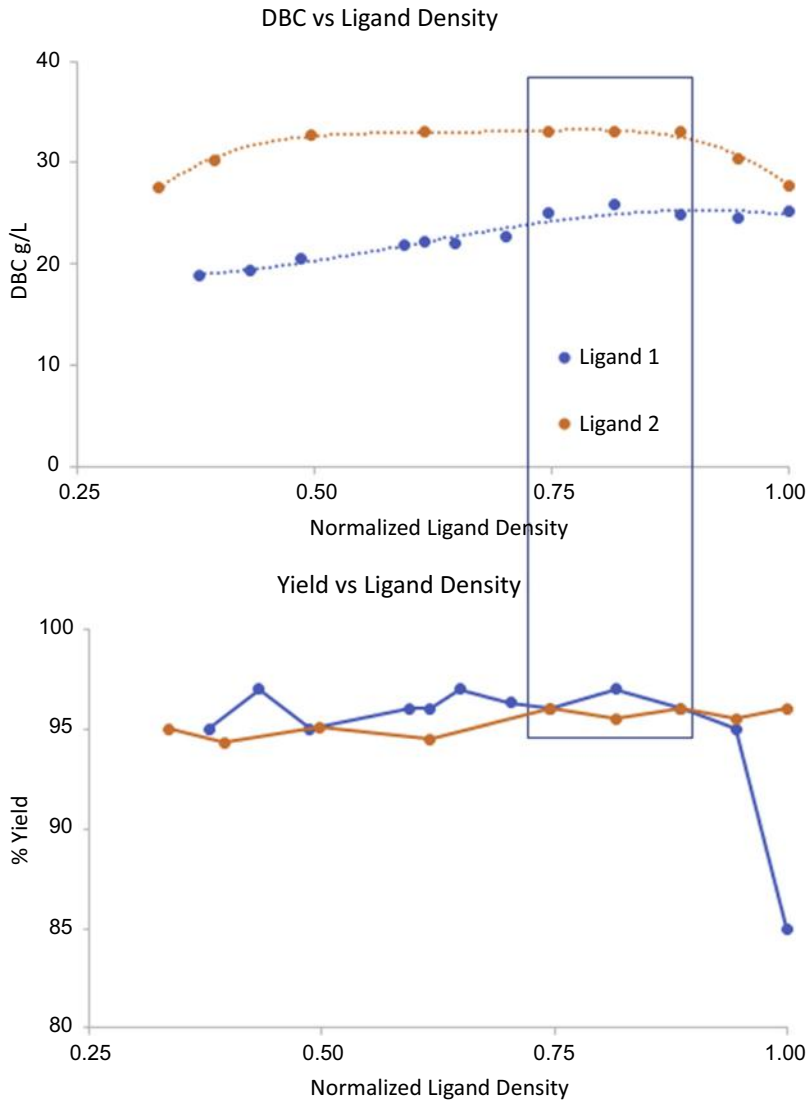


FIG. 3.3 Ligand density was optimized for binding capacity and yield to derive resin manufacturing and release criteria. The boxed area the ligand density range in the final specifications of the resin (the midpoint of this range).

Custom performance specifications with relevant feedstock were also applied was also applied throughout resin storage stability trials, performed at both elevated temperature and cold storage conditions. The latter are scheduled to run for 3 years and at the time of this writing are still in progress.

Industry experience with protein A has highlighted the need to monitor process introduced impurities in the purified drug substance. Thus, analogous to residual protein A assays, an ELISA was developed to measure the peptide affinity ligand. Due to the small

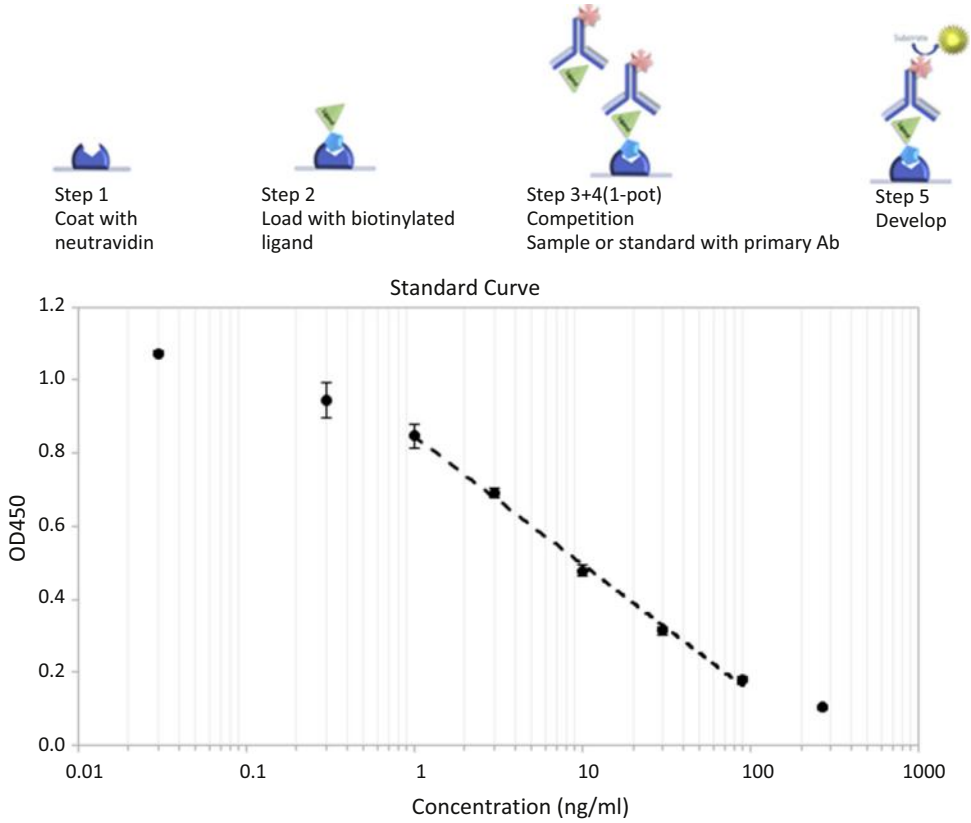


FIG. 3.4 Residual ligand ELISA format and standard curve for the determination of trace amounts of leached ligand (peptide) in Fc-fusion protein samples.

size of the peptide ligand, the assay was developed as a competitive sandwich ELISA, summarized in Fig. 3.4. An example standard curve is also shown in the figure. Different resin lots were prepared and the residual ligand levels in the column eluates were determined to be in the range 8–26 ppm.

This case study details the entire discovery and development of an affinity resin from conception through scale-up in a quality controlled environment. Notably, the performance of the affinity resin was sufficient to replace protein A as the primary capture while simultaneously providing separation of product related impurities.

Case study 2: Fc-fusion protein #2

Similar to the first case study, this program also concerned a bispecific Fc-fusion protein therapeutic. However, unlike the previous case study, which was beset with chain mispairing, cleavage products and aggregates, in this instance the major product-related impurities

were misfolded versions of the fusion protein with an identical molecular mass. The misfolded versions accounted for half of the product and bound the biological target with much lower affinities, thus decreasing potency and increasing safety risks. Conceptually, the biological target is the ideal quality-ensuring affinity ligand, but it was impractical and not suitable for bioprocessing. Thus, the objective for this affinity purification was to mimic the native biological target (cognate receptor) and selectively capture only the therapeutically active isoform (the Drug Substance, DS) thus eliminating the misfolded forms in a single step.

A monoclonal antibody (mAb) recognizing the active site of the DS had previously been identified. It too was not suitable for bioprocess purification but was a critical analytical reagent to discriminate between the active drug and product-related impurities.

Ligand discovery efforts yielded 62 affinity ligands that bound the DS with high affinity and binary selectivity, demonstrating virtually undetectable binding to the product-related impurities (Fig. 3.5A,B. An epitope mapping study demonstrated that Avitide ligands compete with the Mab for drug binding, indicating the two molecules bind to the drug

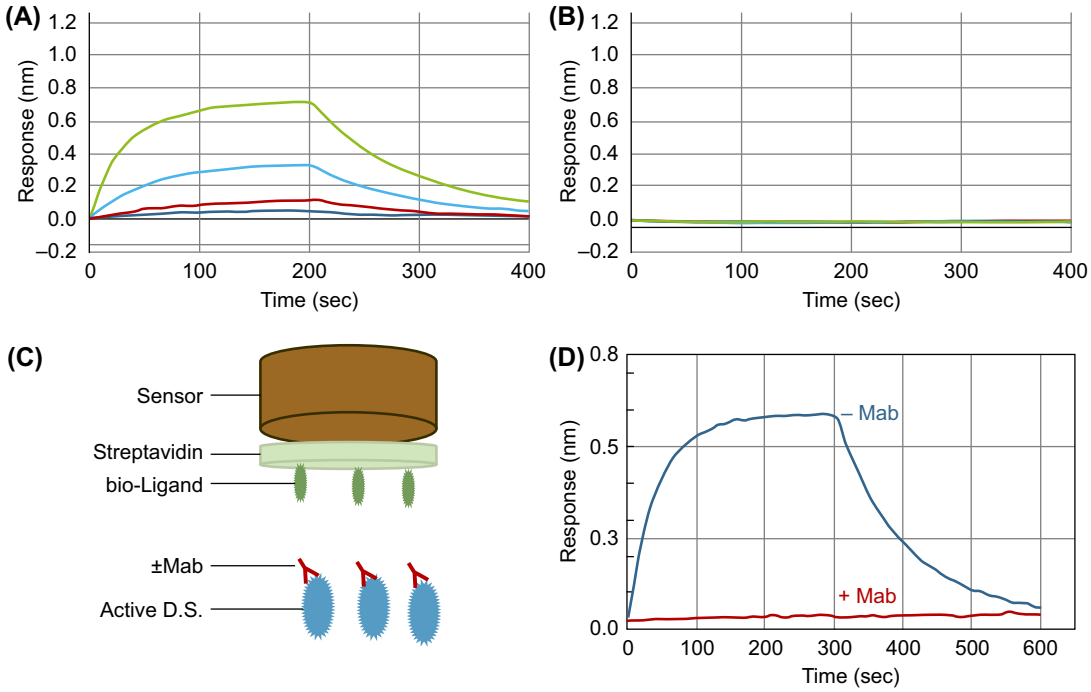


FIG. 3.5 Binding analysis of Avitide ligands *via* biolayer interferometry (BLI). A representative ligand (AVI-1719) was biotinylated, immobilized onto Streptavidin sensors and dipped into a titration of the purified drug substance (A), or the inactive impurity fraction (B). Ligand AVI-1791 does not demonstrate measurable binding to the product-related impurities. Competition experiments were performed to map the epitope of Avitide ligand binding (C–D). Avitide ligands were immobilized onto BLI sensors and dipped into a solution containing either the drug substance (250 nM, blue (dark gray in print version) trace), or a mixture of the drug substance plus a 2-fold molar excess of the active site-specific Mab (red (gray in print version) trace). The Mab-drug mixture completely abrogates the ligand-drug binding interaction for Avitide ligand AVI-1719 (D), indicating the ligand and the Mab compete for the same epitope.

substance at the same site (Fig. 3.5C–D). A total of 21 ligands with the highest affinity toward the DS ($K_D < 250$ nM) and with confirmed active site epitope binding were selected for investigation as affinity chromatography ligands.

Significantly, although 48 different scaffold families were entered into ligand discovery, the 62 best ligands were all drawn from one family, scaffold #45! This extraordinary discovery highlights the need for diversity in ligand architectures and not just high sequence diversity within a particular ligand architecture. This concept underpins Avitide's continued exploration of new ligand architectures. Efficiently processing ligand diversity between and among scaffold families is the primary technical challenge during ligand discovery efforts.

Sequence similarities enabled us to sort the 62 ligands into 5 clades. The other families yielded ~900 ligands with moderate affinity, but these lacked the exquisite selectivity of scaffold #45 hits. Interestingly, scaffold #45 was developed explicitly to fill a perceived gap in our repertoire of biological "lock and key" affinity ligands [6]. The dearth of selective ligands from other scaffold types indicated only scaffold #45 possessed the molecular architecture required to mimic the cognate receptor. Never before had this family dominated ligand discovery to such an extent and although our preference is always to take a diverse set of ligands into resin development, in this instance we did not have that luxury. The significance of this became apparent during resin development.

Initially resin development used protein A-purified material as feedstock, which, depending on the batch, varied from 48% to 58% of misfolded forms. Static binding experiments conducted in filter plates coupled with Mab immunoaffinity HPLC was used to initially screen the 21 affinity resins. MabSelect SuRe™ was used as a control for a non-selective affinity resin. Fifteen affinity resins enriched the correctly folded active form to > 90% (Table 3.4) and, as expected, MabSelect SuRe™ was non-selective. Total binding capacity and NaOH stability were used to further rank the resins. Five affinity resins had unchanged binding capacity after incubation in 0.1 N NaOH for 17 h, however the total binding capacity was 5 g/L or less for all resins. Clearly, this low binding capacity is unacceptable for bioprocessing and consequently, having attained the requisite selectivity and resin lifetime, improving resin binding capacity became the priority.

Efforts to improve binding capacity explored the following-

1. Ligand attachment chemistry
2. Ligand linker/spacer molecules
3. Ligand engineering/optimization
4. Alternative base beads
5. Degree of base bead activation
6. Ligand density
7. Bioprocess changes, e.g. modulating the drug substance loading conditions.

The top 5 ligands were engineered to enable alternative, site-directed chemical attachment to resin, and to incorporate 3 different protein-based linker/spacers. The greatest improvement was observed when the resin was formulated with an oligopeptide linker/spacer (Fig. 3.6A). Significantly, efforts to multimerize the ligands analogous to the multi-domain structure of protein A ligands [7] were catastrophic. Many ligands became intractable, in instances where resins could be prepared the performance was compromised. The effects of ligand density and resin activation were evaluated next. Resins were produced from ligand

TABLE 3.4 High throughput analysis of Avitide ligand selectivity on resin.

Ligand/Resin ID	% Active Form	Step yield (rank)
Load Control	*48*	*N/A*
MabSelect SuRe™	*48*	*N/A*
AVI-1706	90	1
AVI-1705	92	2
AVI-1704	90	3
AVI-1713	90	4
AVI-1703	91	5
AVI-1708	91	6
AVI-1709	91	7
AVI-1712	91	8
AVI-1711	92	9
AVI-1702	89	10
AVI-1718	90	11
AVI-1714	91	12
AVI-1715	91	13
AVI-1707	90	14
AVI-1717	90	15
AVI-1710	88	16
AVI-1721	75	17
AVI-1716	85	18
AVI-1719	79	19
AVI-1722	92	20
AVI-1720	67	21

AVI-1708 with low, medium or high resin activation (5–70 μmol/g), and low, medium or high ligand density (2–30 g/L_{resin}). Binding capacity was evaluated *via* equilibrium binding experiments in batch format for all 9 resins (Fig. 3.6B). Resins produced with lower activation levels and intermediate ligand densities had the highest binding capacity. Base beads from several bioprocess vendors were evaluated that spanned different pore sizes (50–600 nm), material type (agarose, polymethacrylate and polystyrene) and bead sizes (35–90 μm). Agarose-based resins clearly demonstrated superior capacity to hydrophobic-based polymeric media in every case. On average, agarose-based media demonstrated 5-fold higher capacity than polymethacrylate or polystyrene media. A combination of the best attachment chemistry, linker/spacer, activation and ligand density were combined to make a final

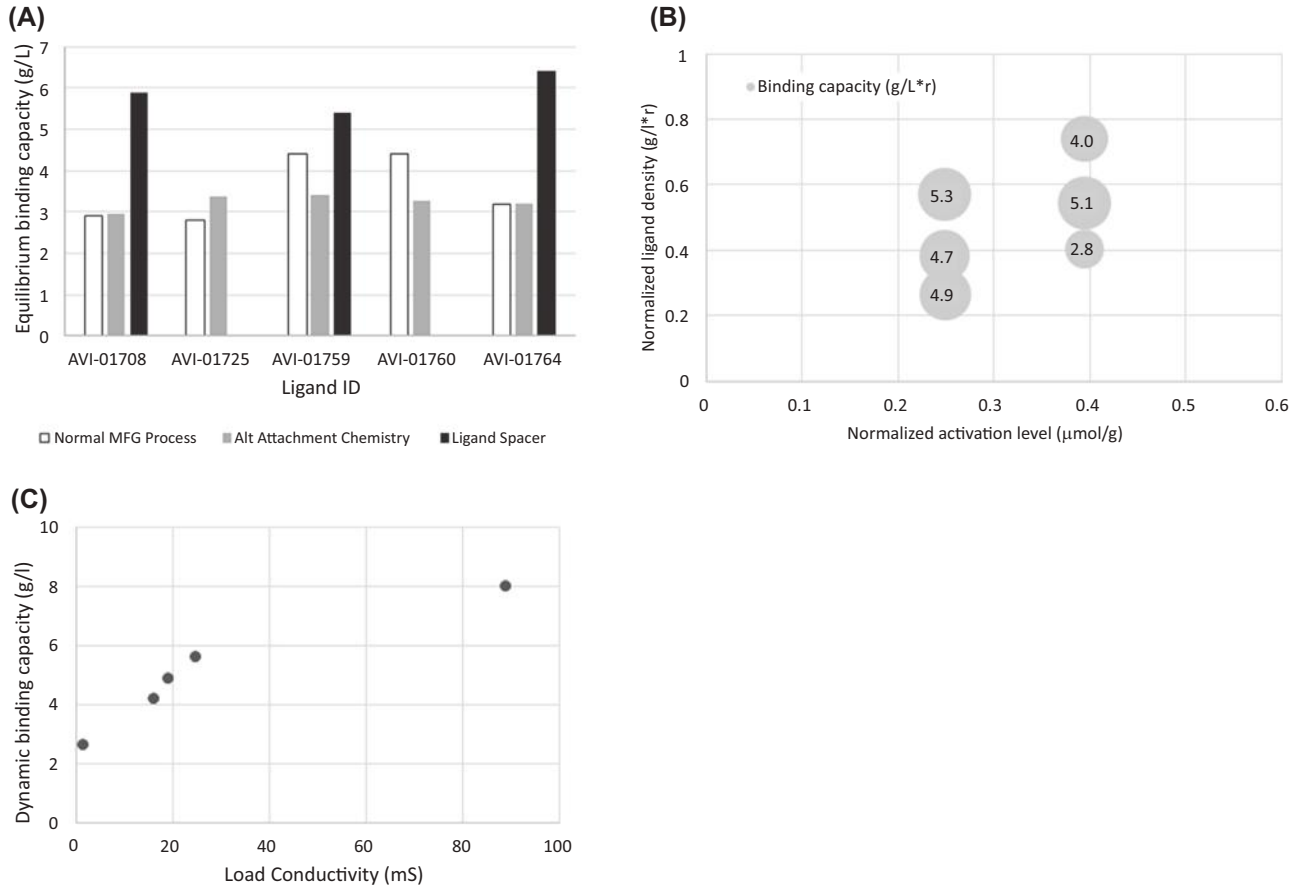


FIG. 3.6 Optimization of resin conjugation and purification process conditions. Equilibrium binding capacity experiments were performed to evaluate the effects of changing ligand coupling chemistry and incorporation of a spacer molecule between the affinity ligand and the resin (A). The attachment chemistry did not have an effect on binding capacity of the resins, but the inclusion of a peptide-based spacer molecule almost doubled the binding capacity for select ligands. The effect of altering activation level and ligand density on binding capacity was evaluated for AVI-1708 (B). The best binding capacity was observed with low levels of resin activation and low to moderate ligand density. High activation levels (Normalized activation level = 1) completely abrogated resin performance. Dynamic binding capacity increases with increasing ionic strength (C). DBC was determined with 3 × 25 mm columns.

version of the AVI-1708 resin for further testing in packed columns. Nonetheless, these efforts were only able to increase binding capacity to 5.3 g/L.

The low binding capacity and resistance to attempts to increase it to > 20 g/L is extraordinary and is worthy of comment. It is noteworthy that MabSelect SuRe™ had a binding capacity of 15 g/L, which is about 1/3 of the expected capacity for a typical IgG. Moreover, given that only half of the bound Fc-fusion protein was correctly folded DS, the effective binding capacity is reduced to 7.5 g/L, marginally higher than that achieved with AVI-1708. Thus, given that AVI-1708 selectively bound only the DS, this resin was a practical replacement for protein A capture.

However, profound improvements to binding capacity were achieved during bioprocess method development and enabled challenging the resin to 25 g/L. Increasing the conductivity of the protein A purified material significantly improved the binding capacity of AVI-1708 (Fig. 3.6C). Interestingly, the identity of the salt plays an important role, as specific ions had a much greater contribution to binding capacity than others; we identified 600 mM Na_2SO_4 as the optimal additive to increase binding capacity of AVI-1708. Crucially, modulation of the load conductivity did not impair the selectivity of AVI-1708 for the authentic drug substance; purification fractions analyzed by the MAb immunoaffinity HPLC analytical method routinely demonstrate enrichment from ~50% active drug substance in the load condition to >95% active drug substance in the eluate. When the resin was challenged to 25 g/L resin and eluted with 30 mM glycine, pH 2, the results demonstrated impressive selectivity and performance. The eluate fraction was measured to be 99% active drug substance, and step yield of the drug substance was 93%. A typical immunoaffinity HPLC result of Avitide purification is reproduced in Fig. 3.7.

This feature led to the preferred operation of the resin as a polish step following protein A capture. A final method for purification of the DS is outlined in Table 3.5 and a typical product quality profile is included in Table 3.6.

Case study 3: A general approach to purification of bispecific antibodies

A diverse array of molecular designs are under development as bispecific antibodies, with some estimates of >60 in clinical development totaling several hundred different bispecific molecules [8–10]. The molecular architectures build upon the standard mAb framework and inevitably involve greater complexity. With that complexity also comes product related impurities [11]. While the purification of traditional mAbs also must contend with the problem of PRI, the magnitude and scope of the separation of the additional product related impurities from the desired drug molecule magnifies the challenge immensely. The nature of the product related impurities depends upon the architecture and can include: heavy chain mispairing, light chain mispairing, free heavy and light chains, $^1/_2$ antibody, $^3/_4$ antibody (missing light chain), oligomers and, particularly if the design includes additional domains, proteolytically cleaved products. For example, co-expression of two antibodies (i.e. 2 heavy chains and 2 light chains) can give rise to up to nine unwanted IgG species in addition to the desired bispecific. To address this problem Avitide has been asked on many occasions to develop affinity resins targeting the paratopes, which complement the existing tools that downstream purification scientists have at their disposal (Fig. 3.8).

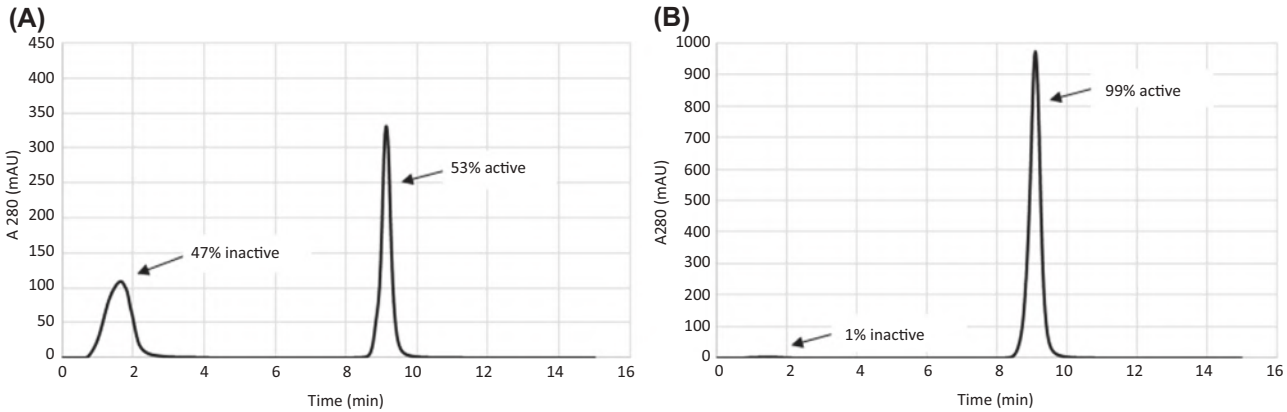


FIG. 3.7 Immunoaffinity HPLC analysis of Avitide affinity purification from a mixed process intermediate was performed to evaluate purification performance. The Mab HPLC column binds only the active drug substance, which is eluted with 30 mM glycine, pH 2.5. Protein A-purified load challenge was loaded onto the HPLC column (A), and the mixture of 47% inactive impurities was resolved from the active drug substance (53%) during the HPLC run. Analysis of purification fractions from Avitide resin AVI-1708(B) demonstrate a selective enrichment of the authentic drug substance to > 99% (B).

TABLE 3.5 Method operating parameters for purification with AVI-1708 resin. Flow rates provided a residence time of 4.2 min.

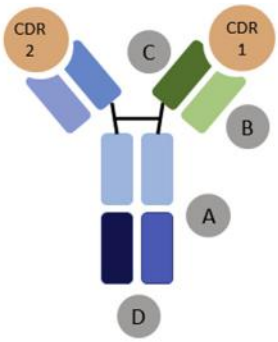
Step	Buffer	CVs
Equilibration	50 mM NaP_i, 600 mM Na_2SO_4, pH 7	3
Load sample	0.49 g/L total Fc drug substance mixture; 50 mM NaP_i, 600 mM Na_2SO_4, pH 7	32
Wash	50 mM NaP_i, 600 mM Na_2SO_4, pH 7	15
Elute	30 mM glycine, pH 2.0	5
Strip	0.1 N NaOH	5
Re-equilibration	50 mM NaP_i, 600 mM Na_2SO_4, pH 7	10

TABLE 3.6 AVI-1708 resin chromatographic performance and product quality analysis of Fc-fusion protein #2.

Parameter	Result
Load type	p rotein A purified process intermediate
Load challenge (g/L_{resin})	25.3 - total Fc 13.5 - active drug substance
Load challenge (% active drug substance)	54
Step yield (%)	93
Eluate purity (% active drug substance)	98

A large motivation for generating paratope (antigen binding site) specific resins is that a single $^1/_2$ antibody may form the basis of a platform to be paired with a multitude of other $^1/_2$ antibodies. A prime example is antibodies targeting CD3, which are 3 times more prevalent in bispecific antibodies than any other targeting domain [12] and are featured in over 130 clinical programs. Significantly, both of the approved bispecific antibodies target CD3. After adopting a preferred CD3 half-antibody, a company may then pair it with several complementary half-antibody domains during preclinical development and candidate selection. Furthermore, this process might be repeated across many programs (disease indications). Thus, none too surprisingly, this was the first paratope binding affinity resin Avitide was sponsored to prepare, initially intended to accelerate candidate selection and pre-clinical development.

Paratope specific resins are attractive because quality is inherent in the design, i.e. the affinity resin ensures that the purified product has a functional paratope. Thus, when two paratope-specific resins are used consecutively the integrity of the purified bispecific is assured. This same concept is used in the purification of κλ-bodies described by Novimmune [13], with the sequential use of kappa- and lambda-light chain selective resins to ensure quality (Fig. 3.9). In contrast, the paratope-specific approach releases the bispecific design from the constraint of requiring two different light chain classes. Moreover, the light chain selective



	Domain	Example
A	CH2-CH3	Protein A
B	Light chain	Kappa-select, lambda-select, Protein L
C	Fab/CH1	Protein G, CH1-XL, Some Protein A variants
D	CH3	FcXL

FIG. 3.8 The major binding sites of commercial resins and the paratope binding ligands. Some protein A and protein G resins show secondary, weaker binding to the Fab domain.

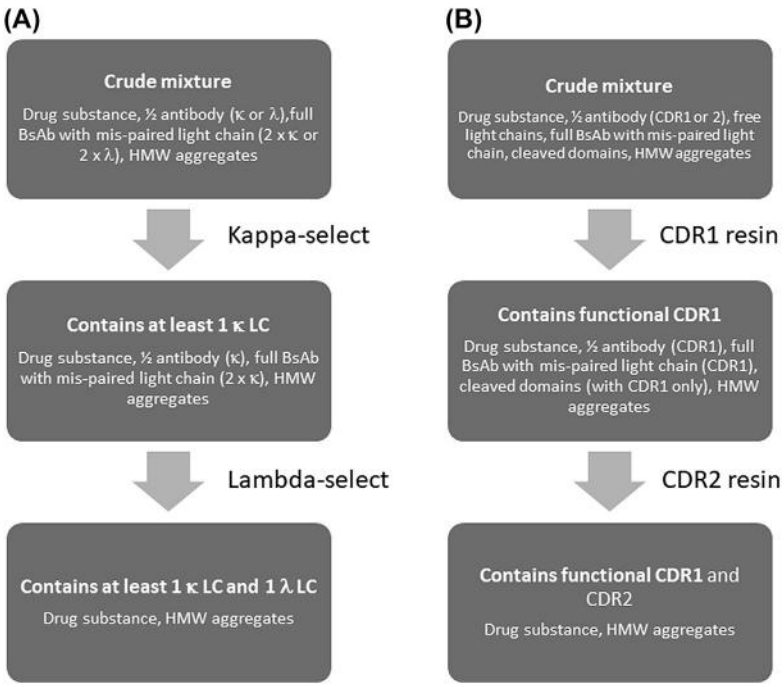


FIG. 3.9 Flow diagrams showing the removal of impurities for the sequential use of (A) Kappa- and LambdaFab Select™ purification and (B) Paratope specific resins.

resins are typically applied after protein A capture to ensure free light chain is removed, thus requiring an extra purification step [13].

The development of paratope specific resins does not require the full bispecific construct or even any knowledge of what the antigen is. Indeed, Avitide has developed 8 paratope specific resins for components of bispecific antibodies and the identity of the antibody domains was not revealed in 6 of those instances.

Demonstrating that the affinity ligands are indeed specific for the paratopes is reasonably straightforward if the antigens are available. Unfortunately, knowing the identity of the paratopes has been rare for many of these projects completed at Avitide. Thus, we often resort to a more circumstantial approach, with the final confirmation being performed by our industry partner. Our approach is to screen a panel of antibody molecules with different paratopes. Identification of sequence similarities between the affinity ligands helps identify families that bind to a similar region of the paratope. Binding competition studies are also used to indicate if the affinity ligands, peptides and small proteins compete with each other for binding locations. Generally, an antibody possesses picomolar affinity to its target, which is in part a reflection of the large surface area of the binding interaction, estimated to be on average 28 Å by 21 Å [14]. In contrast, the small oligopeptide affinity ligands we derive to paratopes have affinities in the range 1−100 nM. These affinities are more favorable for purification purposes, which demands not only binding, but also subsequent release.

The paratope specific affinity ligands also find utility as analytical reagents. The affinity ligands can be incorporated into HPLC columns or binding assays in an ELISA and other formats (e.g., SPR or BLI). The combination of the purification resins and analytical assays that are direct quantitative measures of quality are valuable tools for cell line development and selection.

The development of the anti-CD3 affinity resin illustrates many of these concepts. Using the Fab from the parental antibody, many ligands with affinities < 50 nM were discovered. For comparison purposes, the affinity of a single (Z) domain of the (tetra-Z) protein A ligand of MabSelect SuRe™ is reported to be approximately 10 nM [15,16]. Given the early preclinical status of the program, the performance specifications required for this affinity resin were modest. A successful resin only needed limited CIP cycling and moderate capacities. It is noteworthy that for some bispecific constructs, the clinical dosages are much lower than for traditional antibodies. Consequently, whereas mAbs are frequently produced at a scale with annual production measured in the hundreds of kilograms, with bispecifics it may be at gram scale. This lower annual production requirement relaxes some of the throughput requirements that occur in large scale mAb production.

For development of affinity resin AVI-017, the anti-CD3 MAb was substituted for the authentic pipeline candidates. The affinity resin clearly met the design criteria achieving 26 g/L binding capacity (10% breakthrough) at 4 min residence time (Table 3.7). With clarified CHO cell culture fluid, yields exceeded 90% and even with just a PBS wash the resin was able to achieve a residual HCP level of ∼4000 ppm. At this point the resin was internalized by our partner for implementation. Although we prefer to fully develop a product, we also can accommodate our partner's desire to protect their intellectual property and enter into co-development. The observed HCP clearance compares well with that of protein A and while further process exploration of wash conditions might be expected to decrease this by another

TABLE 3.7 AVI-017 resin chromatographic performance and product quality analysis.

Parameter	Affinity resin
Dynamic binding capacity	26 g/L
Yield	94%
Residual HCP	4050 ppm (2.2 LRV)
Residual DNA	25 ppm (2.4 LRV)
0.1 M NaOH CIP cycling	>10 cycles

order of magnitude [17], the preference was for our partner to develop wash conditions with the authentic pipeline candidates and production streams.

A second example involved two different bispecific antibodies that shared a Fab domain that was similar, but not identical. Moreover, designed mutations in one of the bispecifics meant that it did not bind to protein A, thus demanding a new approach to purification. Within 3 weeks, ligand discovery yielded 110 ligands with affinities ranging from sub-1 nM–650 nM toward this bispecific. These ligands did not bind to the Fc domain or a Fab pool from human sera. 84% (92/110) of the ligands also bound the second bispecific.

In a third example, we sought to discover ligands to a Fab arm that was common to 2 bispecific antibodies in our partner's pipeline. Thus, there were 3 different Fabs across both molecules. In total, 250 different ligands were discovered that bound 1 or more of the Fabs. Ultimately, to address each of the 3 different Fabs, 42 ligands that were resistant to 0.1 M NaOH were selected and resins were prepared from each of these and evaluated by the sponsor company. This included 17 ligands with affinities greater than 11 nM that bound to the paratope of the common Fab, 17 ligands with affinities of greater than 12 nM toward the second Fab and 8 ligands with affinities greater than 20 nM to the third Fab.

In a fourth example, an entirely different approach is described in which the focus was on the impurities with the intention of developing a resin that binds these specifically. This presents an attractive route when the impurities are minor components, enabling small columns to be used to extract the impurity from the drug substance. Moreover, one of the homodimers was identified as causing toxicity and hence it became imperative to ensure the amount of this homodimer was controlled and reduced as low as possible. In alignment with this, we sought ligands that bound the homodimer with high affinity, but bound the heterodimer with much lower affinity. Twenty-four ligands are listed in Table 3.8 with affinity differences greater than 10-fold. There were many more ligands which had affinity differences less than 10-fold. Thus, the great majority of data suggest that the avidity contribution arising from the dimeric presentation of the homodimer is less than 10-fold [18,19]. Clearly, the very large affinity differences shown in Table 3.8 far exceed contributions from avidity alone. It seems likely that these ligands are recognizing a structural difference between the homo-and heterodimer forms. This might arise if the ligands were binding to a paratope with partial overlap of a neighboring domain, but we have not sought to explore this phenomenon in detail. It should be noted that only small amounts of material were required to discover these ligands. Less than 10 mg of the relevant Fab and just a few mg of the heterodimer and homodimer were required.

TABLE 3.8 24 paratope binding ligands with greater than 10-fold affinity difference between homodimer and heterodimer.

K_D (nM)	Ligand type	Homodimer	Heterodimer	Fold difference
1	Peptide	430	7100	17
2	Peptide	15	322	21
3	Peptide	110	>10,000	>100
4	Peptide	35	473	14
5	Peptide	<1	161	>160
6	Peptide	<1	434	>400
7	Peptide	<1	30	>30
8	Peptide	<1	378	>300
9	Peptide	3	97	32
10	Recombinant	5	206	41
11	Recombinant	5	175	35
12	Peptide	6	84	14
13	Peptide	6	239	40
14	Peptide	6	233	39
15	Recombinant	7	167	24
16	Peptide	10	>10,000	>100
17	Peptide	163	>10,000	>60
18	Peptide	130	>10,000	>80
19	Peptide	13	216	17
20	Recombinant	1	>10,000	>10,000
21	Recombinant	11	>10,000	900
22	Recombinant	1	>10,000	>10,000
23	Recombinant	6	>10,000	>1700
24	Recombinant	9	>10,000	>1100

A summary of all of our ligand discovery campaigns toward paratopes of bispecific molecules is shown in Table 3.9. We have speculated that not all mAbs are amenable to this approach. The literature precedent is scant, but this is biased by the feature that mostly small peptides have been explored as suitable ligands yielding low affinity binders [20–23]. With the rich diversity of ligands available to us, we have always generated nanomolar affinity binders to paratopes themselves. Although we look forward to meeting this challenge, it might also be that the modern notion of "developability" means such candidates are culled from pipelines at an early stage and hence this requirement is decreasing in significance.

TABLE 3.9 Results of 8 different ligand discovery efforts toward paratopes.

Example	Target Fab	No. of ligands	K_D range	Preferred ligands
1	Anti-CD3	63	1–50 nM	
2	Unknown 1A	110	1–650 nM	
3	Unknown 1B	92	13–700 nM	All of these also bound 1A
4	Unknown 2	90	1–100 nM	17 with 1–12 nM affinity
5	Unknown 3	93	3–100 nM	17 with 3–11 nM affinity
6	Unknown 4	52	2–100 nM	8 with 2–20 nM affinity
7	Unknown 5	48	1–20 nM	21 with 1–9 nM affinity
8	Unknown 6	24	1–430 nM	>10-fold higher affinity for homodimer

Case study 4: heterodimer specific resins

Several heterodimer bispecific designs have been developed ostensibly around re-engineering of the interface of the CH3 domains (e.g. "knob-in-hole, SEED) [8] representing efforts by different companies to establish proprietary designs. As discussed in the preceding case study, these are plagued by production of appreciable amounts of the two homodimer forms as well as the desired heterodimer. One solution is to rapidly develop resins that target the paratopes, which would provide solutions for individual bispecifics and in some cases families of bispecifics that share one arm. The more substantial solution is to develop a platform resin against heterodimer CH3 domain interface. While more difficult, this approach works independently of the paratopes and enables purification of the heterodimer specifically while removing both of the homodimer forms as well as the $^1/_2$ antibody PRIs. The advantage of the platform approach is that it greatly accelerates preclinical development of future candidate drugs while providing a reliable line of sight to clinical production.

Although it is hypothesized that a CH3 engineered heterodimer may have a different structure from the homodimers, it is rare that this has been explicitly demonstrated [24]. It is even less clear that such differences may be sufficient to develop affinity ligands specific for the heterodimer to be able to effect purification, but Avitide has achieved this for a proprietary heterodimer design. Twenty-five ligands were discovered that demonstrated near binary binding behavior; binding the heterodimer with high affinity but with virtually undetectable binding to the homodimers. The ligands included a peptide and sequences from two protein families. Verification of the ligands binding to the CH3 domains was achieved by showing strong affinity to the Fc domain and no detectable affinity toward the Fab domains. Presumably, the ligands bind at the interface of the CH3 domains, the site of the major structural differences.

All the affinity resins prepared from the ligands were able to enrich the heterodimer, but also highlighted an unanticipated complication with some of the HMW impurities. These included typical dimers and also "1 $^1/_2$ antibodies", some of which are depicted in Fig. 3.10. As is evident from the figure, some of these species are comprised of a correctly folded heterodimer and hence bind to the resin. This complicated the analysis, requiring a

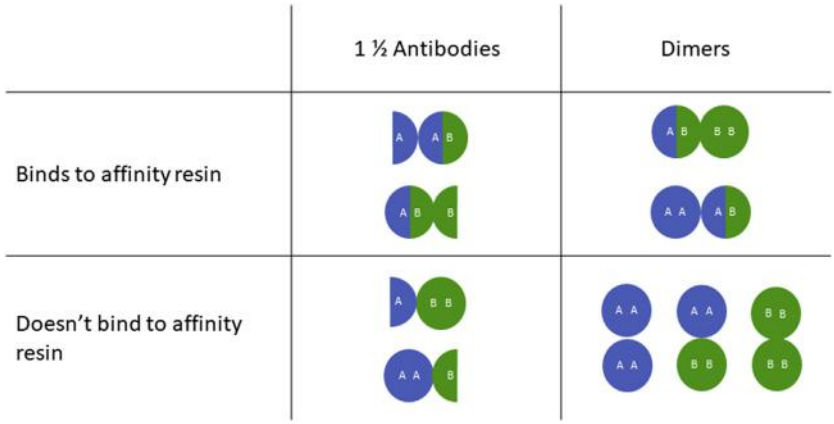


FIG. 3.10 Cartoon representation of some of the dimer and 1 $^{1}/_{2}$ antibodies impurities. Those species containing a properly formed AB heterodimer (boxed) are expected to bind to the affinity column.

combination of intact protein mass spectrometry, capillary electrophoresis and SEC to characterize the products. With this insight and analytical capability Avitide focused on the development of 2 resins, which offered dramatically different solutions (Table 3.10). Both resins could withstand 50 CIP cycles with 0.1 M NaOH. The first, AVI-4891, reduced the PRIs to low levels but had low capacity. It is noteworthy that some of the HMW impurities bound to the resin, but it was possible to selectively elute the homodimer. The second resin, AVI-4933, offered over twice the capacity and reduced the PRIs to less than 2%, except for the HMW impurities (Fig. 3.11). However, in collaboration with the sponsor company we were able to demonstrate removal of these HMW impurities with a subsequent traditional mode chromatography step. Thus, a process based around AVI-4933 as primary capture was the most efficient process.

Each resin is proprietary to the individual company for which it was created, and as such, it is not possible to comment upon whether these resins are applicable to other CH3 engineered designs. However, by virtue of the constant Fc region, this case study illuminates

TABLE 3.10 The proportions of the heterodimer and PRIs after purification of HCCF by MabSelect Sure™ or AVI resins.

Parameter	MabSelect Sure™	AVI-4933	AVI-4891
Heterodimer	55%	80%	96%
Total homodimers	13%	<1%	<1%
Total HMW	17%	17%	1%
Total $^{1}/_{2}$ -Antibody	10%	<LOQ%	1%
Other (Fc/LC/HC)	5%	2%	2%
DBC	>40 g/L	33 g/L	12 g/L
Yield (heterodimer)	>95%	>95%	60%

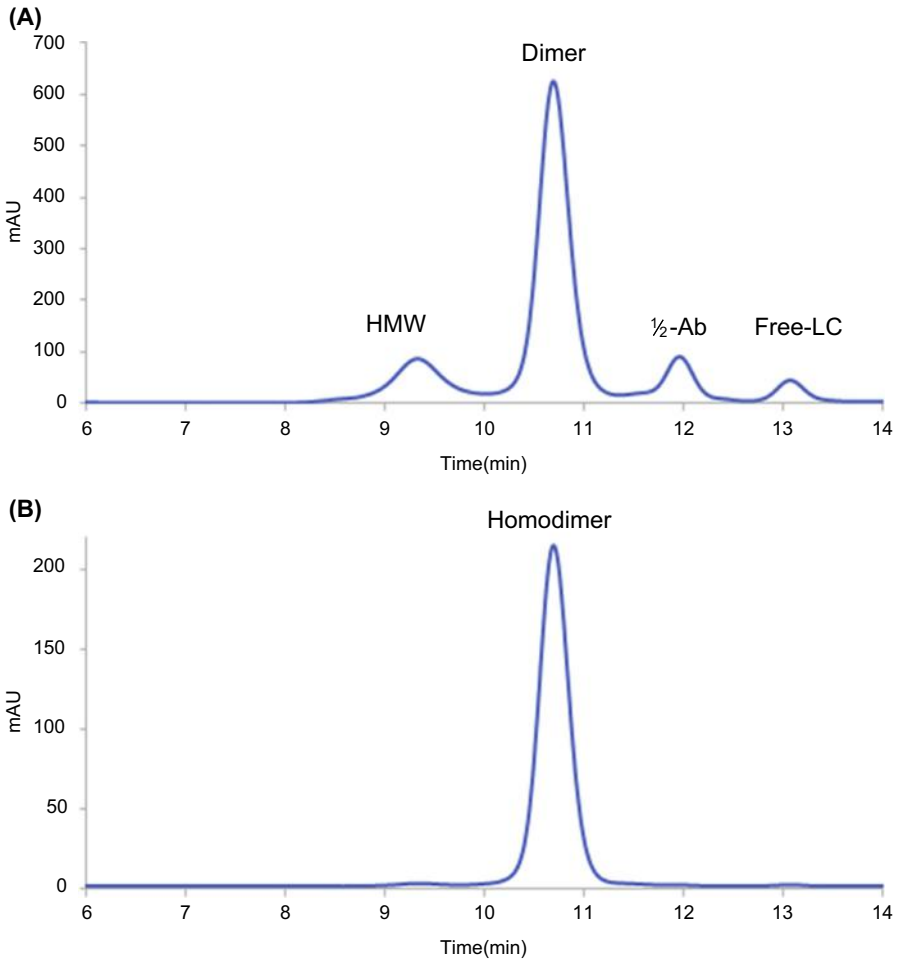


FIG. 3.11 SEC analysis of bispecific purified by (A) Protein A and (B) AVI-4933 resin.

the possibility to create platform resins that can remove these problematic product related impurities. Each proprietary design can now have a corresponding proprietary purification platform. The creation of a reliable purification platform, for each bispecific design, will allow for significant throughput and yield increases due to loosening restrictions on upstream development.

Summary

The diversity and complexity of therapeutic molecules that contain an Fc domain has increased and brought new challenges to purification. While protein A is quite rightly the mainstay of affinity chromatography for molecules comprising Fc, the universality of its

binding selectivity can sometimes pose a liability. As such, it captures product related impurities as equally well as the desired product for the modern complex molecules. To complement the use of protein A, we have developed high fidelity affinity purification that separates the product related impurities from the desired product and demonstrated how it can be used with 4 case studies. The case studies demonstrate the different moieties that might be targeted to differentiate the product from the PRIs, as frequently the most appropriate target is not in the Fc domain. It is not necessary to use these resins just as polish resins, because in many instances these affinity resins can be substituted for protein A as the primary capture while still maintaining the high fidelity among from the product related impurities, providing exceptional purity in a single step.

References

[1] Dow E, Tin C. Key Considerations for Automating Octet Assays: Ligand Screening at Avitide. 2019. https://www.moleculardevices.com/sites/default/files/en/assets/white-paper/biologics/automating-octet-assays-ligand-screening-avitide.pdf.
[2] Mohan, S. Multiplexing Ligand Discovery to Create On-Demand Affinity Purification Columns | SelectScience n.d. https://www.selectscience.net/SelectScience-tv/Videos/multiplexing-ligand-discovery-to-create-on-demand-affinity-purification?videoID=4383#.XBp2ULT_pnA.linkedin (accessed May 15, 2019).
[3] Ghose S, Tao Y, Conley L, Cecchini D. Purification of monoclonal antibodies by hydrophobic interaction chromatography under no-salt conditions. mAbs 2013;5:795–800. https://doi.org/10.4161/mabs.25552.
[4] Aono H, Iliescu I, Cecchini D, Wood S, McCue JT. Mitigation of chromatography adsorbent lot performance variability through control of buffer solution design space. J Chromatogr A 2013;1318:198–206. https://doi.org/10.1016/j.chroma.2013.10.019.
[5] Wahome WZ, Kundu A, Wahome J. Impact of lot-to-lot variability of cation exchange chromatography resin on process performance. Biopharm Int 2008;21.
[6] Du X, Li Y, Xia Y-L, Ai S-M, Liang J, Sang P, et al. Insights into protein–ligand interactions: mechanisms, models, and methods. Int J Mol Sci 2016;17. https://doi.org/10.3390/ijms17020144.
[7] Müller E, Vajda J. Routes to improve binding capacities of affinity resins demonstrated for Protein A chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2016;1021:159–68. doi:10/f8pps5.
[8] Kontermann RE, Brinkmann U. Bispecific antibodies. Drug Discov Today 2015;20:838–47. https://doi.org/10.1016/j.drudis.2015.02.008.
[9] Brinkmann U, Kontermann RE. The making of bispecific antibodies. mAbs 2017;9:182–212. https://doi.org/10.1080/19420862.2016.1268307.
[10] Spiess C, Zhai Q, Carter PJ. Alternative molecular formats and therapeutic applications for bispecific antibodies. Mol Immunol 2015;67:95–106. https://doi.org/10.1016/j.molimm.2015.01.003.
[11] Li Y. A brief introduction of IgG-like bispecific antibody purification: methods for removing product-related impurities. Protein Expr Purif 2019;155:112–9. https://doi.org/10.1016/j.pep.2018.11.011.
[12] Intelligence B. Bispecifics: turning great deals into great drugs. Beac intell 2019. https://register.gotowebinar.com/recording/4792569272921973761.
[13] Greg E, Nicolas F, Jean-francois D, Nicolas F, Giovanni M. Methods of purifying antibodies. US 10047144 B2. 2012.
[14] Ramaraj T, Angel T, Dratz EA, Jesaitis AJ, Mumey B. Antigen–antibody interface properties: composition, residue interactions, and features of 53 non-redundant structures. Biochim Biophys Acta BBA - Proteins Proteomics 2012;1824:520–32. https://doi.org/10.1016/j.bbapap.2011.12.007.
[15] Jendeberg L, Persson B, Andersson R, Karlsson R, Uhlén M, Nilsson B. Kinetic analysis of the interaction between protein A domain variants and human Fc using plasmon resonance detection. J Mol Recognit JMR 1995;8:270–8. https://doi.org/10.1002/jmr.300080405.
[16] Linhult M, Gülich S, Gräslund T, Simon A, Karlsson M, Sjöberg A, et al. Improving the tolerance of a protein a analogue to repeated alkaline exposures using a bypass mutagenesis approach. Proteins 2004;55:407–16. https://doi.org/10.1002/prot.10616.

[17] Shukla AA, Hinckley P. Host cell protein clearance during protein A chromatography: development of an improved column wash step. Biotechnol Prog 2008;24:1115–21. https://doi.org/10.1002/btpr.50.
[18] Rudnick SI, Adams GP. Affinity and avidity in antibody-based tumor targeting. Cancer Biother Radiopharm 2009;24:155–61. https://doi.org/10.1089/cbr.2009.0627.
[19] Vauquelin G, Charlton SJ. Exploring avidity: understanding the potential gains in functional affinity and target residence time of bivalent and heterobivalent ligands. Br J Pharmacol 2013;168:1771–85. https://doi.org/10.1111/bph.12106.
[20] Jiang B, Liu W, Qu H, Meng L, Song S, Ouyang T, et al. A novel peptide isolated from a phage display peptide library with trastuzumab can mimic antigen epitope of HER-2. J Biol Chem 2005;280:4656–62. https://doi.org/10.1074/jbc.M411047200.
[21] Sun T-Y, Wang Q, Zhang J, Wu T, Zhang F. Trastuzumab-peptide interactions: mechanism and application in structure-based ligand design. Int J Mol Sci 2013;14:16836–50. https://doi.org/10.3390/ijms140816836.
[22] Riemer AB, Klinger M, Wagner S, Bernhaus A, Mazzucchelli L, Pehamberger H, et al. Generation of peptide mimics of the epitope recognized by trastuzumab on the oncogenic protein her-2/neu. J Immunol 2004;173:394–401. https://doi.org/10.4049/jimmunol.173.1.394.
[23] Bellofiore P, Petronzelli F, De Martino T, Minenkova O, Bombardi V, Anastasi AM, et al. Identification and refinement of a peptide affinity ligand with unique specificity for a monoclonal anti-tenascin-C antibody by screening of a phage display library. J Chromatogr A 2006;1107:182–91. https://doi.org/10.1016/j.chroma.2005.12.064.
[24] Zhang H-M, Li C, Lei M, Lundin V, Lee HY, Ninonuevo M, et al. Structural and functional characterization of a hole–hole homodimer variant in a "knob-into-hole" bispecific antibody. Anal Chem 2017;89:13494–501. https://doi.org/10.1021/acs.analchem.7b03830.

CHAPTER

4

Recent advances in continuous downstream processing of antibodies and related products

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List of Abbreviations

APIs Active Pharmaceutical Ingredients
BPOG BioPhorum Operations Group
cm Centimeter
CV Column Volume
DNA Deoxyribonucleic Acid
EU Europe
FDA Food & Drug Administration
GMP Good Manufacturing Practice
g/L Grams per Liter
HCCF Harvested Cell Culture Fluid
HILVOP High Intensity, Low-Volume Perfusion
L Liter
mAb Monoclonal Antibody
MVDA Multivariate Data Analysis
NPV Net Present Value
pH Power of Hydrogen
PAT Process Analytic Technologies
PD Process Development
SEC Size Exclusion
SUT Single Use Technology
sq/ft Square Feet
TFF Tangential Flow Filtration
VI Viral Inactivation
US United States
UF/DF Ultrafiltration/Diafiltration
UV-C Ultraviolet Short wavelength

Approaches to the Purification, Analysis and Characterization of Antibody-Based Therapeutics
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Introduction

Through the last century process intensification via continuous manufacturing has made a revolutionary impact on the steel, chemical, food and petrochemical production industries [1], so why has it taken so long for the Biopharmaceutical industry to embrace continuous processing? Only recently has the first mAb made by a fully continuous process entered into clinical trials (BiosanaPharma biosimilar for omalizumab) [2]. Here we review the recent advances in continuous downstream processing of antibodies and related products, including the biopharmaceutical landscape, transformative technologies to enable continuous processing and the current state of the art in continuous bioprocessing as demonstrated by the early adopters.

Introduction: drivers for change in biopharm manufacturing — changing landscape

More than 80 mAb therapeutics have achieved regulatory approval and a total of 12 new mAbs were approved in the EU or US in 2018 [3]. As such, the industry can now be considered mature and like other mature industries cost saving through process intensification comes to the fore [4]. The requirement for reduced cost comes through competition from multiple mAbs for the same indication and from biosimilars. Competition results in unpredictable demand. Thus, reduced cost and increased flexibility will be the key drivers in future bioproduction facilities.

Biosimilars

The success of mAb biotherapeutics is linked to some high profile scientific awards. In 2018 the Nobel Prize for Chemistry was jointly awarded to George P. Smith and Sir Gregory P. Winter for phage display of peptides and antibodies [5]. Adalimumab became the first phage display derived mAb to gain market approval in 2002 [3]. Adalimumab has gone on to become the highest grossing therapeutic with global sales of almost $20 billion per annum [6]. However, patents for Adalimumab along with many other biotherapeutics have recently expired. The worldwide top 10 list of highest grossing biologic therapeutics and their patent expiry dates is shown in Table 4.1 [7,8]. The fact that most of these patents have expired or will expire soon has been the impetus for biosimilar development programs, of which there are more than 1000 currently ongoing [9]. Changes in the bioprocessing landscape are inevitable due to the development of multiple biosimilars for each mAb. The competition in this space is already so high that Momenta has ended its development of an Adalimumab biosimilar based on the likelihood that when legal agreements between the originator firm and the biosimilar companies expire in 2023 the market will already be saturated [10]. Similarly, Sandoz halted its submission for a rituximab biosimilar after the FDA asked for more information, based on the assumption that the market for rituximab will be saturated before the data could be acquired [11].

TABLE 4.1 The list of top 10 list of highest grossing biologic therapeutics worldwide and their patent expiry dates.

Therapeutic	Generic name	Global sales in 2017 (USD$)	Launch date	Patent expiry date (generic expiry date)
Humira	Adalimumab	18.4 billion	2002	Oct 2018 (Dec 2016)
Rituxan	Rituximab	9.2 billion	1997	Nov 2013 (Sep 2016)
Enbrel	Etanercept	7.9 billion	1998	Aug 2015 (Nov 2028)
Herceptin	Trastuzumab	7.4 billion	1998	Jul 2014 (Jun 2019)
Avastin	Bevacizumab	7.1 billion	2004	Jan 2022 (Jul 2019)
Remicade	Infliximab	7.1 billion	1998	Feb 2015 (Sep 2018)
Lantus	Insulin glargine	5.7 billion	2000	2014 (2014)
Neulasta	Pegfilgrastim	4.7 billion	2002	Aug 2017 (Oct 2015)
Avonex	Interferon beta-1a	2.1 billion	1996	2015 (2015)
Lucentis	Ranibizumab	1.5 billion	2006	2022 (Jun 2020)

In the saturated landscape it will be cost that drives success through the ability to penetrate new markets, and so it is no surprise biosimilar companies such as BiosanaPharma are driving innovation in process intensification via continuous processing. The competition also introduces an element of unpredictable demand for both the originator and the biosimilar, putting a premium on flexible manufacturing.

Multiple mAbs for the same indication

In 2018 the Nobel Prize in Physiology or Medicine was awarded to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation." [12]. Since this initial discovery many immune response regulatory checkpoints have been discovered and a series of antibody therapeutics targeting these checkpoints have been developed: For CTLA-4 (ipilimumab) [13], and for PD-1 (cemiplimab, nivolumab, pembrolizumab), the ligand for PD-1 (PD-L1; durvalumab, avelumab, atezolizumab) [14]. The preponderance of therapeutics all acting in the same or similar pathways provides a level of competition that will ultimately result in cost pressures. Currently we see some of these drugs going head to head for the treatment of new indications [15]. This leads to unpredictable demand and drives the requirement for flexible manufacturing.

Intensified upstream process and the downstream bottleneck

An increased demand for biologics has driven the intensification of the upstream process to boost productivities and lower manufacturing costs. Subsequently, titers of fed batch manufacturing processes have increased from 0.2 g/L to >3 g/L [16]. Additionally, Perfusion processes, originally used as a tool to meet the large demand or for production of labile

molecules [1,17], have been revisited due to their increased productivity [18]. Notably, high intensity, low-volume perfusion (HILVOP) processes offer much higher productivities than fed batch production [19].

However, the advances in downstream processing have not kept pace with the increases in titer. Consequently, the processing bottleneck has shifted from upstream to downstream operation [20]. Even high capacity chromatographic capture media cannot cope with the high titers prevalent in modern cell culture processes [21,22]. This deficiency has led to increased interest in multicolumn chromatography which can effectively uncouple column sizing from titer. Columns can be chosen based on flow rate and more columns can be added to the process as titers increase [23].

Small molecule success and regulatory drive

The small molecule pharmaceutical industry is already benefiting from a switch to continuous flow technology [24]. Here, a stream of chemical reactants is continuously flowed into a reactor to yield a desired product [25]. Benefits of this kind of operation include rapid mixing, enhanced heat transfer, intensified mass transfer and ability to carry out exothermic reactions due to high surface-area-to volume ratio [26]. The flow technology equipment allows for rapid scale out avoiding many problems inherent in scaling up batch processes. Reaction profiles that benefit from very high or very low temperature (above 200°C and below −40°C) and higher pressures have been extensively explored using microreactor technology, which has become widely commercially available from a variety of manufacturers. Other advantages include minimization of long-term storage of large stocks of materials and ability to carry out reactions involving hazardous gases in a safe manner. As a result, continuous technology has enabled the pharmaceutical industry to synthesize large numbers of active pharmaceutical ingredients and natural products [26,27].

The FDA's support in terms of approvals and reviews of the new products on the market has resulted in burgeoning acceptance of continuous methods in the small molecule industry. Beginning in 2015, the agency has approved four products made using continuous processes—two cystic fibrosis drugs (Orkambi and Symdeko, from Vertex Pharmaceuticals), a breast cancer drug (Verzenio, from Eli Lilly and Company), and an HIV drug (Prezista, from Janssen Pharmaceuticals) [28]. This acceptance and encouragement from the FDA for continuous manufacturing of small molecule drugs paves the way for continuous bioprocessing. The FDA are actively encouraging an update of the biomanufacturing process as it holds the possibility of "improving the overall quality of products and availability to patients" [29]. As a part of the push for a Quality by Design (QbD) approach, the FDA has charged the Emerging Technology Team to help early adopters of continuous manufacturing to "help and resolve implementation challenges and navigate the application review process for products made with these modern methods" [30].

Pros and cons of continuous processing

Given the landscape of the biopharmaceutical industry along with government and societal pressure to reduce the cost of drugs [31,32] it should be no surprise that there has been a focus on process intensification. There are potentially significant improvements in process economics despite the cost of goods being a relatively insignificant amount of the

sale price [33]. There have been a series of cost of goods analyses for continuous processing relying on a number of methods [34–40]. However, the outcome of these is strongly dependent on the assumptions used and as no continuous manufacturing process has been implemented and every companies manufacturing scenario is different, the direct economic advantages remain speculative; this is a viewpoint shared by the BioPhorum Operations Group [22].

Although many aspects of continuous processing are not readily monetarily quantifiable, there are features that may still facilitate a compelling case for implementation. If we consider swift and even flow of product through the manufacturing process we can immediately understand that this new technology has the potential to reduce the overall manufacturing time and to subsequently improve product quality [41,42]. In a batch process, all the material is processed through a unit operation and collected in a hold tank before starting the next operation. On the other hand, the continuous approach is contingent on minimizing the surge tanks between unit operations and starting the following unit operation as soon as material is ready to process. Thus, in continuous bioprocessing, all unit operations operate in parallel, maximizing equipment and facility utilization. For a batch process where, perhaps, just one or two unit operations are performed each day, it may take >1 week for the product to go from the bioreactor to formulation. In continuous processing we see case studies where the product from the bioreactor is processed to formulation in <20 h [43,44]. This speed of operation is especially important when it comes to processing unstable biologics. In this case, continuous manufacturing may be the only way to bring these products to the market [45].

Increased equipment utilization enables further benefits: Despite the decreased overall process time, each individual unit operation can be performed for a longer period of time. This introduces the opportunity to operate at a lower flow rate and/or perform more purification cycles leading to smaller equipment and a reduced footprint [46].

In batch capture chromatography, for example, the bioreactor is normally processed with 2–4 chromatography cycles. However, in continuous chromatography the columns can be operated with shorter residence times, enabling the use of smaller columns which can be cycled more often to more effectively use the lifetime capability of the resin. The smaller columns are available pre-packed, with an internal diameter up to 80 cm [47], further de-risking the chromatography operation.

The smaller equipment also enables the implementation of single use (SU) flowpaths, which can become prohibitively expensive or technically unfeasible at the larger scale. SU brings added benefits: reduced cleaning and its validation along with simpler facilities including modular 'ballroom' floorplans which are cheaper and quicker to build and are suited to rapid changeover between different products. This type of facility can be leveraged as a one size fits all processing suite, where the same equipment can be used through the product development cycle, toxicology, clinical trial and through to full manufacturing. This flexibility is enhanced through the ability of perfusion processes to adjust the amount of product manufactured by operating for different time periods. Thus, costly and time-consuming scale up can be avoided, perhaps enabling faster access to the drug.

Another advantage of increased cycling is that continuous processes operate in a steady state, which has proven for other industries to improve reliability than batch operations [48]. Steady state operation also brings with it the ability to generate much more data that can be used to predict, though statistical methods such as MVDA, when a process is drifting

from a desired state before the process fails. Thus, continuous processing may drive increased quality through dynamic control.

Nevertheless, these advances are dependent on overcoming significant technical hurdles. Ultimately the progress comes from not just the transfer of individual unit operations to continuous mode, but also the coupling of the unit operations to develop a process. Below we will discuss some transformative technologies and how the early adopters are piecing together their own continuous downstream purification platforms.

Key enabling technologies for continuous processing

Acoustic wave separation

Significant investments in process development are yielding higher intensity, higher productivity cell cultures. In many situations, these increases in productivity also increase the biomass within a bioreactor. For batch upstream processes centrifugation or single-use depth and traditional filtration media are used to reduce the cells and cell debris from the drug product. However, at high cell densities centrifugation becomes less robust and traditional filtration media can clog prematurely, necessitating increased filtration footprint which cannot always be accommodated in current facilities [49]. For perfusion processes cell retention is typically performed via hollow fiber tangential flow filtration, either with unidirectional or alternating flow. However, it is common for product transmission across the hollow fiber to decrease over time and for product to become trapped in the bioreactor [50].

Therefore, the challenge of high cell densities has led to the exploration of alternate primary clarification technologies, including acoustic wave separation. For more than 50 years, nonlinear acoustics have been used in a variety of industries; from medical ultrasound to underwater detection, microfluidics to acoustic levitation [51,52]. These applications are governed by a well characterized set of physical wave equations [53,54]. In broad terms, acoustics can be precisely and intelligently controlled to coalesce and remove particles from a carrier fluid in a continuous, non-fouling, scalable process. This unique aspect of the technology makes it suitable for inclusion as the primary clarification step of a truly continuous drug manufacturing facility [55].

The core processive portion of an acoustic separator, a piezoelectric transducer and acoustic reflector, spans a fluid path through which cell culture fluid passes (Fig. 4.1). In Pall's Acoustic Wave Separator, a 3D acoustic standing wave is established between the transducer and the reflector [56]. Inside the single-use resonator, the standing wave sets up a highly tuned pressure field with predictably arranged nodes of high and low pressure in which particles coalesce based on their density relative to the cell culture fluid. Coalescing multiple particles into a larger clump enables gravity to readily pull clustered particles out of suspension, where they are collected and removed or added back into the process (depending on the particular fluidics and demands of the processing step).

When used in the clarification of a fed-batch process, the cell-laden bioreactor fluid is passed through the pressure field in the resonator. By coalescing and removing the particulates from the fluid, the flow path funnels the cells away from the mAb-laden permeate, allowing them to be continuously pumped into a concentrated waste stream — the permeate

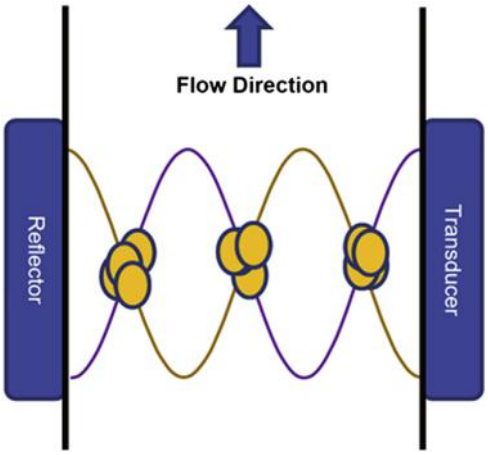

FIG. 4.1 The illustration of acoustic wave separation for cell lysate clarification. The cells, shown in yellow (light gray in print version), are retained and the product freely flows across the 3D acoustic standing wave.

being passed down to subsequent processing steps as a clarified fluid. In a perfusion process, where retention (and not disposal) of the cells is required, a tangentially recirculating bioreactor feed stream passes beneath the acoustic field, selectively allowing mAb to pass through the acoustics unhindered as permeate, while the cells are returned to the bioreactor for further culture. In both applications, the propriety acoustic algorithm and carefully engineered flowpath work to separate mAb from the mAb producing cell culture in a continuous, gentle, and efficient manner.

Multicolumn chromatography

Multicolumn chromatography is a key enabling technology in the move toward continuous processing. There has been considerable focus on intensifying the Protein A capture chromatography step. This is in part because of the relative expense of Protein A resin [57], which represents a significant proportion of the downstream processing cost [58]. A common feature of Protein A resins is that their capacity for mAb is strongly dependent on the residence time of the load step [59]. Protein A membranes have been shown to uncouple this dependency. However, due to the lower surface area of membranes capacities have lagged behind resin by two-fold or more [60]. To maximize capacity, Protein A resins are typically loaded at residence times of 4 min or greater [61]. High residence time leads to relatively low productivity, which is often expressed as grams of product purified per liter of resin per hour. In a typical batch manufacturing scenario, the Protein A column is sized based on the capacity to process the bioreactor with two to four chromatography cycles, leading to large columns and significant capital expenditure [62].

To address the shortcomings of the batch Protein A step, a range of continuous chromatography solutions have been proposed. Most of these solutions rely on loading more than one column at a time, bringing about increased capacity and productivity [63]. Loading can be performed more quickly (lower residence time) because secondary and tertiary columns can be used to capture unbound product. This opens the possibility to reduce the

amount of resin required in a commercial bioprocess and improve manufacturing economics through quicker cycling of the resin [23,40,64–68].

A number of companies have developed continuous chromatography systems for the biopharmaceutical market based on loading more than one column at a time. The systems are differentiated primarily on the number of columns which can be operated. For example, the Chromacon Contichrom Cube has 2 columns, GE PCC 3 or 4 columns, Novasep Bio SC 6 columns, Semba Octave 8 columns and Pall BioSMB GMP 8 or PD 16 columns. All of these systems rely on improving efficiency by overloading the primary load column past the product breakthrough point. Product loss is avoided through capture of product that breaks through the first column via subsequent columns. Column overloading can be visualized as breaking up a larger column into smaller parts to as to more actively utilize the mass transfer zones [34]. All columns go through the same operation and product quality is consistent through repeating chromatography cycles. To understand the impact of the number of columns and the operation of the different systems it is useful to consider the chromatography cycle as 2 phases, load and non-load. The later includes all the non-loading steps of the chromatography cycle including wash, elute, regeneration and re-equilibration. The conceptually simplest operation is with three columns, where at any point of time two chromatography columns are loaded in series, while the third column is going through the non-load steps. All of the columns go through the cyclical chromatography operation. The column that receives load directly will at some point be saturated with product. At this point, the loading to this column can cease and the column is relegated to the non-load steps. The second load column that was receiving the flow-through of the first column now receives load directly and the regenerated column is placed after the primary capture column, to prevent product loss. At modest titers, 1 g/L, the load step is rate limiting [23]. Capacities of >40 g/L can be achieved with loading at 1min residence time[23]. The non-load steps can also be performed at 1 min residence time, and typically 20 to 30 CV are required, to wash, elute, clean and regenerate a Protein A column. As titers increase the load time is reduced. At 10 g/L, perhaps only a 4 min load time is required with a 40 g/L capacity and a 1 min residence time of loading [69]. The non-load steps cannot be performed within this time frame and become the rate limiting step, Fig. 4.2 There are multiple possible solutions to combat this phenomenon including slowing or stopping the load, which impacts productivity. Another option is to add more columns to perform the non-load steps of the process. This drives productivity at higher titers.

The Continuous Countercurrent Tangential Chromatography technology developed by Chromatan represents a notable alternative approach to multicolumn chromatography [70]. In this approach, resin is pumped around the system, reducing the dependence on packed bed columns. The advantage of this is that it facilitates a true continuous elution, unlike the column methods which supply discrete eluates. This represents a further step away from the current batch column process. One drawback of this method centers around resin stability and lifetime, which must be validated. Additionally, elution concentrations appear to be modest compared to column based chromatography, impacting the productivity of the downstream unit operations.

Other continuous chromatography solutions have been explored. These include annular, simulated moving bed (SMB) and its variant Multi-Column Counter-current Solvent

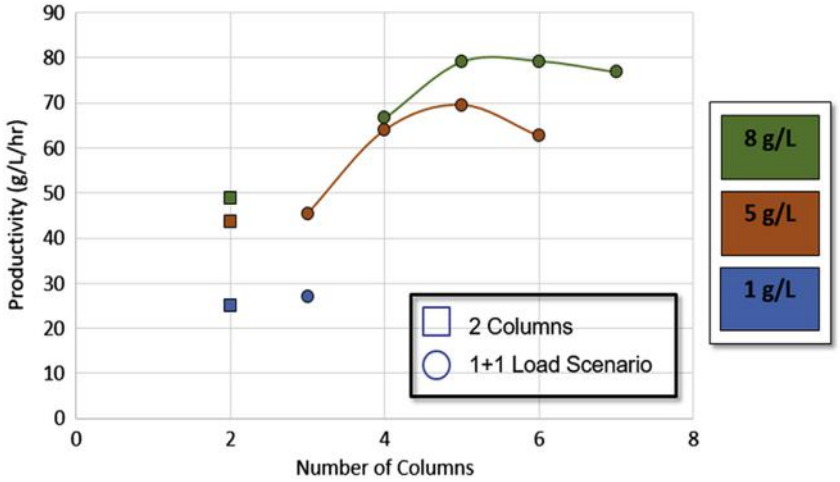

FIG. 4.2 The productivity comparison of scenarios with different loading configuration in multi-column chromatography. The 2 columns scenario, as advocated by Chromacon, depends upon a system to operate a total of two columns. The columns are intermittently interconnected for the load step, and disconnected for one of the columns to undergo the non-load steps. The 1 + 1 scenarios, with two columns in series, is a commonly used loading scenario for when 3 columns or more are available. The 2 column scenario gives greatly enhanced productivities over single column, but as titers increase productivities can be increased through the addition of more columns.

Gradient Purification (MCSGP). These methods have generated limited interest in bioprocessing. Annular chromatography relies upon packing separation-medium within the annular space of two concentric cylinders [71]. The column is slowly rotated, whereas the feeding arrangement and the fraction collector are kept stationary. Currently there are no commercially available systems for operating continuous annular chromatography. This is perhaps because the technical challenges of evenly packing the separation matrix and sealing the rotating parts. SMB is performed by periodically switching column inlets, forming a simulated countercurrent of separation matrix and liquid [72]. SMB is ideally suited for binary separations of chiral compounds, which has led to common application to intermediates in synthesis of natural products. There are no current bioprocesses using SMB, however it may have utility for processes where (SEC) size exclusion chromatography is essential. MCSGP is a variant of SMB where partially purified fractions are recycled to the load [73]. This offers the possibility of high purity and high yield, but reloading material can lead to low productivities especially when applied to SEC.

Low pH virus inactivation

Low pH virus inactivation (VI), a key step in assuring product safety, is normally performed directly after the Protein A capture step [74]. In current batch manufacturing processes VI is mostly performed manually: Titrations are performed by pumping in acid and base and the pH of the product is often verified by sampling and testing via a calibrated benchtop pH meter. This process is not easy to automate although batch systems are available from GE, Sartorius and Pall.

As combined perfusion upstream processes and continuous capture downstream processes are now considered, this places a focus on the VI unit operation. A number of different possibilities for VI are being explored. These can be categorized into 3 classes:

Continuous stirred tank reactors

The only commercially available continuous low pH virus inactivation solution is the Cadence VI system from Pall [75]. The Cadence VI relies on a two-tank system for the operation of semi continuous VI. The functions of the tanks alternate, one tank receives new eluates from the upstream process. The other tank is used to operate the low pH inactivation step: When the process is complete this tank is emptied and can then receive new eluates. Meanwhile the eluates collected in the other tank can be processed through the low pH step. This way product is collected and processed within the same tank. The Cadence VI system can be considered to perform an automated version of the current batch process and this may offer a relatively simple path to performance validation. However, there are several risks to this approach and these are highlighted by the biopharma users group. Many of these risks were identified in the development of the system and have been mitigated or reduced through the system design and testing.

These key risks include contamination of inactivated product with non-inactivated product through dead legs (isolated branches or tubing sections with one end capped) and hanging drops. This risk has been mitigated by introducing liquids into the low point of the mixer, eliminating splashing and through the use of Artesyn valves which have a minimal hold up volume and a recirculation loop. These features also reduce the risk of acid and base back mixing, eliminating the opportunity for pH drift during the hold step. Another key risk is the pH probe, which has to remain finely calibrated for prolonged periods of time. At Pall, pH probes were tested with a robotic arm to transfer the probes between low and high pH buffers to mimic the VI. Testing showed the pH probe drift was minor, 0.05 pH units in 48 h, making these probes suitable for Continuous VI.

Cyclical flow reactors

The challenges of automating the current batch VI process and the desire to make something more continuous has led several groups to pursue cyclical (plug) flow reactors [76–78]. Here the required low pH hold time is provided by a tortuous flow path. To minimize axial distribution of the flow and to minimize the distribution of residence time product spends in the plug-flow device a tube with alternating coiling direction has been developed to introduce Dean vortices and promote homogeneity. Despite some patent activity [79,80,81], there is no commercially available solution. There remain some technical challenges and risks as identified by the Biopharma users forum. The biggest challenges center around titration and pH measurements. Eluates from Protein A vary in protein conductivity and pH across the elution peak. This variability has been addressed by pooling material before titration, but this still presents a challenge of ensuring material is homogenous enough to reach the inactivation pH before entering the plug flow reactor. This risk is amplified as pH probes have a hysteresis: The possibility remains for product to not achieve the inactivation pH, under a constant flow situation, and pass by the pH probe undetected.

Column based strategies

The axial dispersion and subsequent broad residence time of the plug flow approach may be addressed by using column based approaches to supply the necessary hold time at low pH. A variety of media have been applied, including Protein A, Cation Exchange, Size Exclusion and inert beads [41,82–84]. The Protein A and Cation Exchange approaches are compelling. Using these enables low pH VI to be combined into a required chromatography step, which may be considered a further advancement toward process intensification. However, there are downsides to the approach. High conductivity buffers are required to maintain mAb - Protein A binding at low pH. It is unclear how these buffers, and the typical increase in turbidity that is seen post inactivation, will have on the lifetime of the resin. Additionally, it would be challenging to claim virus clearance for both the Protein A step and the low pH inactivation.

The drawback to using SEC is media compressibility. Consequently, SEC can only be operated at low linear velocities [85]. Thus, adding the requirement for a relatively large diameter packed column to be introduced into the process. An improvement to this approach may be the use of inert beads. These have advantage of scaling by increasing the length of the column which adds more theoretical plates and lowers dispersion.

Alternatives to low pH virus inactivation

Low pH can be substituted for other methods of virus inactivation. Notably solvent/detergent inactivation can use the same equipment [86]. The advantage of solvent detergent inactivation is that it can be applied to pH sensitive proteins.

Another alternative approach for virus inactivation is UV-C irradiation [87]. UV-C irradiation along with high temperature has been applied to cell culture material to reduce virus load entering the process. UV-C has been developed into commercial systems by Bayer and commercialized by Sartorius. The systems employ a coiled flowpath to ensure consistent mixing and UV-C exposure. This approach is ideally suited to continuous manufacturing. Nothing is added and the inactivation can take place with continuous flow. Despite the advantages, the UV-C systems no longer appear to be marketed. UV-C induces pyrimidine dimers in DNA. Such lesions can arrest DNA polymerase, preventing replication. However, UV-C irradiation can also damage proteins through photolysis or the generation of reactive species, resulting in protein thiol oxidation [88]. Perhaps because of the potential for protein damage, UV-C irradiation has found limited applications in bioprocessing.

Single-pass tangential flow filtration

Single-pass tangential flow filtration (SPTFF)

Conventional TFF is ubiquitously applied to the formulation of biopharmaceuticals. The process is typically ultrafiltration (UF), concentrating protein by de watering followed by diafiltration (DF) to place the product into the correct buffer system and UF to achieve the final concentration. For biomolecules the formulation concentration is characteristically greater than 100 g/L to minimize the volume of product to be injected into the patient [89]. The downside of conventional TFF is multiple passes through the device are required,

which necessitates numerous passes through a pump and TFF, which can generate shear forces that can damage the product. Additionally, the multiple pump passes necessitate recirculation to a hold tank using a relatively large feed pump and large diameter system pipework. This is fundamentally a batch operation.

While many advancements in membrane properties and materials of construction have occurred since the introduction of TFF more than four decades ago, the principles of TFF have not changed substantially over that period. Therefore, SPTFF Technology represents the first significant change in the practice of TFF since its invention, simplifying the purification process and creating new capabilities not possible with conventional TFF. As such, it takes TFF technology to a new degree of manufacturing capability, robustness, sophistication and enables truly continuous operation.

SPTFF addresses the drawbacks of conventional TFF, by delivering high conversions in a single pass, thereby providing the performance of TFF with the simplicity of direct flow filtration. As shown in Fig. 4.3, and in contrast to conventional TFF processes, SPTFF processes are continuous and do not have recirculation loops, eliminating the need for in-process tanks and enabling the processing of a stream as soon as available without the need to accumulate a batch.

Typically TFF and SPTFF are operated with the same kinds of modules using the same cassette geometry, including the same membrane and the same feed and permeate screens. However, SPTFF modules are configured to have much longer flow paths, are driven with somewhat lower flow velocities and somewhat higher pressures, and have a flow channel whose cross-section changes along the flow path. This combination of properties and features enables high conversions per pass, with equal to or greater productivity as compared to conventional TFF [90].

SPTFF processes perform the same separations as TFF processes; whatever is done today with TFF process can also be performed with SPTFF. In an SPTFF process the complete separation occurs in a single-pass along the flow path of the module over a period of a few minutes under steady-state conditions. In contrast, in a conventional TFF process the separation occurs gradually over typically 3–4 h of processing time. This attribute can result in increased yields for sensitive products, which lend themselves to continuous processing [91].

In line concentration via SPTFF has been applied at a range of positions in bioprocesses, including the interface between upstream and downstream processes, where it can be used to uncouple them completely [92], increasing the productivity of capture chromatography for perfusion processes that have modest titers [90], buffer exchange prior to chromatography, or concentration/buffer exchange for final formulation prior to sterile filtration.

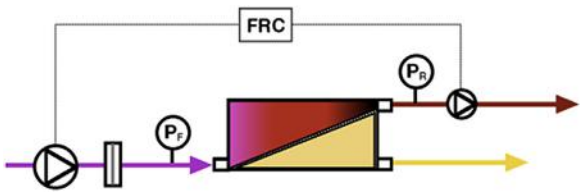

FIG. 4.3 P&ID of SPTFF process.

SPTFF for in line diafiltration (ILDF)

SPTFF has been adapted for diafiltration applications to enable continuous buffer exchange [93]. An ILDF process is essentially a series of SPTFF concentration steps within a single device, the effluent of each concentration step is diluted with the DF buffer and then re-concentrated through the next stage. Such modules have a DF-distributor, a fluidic device that distributes the DF buffer appropriately into each DF stage.

In contrast to a SPTFF concentration module, the flow path of ILDF modules is typically of constant cross-section since the changes in the fluid properties do not change as much as in a SPTFF concentration module.

Fig. 4.2 is a P&ID of an ILDF process showing the additional DF stream, requiring a second Flow rate control (FRC) to control the DF rate. In an ILDF process the effluent stream may or may not be at the same concentration as the feed stream. In the example shown below it is assumed that there is no concentration occurring during the ILDF process, and therefore, that FRC maintains a concentration factor of 1 (Fig. 4.4).

Just as for diafiltration in conventional TFF, the larger the DF volume the larger the removal factor, defined as the reduction in the concentration of the undesirable solute(s). Three logs or more of buffer exchange can be performed via a single pass through an ILDF device with six identical stages of concentration and dilution.

One drawback of performing the diafiltration by multiple dilution and re-concentration (batch diafiltration) steps, albeit continuously, is that the buffer consumption can be higher than in a conventional TFF diafiltration process. If desired, the efficiency of buffer usage can be improved by recycling the permeate from some of the diafiltration stages into the DF feed stream for the earlier stages. This is known as counter current DF [94].

SPTFF concentration and ILDF devices can be used in conjunction to perform continuous formulation via UF/DF/UF.This is a key advance in being able to operate a completely continuous process [95].

Updates from early adopters

The early adopters of continuous processing have taken many different routes to intensification. These decisions stem from the business case at each company, revolving around current manufacturing demand and capability versus predicted future requirements due to the drug pipeline. Here we aim to provide an update of the work from the leading early adopters, although it should be made clear, not all companies share many or even any details of their plans for intensification as they may consider it sacrificing their competitive advantage. Given this caveat the list is not exhaustive.

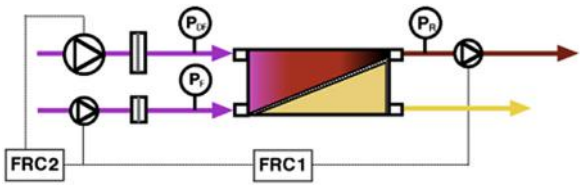

FIG. 4.4 P&ID of SPTFF/DF process.

Alvotech

Alvotech is focused on the development and manufacturing of biosimilars and one of their key pillars is to "Invest in highest quality and differentiation strategies to develop a value added and patient friendly product" [96]. The manufacturing platform at Alvotech is based upon a 1000 L perfusion bioreactor operating for up to 45 days, but based on batches of 15 day operation. Cell retention is via an ATF and the downstream process includes continuous capture with a 3 column process using the Cadence BiosMB followed by Cadence VI (Andrew Falconbridge, World Biopharm Forum, 2019).

Amgen

The road to process intensification for Amgen has been driven by higher intensity perfusion processes. Amgen claims that a 2000 L perfusion bioreactor with a volumetric productivity of 2.5 g/L-day can produce 50 kg of Drug Substance in 15 days. This is the equivalent to the output of a single 15,000 L fed batch bioreactor [97]. Amgen has combined perfusion upstream with a downstream process that minimizes hold tanks between steps to reduce footprint and increase productivity. Leveraging these advances, Amgen has built a 120,000 sq/ft manufacturing facility in Singapore. Built at a fraction of the price of a conventional plant, Amgen's Singapore facility occupies about one-fifth the size of a traditional pharma plant (about 170 thousand sq feet compared to 750 thousand sq feet at Amgens' Rhode Island Plant). One building containing manufacturing, controlled temperature warehouse, administration, quality control and clean utilities, was built in 18 months. The Plant's ballroom design, incorporating single-use systems to shorten the flow-path, obviated the need for clean-in-place systems and offers a high degree of flexibility.

Continuous bioprocessing has enabled much of Amgen's success. The upstream feed is directly fed into two column chromatography units separated with a small surge tank. The outflow of column 2 is fed directly into a viral filtration and UF/DF unit. The total process time to be carried is just 10−14 h.

However, some challenges of using single use systems at Amgen came from creation of a complex supply chain. To assess the biological impact of polymer films required Amgen to correlate raw material variability to process parameters. Characterization studies had to be done to understand the variability of bags used for bioprocessing. Establishing quality control testing to ensure consistent performance became necessary. Single use systems further required continuous monitoring and frequent verification of performance. The success of the Singapore facility is highlighted by the recent news that Amgen has broken ground on another next generation biomanufacturing plant in Rhode Island [98].

BiosanaPharma

As already discussed, BiosanaPharma recently became the first company to go to the clinic with a mAb produced by a fully continuous process. BiosanaPharma has named this process 3C, presumably because it utilizes a **c**ontinuous upstream perfusion bioreactor, combined with **c**ontinuous centrifugation to control cell viability and a **c**ontinuous downstream process. BiosanaPharma claims that "*The 3C technology platform is a high productivity, flexible, small footprint* (50m^2) *manufacturing platform capable of making 1 kg of drug substance antibody* per *week at a*

50 L *bioreactor scale. Batch processing is made continuous with multicycle counter current operation. The upstream process is based on High Cell Density continuous perfusion culturing with alternating bioreactor use. The downstream process comprises protein A and cation exchange in BioSMBs combined with flow through filtration. The process has GMP status and is designed to run for up to 2 months. All steps run in parallel while interconnected* via *smart software (21 CFR part II) for flow control and data acquisition.* BiosanaPharma may be unique in that they developed their continuous process in conjunction with a third party, Mycenax in Taiwan, where the proof of concept processes were performed at the 50 L scale − the same scale that is planned for the final manufacturing process [2].

Boehringer ingelheim (BI) and pfizer

BI and Pfizer have collaborated to realize a next generation manufacturing platform [99]. The goals of the platform include a fully disposable flow path, process intensification through continuous operation and a high degree of automation; all combined to provide a high degree of flexibility and agility for multi-product facilities. At the heart of the process is the next generation perfusion platform, HILVoP [19]. This is a non-steady state process where cell densities may reach up to 200 million cells/mL. BI/Pfizer claim the process yields 10 times more product than batch and 5x more product than steady state perfusion over a similar time. This represents up to 76 g/L of bioreactor over 14 days of operation. Product harvest continues over 10 days and cells are retained via hollow fiber TFF. The downstream process appears to be operated entirely using systems that have been developed in house. Chromatography is performed using an iSKID which supports alternating Protein A capture chromatography. While one column is being loaded the other column operates the non-load steps. This way continuous loading can be achieved. However, for simplicity the columns are only loaded one at time and the capacity of the resin is the same as for a batch operation. BI/Pfizer have also developed a novel solution to low pH VI, the "Jig in a box" (JIB) tortuous flow path continuous system [77].

Overall, BI/Pfizer claims that their next generation platform results in a 10-fold increase in productivity over batch operation, with the ability to produce 30−60 Kg of product per 14 day-2000 L bioreactor perfusion campaign.

Enzene

A subsidiary of Alkem Laboratories, Enzene aims to deliver cost-effective biosimilars through developing disruptive technology platforms and advanced analytics in its state-of-the-art manufacturing facility in Pune, India. By building a fully automated continuous cGMP compliant manufacturing plant for mAb production, Enzene aims to disrupt the affordability of biotech products globally. In this vain, Enzene has acquired a BioSC Pilot instrument from Novasep and aims to integrate this system into its tangential flow filtration and viral inactivation train.

Enzene claims 10 times greater cumulative productivity of perfusion versus a fedbatch culture method [100]. Approximately 5 times reduction in CAPEX through smaller factory footprint and reduction in OPEX by half through lower consumption of raw materials, steam WFI and electricity has been the driver behind Enzene's push into continuous manufacturing

space. They encountered some obstacles toward implementation of continuous manufacturing methodology such as necessity of find the right combination of the process, automation and PAT. For Enzene, the risk of bioreactor and chromatography line contamination and comes to the fore in the interplay of upstream and downstream trains. However, the savings incurred by maximal utilization of Protein A resin has been a major factor toward implementation of continuous manufacturing technology [101].

The Enzene example teaches us that more and more companies will invariably choose to implement continuous manufacturing in the biologics sector because the competitive advantage gained by competitors will be a difficult obstacle toward profiting from developing and manufacturing new therapeutics and biosimilars.

Genzyme

The Implementation of a perfusion process coupled with ATF technology for cell retention and Protein A capture via a 3C-PCC system (GE Healthcare) by Genzyme has been described previously [102]. To further develop this manufacturing platform, Genzyme has recently undertaken building of a Biologics R&D development facility. This new ~20,000 square feet facility, named for its location on New York Avenue in Framingham MA, the 31NYA Pilot Plant consists of two operational spaces which include a single use technology suite with wave bags and stirred SUBs up to 500 L size [103]. The goal of the facility is to evaluate and confirm selection of disposable bioreactor, cell retention technology and periodic counter-current chromatography design. Assessment of process manufacturability, demonstration of 'closed system' operation and performing 'at scale' studies to support continuous manufacturing process development are also mentioned as elements of the 'proof of concept' endeavor Genzyme aims to implement. The amount of effort and investment shows the Genzyme is serious about implementing continuous downstream manufacturing as it expands its biologics portfolio [104,105].

Just Biotherapeutics

Just Biotherapeutics brands itself as "integrated design company focused on technologies that will accelerate development of biotherapeutics and substantially reduce their manufacturing cost" [106]. Their aim is to deliver access to important biologics in the global marketplace using an integrated design approach.

Just Biotherapeutics presented proof of principle for continuous processing at manufacturing scale (ACS Orlando FL 2019). Their continuous process begins with media concentrates from a 500 L perfusion bioreactor. Cells were retained via the use of a hollow fiber tangential flow device. The clarified feed was directed to a 200 L mixer and the capture step was performed via a Pall BioSMB Process 80 operating a three column Protein A process. The elution pool was treated with low pH and neutralized on a Pall Virus Inactivation System, Cadence VI. The downstream continuous process from Protein A through viral inactivation was performed for 6 days, but there are future plans to demonstrate this process for a longer duration.

This work shows the ability to put together a continuous process using off the shelf equipment and highlights the synergies of being able to combine the upstream and downstream processes in a continuous way.

Merck

The motivation at Merck & Co. Inc. (Kenilworth, NJ) for continuous processing comes from their relatively low installed bioreactor capacity and turning this "disadvantage into a competitive advantage". To this end, Merck continue to be one of the pioneers of integrated

Continuous bioprocessing. We last reported on the activity at Merck in 2017 [102] focusing on their protein refinery operations lab (PRO Lab) and its operation of a combined upstream and downstream continuous process over a sixty-day period. The process is fully automated by control system Delta V and consists of a 10 L perfusion bioreactor connected to a continuous downstream process which includes Cadence BioSMB PD for Protein A capture and a plug flow reactor for low pH virus inactivation. Two polishing chromatography steps, one of which is performed on the Cadence BioSMB PD, are coupled to virus filtration and formulation [28]. Since then Merck have continued their activity in the lab working toward Quality by Design and process analytical technologies with the aim of enabling real time product release. Additionally, Merck have worked on the scale up of the Protein A capture step, transferring a process from single column to the multicolumn Cadence BioSMB PD and finally scaling up to the Cadence BioSMB Process all within three weeks. This process comparison showed that quality attributes remain unchanged, but the continuous processes were >3 times more productive [107] (Table 4.2).

Novartis

Novartis claims to be operating at close to full manufacturing capacity [108]. This provides a need to add capacity and an opportunity to innovate the manufacturing platform. The global manufacturing goals include cost reduction, maximizing net present value (NPV), enabling fast development, minimizing technology transfer risk and enabling flexibility. To this end, Novartis envisages a repetitive manufacturing facility that could produce a broad range of molecules. Novartis aims to use a single scale of equipment at all stages from development through manufacturing to eliminate the need for scale up.

The Novartis future manufacturing platform is based upon stainless steel, due to its proven robustness. The upstream process is based on perfusion cell culture operating at high cell density. This is coupled to a continuous capture process using the AKTA PCC

TABLE 4.2 Performance data of the cadence BioSMB PD and process systems.

	Batch benchmark	PD system	Process system
Eluted mAb concentration	9.54 g/L	13.84 g/L	13.85 g/L
mAb yield	98%	97%	97%
Aggregate	0.45%	0.72%	0.65%
LRV[a] DNA	n.a.	4.16	5.02
LRV HCP	2.4	2.6	2.5
Specific productivity	16 g/L/h[b]	56 g/L/h	56 g/L/h

[a]*Log reduction value.*
[b]*g mAb/L. KANEKA KanCapA sorbent/h.*

followed by repetitive batch VI. Surge tanks between operations are minimized to around a 5 min hold time to balance the flow between operations.

With this kind of facility Novartis predicts that a traditional batch manufacturing facility with 6×11000 L bioreactors, producing 4.5 tons per year, could be replaced by an 8×1000 L facility. The continuous facility would take just 2 years to build, 3 years less than the batch facility, resulting in a 50% reduction in cost of goods (Joel Schultz, World Biopharm Forum, 2019).

Conclusion: tipping point

We see the developments in the biopharmaceutical landscape lead the industry to inch ever closer toward the fabled tipping point [109], where the benefits of continuous processing will outweigh the risks. It is perhaps no surprise that the early adopters of continuous processing, described here, are leveraging perfusion to intensify the upstream process to produce more product per volume of bioreactor. Perfusion processes also enable the vision of one size fits all processing, which may eliminate the need for scale-up through operating for a longer time to produce more material. Continuous downstream processing is then a logical extension of the perfusion approach, obviating the need to pool product and purify it as discrete batches. To intensify the downstream process, it appears there are options for each unit operation. In particular, there are many varied approaches to capture chromatography. The cycle of continuous improvement leads us to believe that the multi column methods will be adopted to handle the increasing product titers.

For biosimilars and updated processes of already approved drugs, the time to market may be much less important than reduction in cost. It is perhaps of no surprise that the biosimilar companies are leading the way in continuous processing. The expectation is that biosimilar companies will overcome the technical and regulatory hurdles, lowering the barrier of adoption to continuous processing. At this point, the originator companies, especially those reaching the limits of their current capacity, will have no choice but to rapidly adopt continuous processing.

References

[1] Konstantinov KB, Cooney CL. White paper on continuous bioprocessing. J Pharmaceut Sci 2014;104(Issue 3):813–20.
[2] BiosanaPharma. 2019. www.biosanapharma.com/news/. [Accessed 27 August 2019].
[3] Kaplon H, Reichert JM. Antibodies to watch. mAbs 2019;11(2):219–38. https://doi.org/10.1080/19420862.2018.1556465.
[4] Sigvaldadottir A, Taylor A. Rethinking competitive strategy in mature industries. Lund U. May: MSc Management, School of Economics and Management; 2016.
[5] The Royal Swedish Academy of Sciences. Scientific background on the Nobel Prize in Chemistry 2018: directed evolution of enzymes and binding proteins. https://www.nobelprize.org/uploads/2018/10/advanced-chemistryprize-2018.pdf.
[6] Abbvie News Center. AbbVie reports full-year and fourth-quarter 2018 financial results. 2019. https://news.abbvie.com/news/abbvie-reports-full-year-and-fourth-quarter-2018-financial-results.htm. [Accessed 23 August 2019].
[7] The Balance. Top 10 biologic drugs in the United States. 2019. www.thebalance.com/top-biologic-drugs-2663233/. [Accessed 23 August 2019].

[8] Patent expiry dates for biologicals: 2018 update. Generics and Biosimilars Initiative Journal 2018;7(1):29–34.
[9] Biosimilar Development. Biosimilars pipeline shows remarkable, sustained growth. 2019. www.biosimilardevelopment.com/doc/biosimilars-pipeline-shows-remarkable-sustained-growth-0001. [Accessed 23 August 2019].
[10] BioProcess International. Momenta drops Humira biosimilar, pays GSK $43m in manufacturing hit. 2019. https://bioprocessintl.com/bioprocess-insider/global-markets/momenta-drops-humira-biosimilar-pays-gsk-43m-in-manufacturing-hit/?utm_source$^{1}/_{4}$ hs_email&utm_medium$^{1}/_{4}$ email&utm_content$^{1}/_{4}$ 75824514&_hsenc$^{1}/_{4}$ p2ANqtz-86RbBYM8V2kHMauuRj_x_IL2U3LmRDLBLr_pOyj6hAi2Z-20gU07PBEtzaVfMIuzQrYiRIXaZnZU1qq8lijhLKPvaQnkKYk3EXTJ4QOBhnK6GUay4&_hsmi$^{1}/_{4}$ 75824514. [Accessed 23 August 2019].
[11] BioSpace. What's going on with the biosimilar market?. 2018. www.biospace.com/article/what-s-going-on-with-the-biosimilar-market-/. [Accessed 23 August 2019].
[12] The Royal Swedish Academy of Sciences. The Nobel prize in Physiology or medicine. NobelPrize.org. Nobel Media AB 2018. Fri; 2018. www.nobelprize.org/prizes/medicine/2018/summary/. [Accessed 23 August 2019].
[13] Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. Blood 2018;131:58–67. https://doi.org/10.1182/blood-2017-06-741033.
[14] Tan S, Zhang CWH, Gao GF. Seeing is believing: anti-PD-1/PD-L1 monoclonal antibodies in action for checkpoint blockade tumor immunotherapy. Signal Transduct. Target. Ther. 2016;1:16029. https://doi.org/10.1038/sigtrans.2016.29.
[15] Science Translational Medicine. Keytruda vs. Opdivo: No contest. 2016. https://blogs.sciencemag.org/pipeline/archives/2016/10/10/keytruda-vs-opdivo-no-contest>. [Accessed 23 August 2019].
[16] Rader RA, Langer ES. 30 years of upstream productivity improvements. BioProcess Int 2015;13:10–5. 4.
[17] Bonham-Carter J, Saturda JS. A brief history of perfusion biomanufacturing. BioProcess Int 2011;9:24–32.
[18] Bielser JM, Wolf M, Souquet J, Broly H, Morbidelli M. Perfusion mammalian cell culture for recombinant protein manufacturing – a critical review. Biotechnol Adv 2018;36:1328–40. https://doi.org/10.1016/j.biotechadv.2018.04.011.
[19] Gagnon M, Nagre S, Wang W, Hiller GW. Shift to high-intensity, low-volume perfusion cell culture enabling a continuous, integrated bioprocess. Biotechnol Prog 2018;34:1472–81. https://doi.org/10.1002/btpr.2723.
[20] Healthcare industry BW. Downstream processing: bottleneck purification process. 2009. www.gesundheitsindustrie-bw.de/en/article/dossier/downstream-processing-bottleneck-purification-process. [Accessed 23 August 2019].
[21] Langer ES. Alleviating downstream process bottlenecks. Genetic Engineering & Biotechnology News; 2011.
[22] T. Roadmapping. Biomanufacturing technology roadmap. <www.biophorum.com/continuous-downstream-processing-for-biomanufacturing-an-industry-review/>; [accessed 23.08.2019].
[23] Pagkaliwangan M, Hummel J, Gjoka X, Bisschops M, Schofield M. Optimized continuous multicolumn chromatography enables increased productivities and cost savings by employing more columns. Biotechnol J 2018. https://doi.org/10.1002/biot.201800179.
[24] Reizman BJ, Cole KP, Hess M, Burt JL, Maloney TD, Johnson MD, et al. Small-volume continuous manufacturing of merestinib. Part 2. Technology transfer and cGMP manufacturing org. Process Res. Dev. 2019;23(5):870–81. https://doi.org/10.1021/acs.oprd.8b00442.
[25] Borukhova S, Hessel V. Continuous manufacturing of active pharmaceutical ingredients via flow technology. In: Kleinebudde P, Khinast J, Rantanen J, editors. Continuous manufacturing of pharmaceuticals. Hoboken (New Jersey, US): John Wiley & Sons; 2017. p. 127–69.
[26] Pastre JC, Browne DL, Ley SV. Flow chemistry syntheses of natural products. Chem Soc Rev 2013;42:8849–69.
[27] Poechlauer P, Colberg J, Fisher E, Jansen M, Johnson MD, Koenig SG, Lawler M, Laporte T, Manley J, Martin B, O'Kearney-McMullan A. Pharmaceutical roundtable study demonstrates the value of continuous manufacturing in the design of greener processes org. Process Res Dev 2013;17(12):1472–8.
[28] Macdonald G. J Genet Eng Biotechn N 2019;39(1). https://www.genengnews.com/insights/continuous-processing-shows-continuous-progress/. [Accessed 21 August 2019].
[29] U.S. Food and Drug Administration. Quality Considerations for Continuous Manufacturing Guidance for Industry. https://www.fda.gov/media/121314/download.

[30] U.S. Food and Drug Administration. www.fda.gov/news-events/press-announcements/fda-statement-fdas-modern-approach-advanced-pharmaceutical-manufacturing/. [Accessed 21 August 2019].
[31] Kelly B. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. mAbs 2009;1(5):443–52. pmid:20065641.
[32] Base P, Joglekar G, Rai S, Suresh P, Vernon JA. Analysis of manufacturing costs in pharmaceutical companies. J. Pharm. Innov. 2008;3:30–40.
[33] Hoover Institution One Hundred Years. An overlooked key to lower drug prices. 2018. www.hoover.org/research/overlooked-key-lower-drug-prices. [Accessed 23 August 2019].
[34] Jagschies G. Continuous capture of mAbs—points to consider and case studies. 2018. https://doi.org/10.1016/B978-0-08-100623-8.00028-1.
[35] Vernon JA, Keener HW, Trujillo AJ. Pharmaceutical manufacturing efficiency, drug prices, and public health: examining the casual links. Drug Inf J 2007;41:229–39.
[36] Stock LR, Bisschops M, Ransohoff TC. The potential impact of continuous processing on the practice and economics of biopharmaceutical manufacturing. 2014. p. 479–94. https://doi.org/10.1002/9783527673681.ch19.
[37] Pollock J, Coffman J, Ho SV, Farid SS. Integrated continuous bioprocessing: economic, operational, and environmental feasibility for clinical and commercial antibody manufacture. Biotechnol Prog 2017;33(4):854–66. https://doi.org/10.1002/btpr.2492.
[38] Hummel J, Pagkaliwangan M, Gjoka X, Davidovits T, Stock R, Ransohoff T, Gantier R, Schofield M. Modeling the downstream processing of monoclonal antibodies reveals cost advantages for continuous methods for a broad range of manufacturing scales. Biotechnol J 2019;14:1700665. https://doi.org/10.1002/biot.201700665.
[39] Pollard D, Brower M, Abe Y, Lopes A, Sinclair A. Standardized economic cost modeling for next-generation MAb production. Bioprocess Int 2017;14(8):14–23.
[40] Xenopoulos A. A new, integrated, continuous purification process template for monoclonal antibodies: process modeling and cost of goods studies. J Biotechnol 2015;213:42–53. https://doi.org/10.1016/j.jbiotec.2015.04.020. PMID: 25959171.
[41] Arnold L, Lee K, Rucker-Pezzini J, Lee JH. Implementation of fully integrated continuous antibody processing: effects on productivity and COGm. Biotechnol J 2019;14. https://doi.org/10.1002/biot.201800061. 1800061.
[42] Chandler JR AD. The visible hand. Cambridge, MA: Belknap Press; 1977.
[43] Schmenner RW. Looking ahead by looking back: swift, even flow in the history of manufacturing. Prod Oper Manag 2001;10:87–96. https://doi.org/10.1111/j.1937-5956.2001.tb00069.x.
[44] Gjoka X, Gantier R, Schofield M. Platform for Integrated Continuous Bioprocessing: continuous processing of 100 g of monoclonal antibody in 24 hours has been demonstrated using lab-scale equipment. Biopharm Int 2017;30(7):26–32.
[45] Croughan MS, Konstantinov KB, Clooney C. The future of industrial bioprocessing. Batch or continuous? 2015;112(4):648–51. https://doi.org/10.1002/bit.25529.
[46] Cazeault C. Integrated continuous biomanufacturing: quality and regulatory considerations integrated continuous biomanufacturing. 2013. http://www.engconf.org/staging/wp-content/uploads/2013/12/1.-CCazeault-Oct-2013.pdf.
[47] Repligen R. Announces market launch of OPUS® 80R, reinforces technology leadership in pre-packed chromatography. 2018. www.globenewswire.com/news-release/2018/03/20/1442605/0/en/Repligen-Announces-Market-Launch-of-OPUS-80R-Reinforces-Technology-Leadership-in-Pre-Packed-Chromatography.html/. [Accessed 23 August 2019].
[48] Smith C. Process control for the process industries Part 2: steady-state characteristics CEP magazine. 2017.
[49] Biopharma Reporter. Amgen: industry must look to next generation continuous cell harvesting tech. 2017. https://www.biopharma-reporter.com/Article/2017/10/17/Amgen-Industry-must-look-to-next-gen-continuous-cell-harvesting-tech. [Accessed 26 September 2019].
[50] Kelly W, Scully J, Zhang D, Feng G, Lavengood M, Condon J, Knighton J, Bhatia R. Understanding and modeling alternating tangential flow filtration for perfusion cell culture. Biotechnol Prog 2014;30:1291–300.
[51] Hamilton MF. Fundamentals and applications OF nonlinear acoustics. American Society of Mechanical Engineers, Applied Mechanics Division, AMD 1986;77(1):1–28.
[52] Gallego-Juarez JA. Nonlinear acoustics and industrial applications. AIP Conference Proceedings 2000;524:45.
[53] Crighton DG. Model equations of nonlinear acoustics. Annu Rev Fluid Mech 1979;11:11–33.

[54] Blackstock DT. Fundamentals of physical acoustics. J Acoust Soc Am 2001;109:1274.
[55] Biopharma Asia. 2017. https://biopharma-asia.com/magazine-articles/integrated-disposable-clarification-solution-for-continuous-bioprocessing. [Accessed 26 September 2019].
[56] Acoustic Wave Separation. How it works. 2019. https://biopharma-asia.com/videos/acoustic-wave-separation-how-it-works-pall-corporation/. [Accessed 26 September 2019].
[57] Danielson A. Biopharmaceutical processing: development, design, and implementation of manufacturing processes 2018 17 Affinity Chromatography https://doi.org/10.1016/B978-0-08-100623-8.00017-7.
[58] Rathore A, Pathak M, Ma G, Bracewell DG. Re-use of Protein A resin: fouling and economics. Biopharm Int 2015;28(3):1–6.
[59] Pabst TM, Thai J, Hunter AK. Evaluation of recent Protein A stationary phase innovations for capture of biotherapeutics. J Chromatogr A 2018 15;1554:45–60. https://doi.org/10.1016/j.chroma.2018.03.060. Epub 2018 Apr 7.
[60] Dancette OP, Taboureau J, Tournier E, Charcosset C, Blond P. Purification of immunoglobulins G by protein A/G affinity membrane chromatography. J Chromatogr B 1999;723(1–2):61–8.
[61] Fahrner RL, Iyer HV, Blank GS. The optimal flow rate and column length for maximum production rate of protein A affinity chromatography. Bioprocess Eng 1999;21(4):287.
[62] Franzreb M, Muller E, Vajda J. Cost estimation for protein a chromatography: an in silico approach to mab purification strategy. BioProcess Int 2014;12.
[63] Bisschops M, Frick L, Fulton S, Ransohoff T. Single-use, continuous-countercurrent, multicolumn chromatography. BioProcess Int 2008;7:18–23.
[64] Utterback JM. Mastering the dynamics of innovation. Boston, MA: Harvard Business School Press; 1994.
[65] Baur D, Angarita M, Müller0-Späth T, Steinebach F, Morbidelli M. Comparison of batch and continuous multicolumn protein A capture processes by optimal design. Biotechnol J 2016;11:920–31. https://doi.org/10.1002/biot.201500481.
[66] Bisschops M, Brower M. The impact of continuous multicolumn chromatography on biomanufacturing efficiency. Pharm. Bioprocess 2013;1(4):361–72.
[67] Girard V, Hilbold NJ, Ng CK, Pegon L, Chahim W, Rousset F, Monchois V. Large-scale monoclonal antibody purification by continuous chromatography, from process design to scale-up. J Biotechnol 2015;213:65–73.
[68] Dutta AK, Tan J, Napadensky B, Zydney AL, Shinkazh O. Performance optimization of continuous countercurrent tangential chromatography for antibody capture. Biotechnol Prog 2016;32:430–9. https://doi.org/10.1002/btpr.2250.
[69] Utturkar A, Gillette K, Sun C, Pagkaliwangan M, Quesenberry R, Schofield M. A direct approach for process development using single column experiments results in predictable streamlined multi-column chromatography bioprocess. Biotechnol J 2019;14. https://doi.org/10.1002/biot.201800243.
[70] Chromatan. Single-use column free purification. 2019. www.chromatan.com/. [Accessed 22 August 2019].
[71] Hilbrig F, Freitag R. Continuous annular chromatography. J Chromatogr B 2003;790(1–2):1–15.
[72] Juza M, Mazzotti M, Morbidelli M. Simulated moving-bed chromatography and its application to chirotechnology. Trends Biotechnol 2000;18(3):108–18.
[73] Steinbach F, Muller-Spath T, Morbidelli M. Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production. Biotechnol J 2016;11:1126–41. https://doi.org/10.1002/biot.201500354.
[74] Brorson K, Krejci S, Lee K, Hamilton E, Stein K, Xu Y. Bracketed generic inactivation of rodent retroviruses by low pH treatment for monoclonal antibodies and recombinant proteins. Biotechnol Bioeng 2003;3:321–9. https://doi.org/10.1002/bit.10574.
[75] CadenceTM Virus Inactivation System. https://shop.pall.com/us/en/biotech/continuous-processing/viral-inactivation/cadence-virus-inactivation-system-zidimmfdc1e/. [Accessed 22 August 2019].
[76] Klutz S, Lobedann M, Bramsiepe C, Schembecker G. Chem. Eng. Process. Process Intensif. 2016;102:88.
[77] Parker SA, Amarikwa L, Vehar K, Orozco R, Godfrey S, Coffman J, Shamlou P, Bardliving CL. Design of a novel continuous flow reactor for low pH viral inactivation. Biotechnol Bioeng 2018;115(3):606–16. https://doi.org/10.1002/bit.26497.
[78] Gillespie C, Holstein M, Mullin L, Cotoni K, Tuccelli R, Caulmare J, Greenhalgh P. Continuous in-line virus inactivation for next generation bioprocessing. Biotechnol J 2019;14:1700718. https://doi.org/10.1002/biot.201700718.

[79] Schwan P, Vester A, Lobedann M. US20190117803A1, https://patents.google.com/patent/US20190117803A1/pt./. [Accessed 27 August 2019].
[80] Xenopoulos A. US9809799B2, https://patents.google.com/patent/US9809799B2/zh/. [Accessed 27 August 2019].
[81] Fiadeiro MA, Coffman J, Fahrner RL, Kublbeck JA, Orozco R, Salm JR. US20190071647A1, https://patents.google.com/patent/WO2017156355A1/pt./. [Accessed 27 August 2019].
[82] Bolton GR, Selvitelli KR, Iliescu I, Cecchini DJ. Inactivation of viruses using novel protein. A wash buffers 2015;31(2):406–13. https://doi.org/10.1002/btpr.2024.
[83] Soice N, Hubbard JD, Zhang Y, Hamzik J. Methods for purifying a target protein from one or more impurities in a sample. 2011. Patent # C07K 1/22 (20060101); C07K 16/00 (20060101); B01D 15/08 (20060101) ed, United States.
[84] Martins DL, Sencar J, Hammerschmidt N, Tille B, Kinderman J, Kreil TR, Jungbauer A. Continuous solvent/detergent virus inactivation using a packed-bed reactor. Biotechnol J 2019;14:1800646. https://doi.org/10.1002/biot.201800646.
[85] Rathore AS, Kumar D, Kateja N. Recent developments in chromatographic purification of biopharmaceuticals. Biotechnol Lett 2018;40(6):895–905.
[86] Sommerfeld S, Strube J. Challenges in biotechnology production—generic processes and process optimization for monoclonal antibodies. Chem Eng Process 2005;44(10):1123–37.
[87] American pharmaceutical review. UV-C irradiation: a new viral inactivation method for biopharmaceuticals. 2014. www.americanpharmaceuticalreview.com/Featured-Articles/169257-UV-C-Irradiation-A-New-Viral-Inactivation-Method-for-Biopharmaceuticals/. [Accessed 22 August 2019].
[88] Chan HL, Gaffney PR, Waterfield MD, Anderle H, Matthiessen PH, Schwarz HP, Turecek PL, Timms JF. Proteomic analysis of UVC irradiation-induced damage of plasma proteins: serum amyloid P component as a major target of photolysis. FEBS Lett 2006;580(13):3229–36.
[89] Shire SJ, Shahrokh Z, Liu JUN. Challenges in the development of high protein concentration formulations. J Pharmacol Sci 2004;93:1390–402. https://doi.org/10.1002/jps.20079. PMID:15124199 [PubMed] [CrossRef] [Google Scholar].
[90] Engin Ayturk PhD. Genetic engineering and biotechnology news. 2016.
[91] Casey C, Gallos T, Alekseev Y, Ayturk E, Pearl S. Protein concentration with single-pass tangential flow filtration (SPTFF). J Membr Sci 2011;384(1–2):82–8.
[92] Brinkmann A, Elouafiq S, Pieracci J, Westoby M. Leveraging single-pass tangential flow filtration to enable decoupling of upstream and downstream monoclonal antibody processing. Biotechnol Prog 2018;34:405–11. https://doi.org/10.1002/btpr.2601.
[93] http://www.biopharminternational.com/pall-debuts-new-inline-diafiltration-modules-continuous-bioprocessing-0.
[94] Nambiar AMK, Li Y, Zydney AL. Countercurrent staged diafiltration for formulation of high value proteins. Biotechnol Bioeng 2018;115:139–44. https://doi.org/10.1002/bit.26441.
[95] Rucker-Pezzini J, et al. Single pass diafiltration integrated into a fully continuous mAb purification process. Biotechnol Bioeng 2018;115:1949–57.
[96] World Biopharm Forum. http://www.subramanian.org.uk/wp-content/uploads/2019/04/World-Biopharm-2019_final-program.pdf/. [Accessed 22 August 2019].
[97] Applying new biologic manufacturing technologies. BiosanaPharma; 2018–19 [accessed 22.08.19[87], www.casss.org/resource/resmgr/BADG_Speaker_Slides/0216_BADG_MireSluisTonySlide.pdf/. [Accessed 22 August 2019]. www.biosanapharma.com/.
[98] Amgen news releases. 2019. www.amgen.com/media/news-releases/2018/07/amgen-breaks-ground-on-next-generation-biomanufacturing-plant-in-rhode-island/. [Accessed 26 September 2019].
[99] A next generation platform for integrated continuous. 2018. https://issuu.com/luxevents2/docs/dr_jens_vogel_of_boehringer_ingelhe/. [Accessed 22 August 2019].
[100] Newswire CPR. Enzene biosciences chooses Novasep BioSC® Pilot chromatography for its automated end to end continuous manufacture of mAb. 2019. www.prnewswire.com/in/news-releases/enzene-biosciences-chooses-novasep-biosc-r-pilot-chromatography-for-its-automated-end-to-end-continuous-manufacture-of-mab-824544791.html/. [Accessed 21 August 2019].

[101] Fully continuous biosimilar manufacturing framework: a case study. BrightTALK; 2018. www.brighttalk.com/webcast/10519/305539/fully-continuous-biosimilar-manufacturing-framework-a-case-study?/. [Accessed 21 August 2019].
[102] Bisschops M, Schofield M, Grace J. Two mutually enabling trends; continuous bioprocessing and single-use technologies. In: Subramanian G, editor. Continuous biomanufacturing - innovative technologies and methods: innovative technologies and methods. Weinheim (Germany): Wiley-VCH Verlag GmbH & Co; 2018. p. 149–69.
[103] Better Engineered Solutions. Sanofi (Genzyme) 31NYA Pilot plant, MA. 2019. [Accessed 21 August 2019].
[104] European Biotechnology. Sanofi gets the EU's ok for caplacizumab. 2018. https://european-biotechnology.com/up-to-date/latest-news/newssanofi-gets-the-eus-ok-for-caplacizumab.html?L=0&cHash=805114c23a5ffa3797ac5938a0c97702/. [Accessed 21 August 2019].
[105] European Biotechnology. Sanofi takes over Bioverativ in US$11.8bn deal. 2018. https://european-biotechnology.com/up-to-date/latest-news/newssanofi-gets-the-eus-ok-for-caplacizumab.html? L$^{1}/_{4}$ 0&cHash$^{1}/_{4}$ 805114c23a5ffa3797ac5938a0c97702/. [Accessed 21 August 2019].
[106] Just Biotherapeutics. http://www.justbiotherapeutics.com/. [Accessed 23 August 2019].
[107] Pinto N, Brower M, Gospodarek A, Napoli W, Pollard D, Richardson D. Continuous bioprocessing and process analytical technologies: a path towards quality by design. In: "Integrated continuous biomanufacturing III", suzanne Farid, University College London, United Kingdom Chetan Goudar, Amgen, USA Paula Alves, IBET, Portugal Veena Warikoo. USA: Axcella Health, Inc.; 2017. ECI Symposium Series, https://dc.engconfintl.org/biomanufact_iii/68.
[108] Novartis-MIT center for continuous manufacturing. 2019. https://novartis-mit.mit.edu/. [Accessed 22 August 2018].
[109] Warikoo V, Godawat R, Brower K, Jain S, Cummings D, Simons E, Konstantinov K. Integrated continuous production of recombinant therapeutic proteins. Biotechnol Bioeng 2012;109(12):3018–29. https://doi.org/10.1002/bit.24584'.

CHAPTER

5

Recent advances in antibody-based monolith chromatography for therapeutic applications

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List of abbreviations

CDI Carbonyldiimidazole
CHO Chinese hamster ovary
CIM Convective interaction media
EPO Erythropoietin
FITC Fluorescein isothiocyanate
GC Gas chromatography
GMA/EDMA Glycidyl methacrylate/ethylene glycol dimethacrylate
HSA Human serum albumin
HPLC High performance liquid chromatography
IEC Ion-exchange chromatography
IgG Immunoglobulin G
mAb Monoclonal antibody
NT-proBNP N-terminal pro-brain natriuretic peptide
PHEMA Poly(hydroxyethyl methacrylate)
RP HPLC Reversed-phase HPLC
SEC Size-exclusion chromatography
TEMED Tetramethylethylenediamine

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Approaches to the Purification, Analysis and Characterization of Antibody-ased Therapeutics
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Introduction

In biotherapeutics research and development, chromatography is the leading technology employed for the purification of the final product, as purity is an unassailable need [1]. Particularly, if the therapeutic agent is an antibody based product, affinity chromatography is traditionally selected based on the highly specific interaction of the antigen with the antibody, allowing for high purity in a single step. This type of affinity chromatography is commonly classified as immunoaffinity chromatography. Due to costs of goods considerations, the high selectivity of this type of chromatography is always associated with elevated costs of production.

To optimize the costs in the downstream processing of therapeutic agents; a faster, reusable, reproducible, and efficient immunoaffinity chromatography process should be selected. Monoliths, being the fourth generation in chromatographic supports, are considered as an optimal candidate to fulfill these requirements. In this chapter, different aspects of antibody-based monolith chromatography for therapeutic applications will be covered, including: general principles and fundamentals; advantages and limitations; strategies for the purification and immobilization of antibodies in monoliths; and their applications and possible future directions in the clinical, biomedical, and pharmaceutical fields.

State of the art of antibody-based monolith chromatography

In this section, two main aspects will be examined: (1) Generalities and fundamentals of antibody-based monolith chromatography and (2) their advantages and limitation in comparison to conventional chromatographic supports.

Generalities and fundamentals

Antibody-based monolith chromatography is a type of affinity based liquid chromatography that uses antibodies as ligands coupled to the monolithic stationary phase. The retention of biomolecules in this method is based on the specific, reversible interaction between the antibody and its antigen. This technique is utilized to enrich, isolate or analyze the biomolecule from a complex matrix (e.g. blood, plasma, etc.), or to study interactions in biological systems. Monoliths are a sponge-like chromatographic media that consist of a single, continuous, highly interconnected, and porous organic or inorganic stationary separation block. This stationary phase supports high convective flow rates through the interconnected channels, which enable laminar flow of the mobile phase with low-shear and without interparticular voids [2]. This outstanding mass transfer feature differs monoliths and traditional bead based resin chromatography, where the mass transfer occurs through diffusion, making purification of large molecules usually very time consuming.

The fundamental elements that must be taken into account for the performance of a monolith are: (1) type of support, (2) immobilization strategy, and (3) validation parameters. In the particular case of antibody-based monoliths, the following fundamental elements apply:

(1) The type of support should have low non-specific binding for sample components, be easy to modify for antibody attachment, and be stable under the operating conditions

to be employed with the monolith [3]. The support's material should guarantee the optimal balance between surface area, porosity, as well as pore size and distribution [4]. Regarding the support material, the following elements have been reported previously for antibody-based monoliths:

(a) Glycidyl methacrylate/ethylene glycol dimethacrylate (GMA/EDMA) copolymers are organic polymers most frequently used to prepare antibody-based monoliths, including the commercially convective interaction media (CIM®) from BIA Separations (Slovenia). GMA/EDMA are highly utilized because GMA monomer contains epoxy groups, which will facilitate the immobilization of the antibody as will be discussed later in the immobilization strategy section.

(b) Agarose, a polysaccharide containing repeating units of D-galactose and 3,6-anhydro-L-galactose [5], is a popular support for affinity separation which is prepared by casting an agarose emulsion. Agarose monoliths have good chemical stability over a broad pH range and have low non-specific binding to most proteins and biological ligands [5].

(c) Silica, an inorganic-based monolith prepared by the sol-gel method, usually employing tetramethoxysilane, poly(ethylene oxide), and water.

(d) Cryogels, extremely porous media prepared by polymerization below −10 °C of a mixture of acrylamide, allyl glycidyl ether, N,N′-methylenebis (acrylamide), TEMED (tetramethylethylenediamine), and ammonium persulfate in an aqueous buffer [6]. These types of monoliths are not rigid, allowing for mechanical compression or temperature-induced shrinkage for elution [7].

(2) Immobilization strategy: the attachment of the antibody to the monolith should minimize leakage of the ligand and it should not alter or reduce the binding capacity for the antibody, nor block its binding site. The reported strategies for coupling antibodies within monolithic supports can be divided into two main groups: (a) covalently attached to the support, either directly through various coupling techniques or indirectly through biospecific adsorption, or (b) noncovalent attachment methods, including entrapment or molecular imprinting. These two alternatives will be further discussed in Recent advances in immobilization of antibodies section.

(3) Validation parameters: since the optimal performance of monoliths depend on the balance between morphological, mechanical, and physicochemical features, their validation must be evaluated employing multiple criteria:

(a) selectivity and binding capacity for the target molecule;
(b) mechanical, morphological, and chemical stability;
(c) minimal non-specific molecular adsorption;
(d) stability under cleaning and sterilization protocols for recycling purposes;
(e) short processing time with high throughput capacity; and
(f) scalability.

The criteria for the selection of an appropriate antibody-based monolith are summarized in Fig. 5.1A. Fig. 5.1B depicts an example of the use of an immunoaffinity monolith to isolate stem cells. The principle for the recovery of stem cells is to immobilize a specific monoclonal antibody (mAb), which is the complementary ligand of the surface antigen in the stem cell of

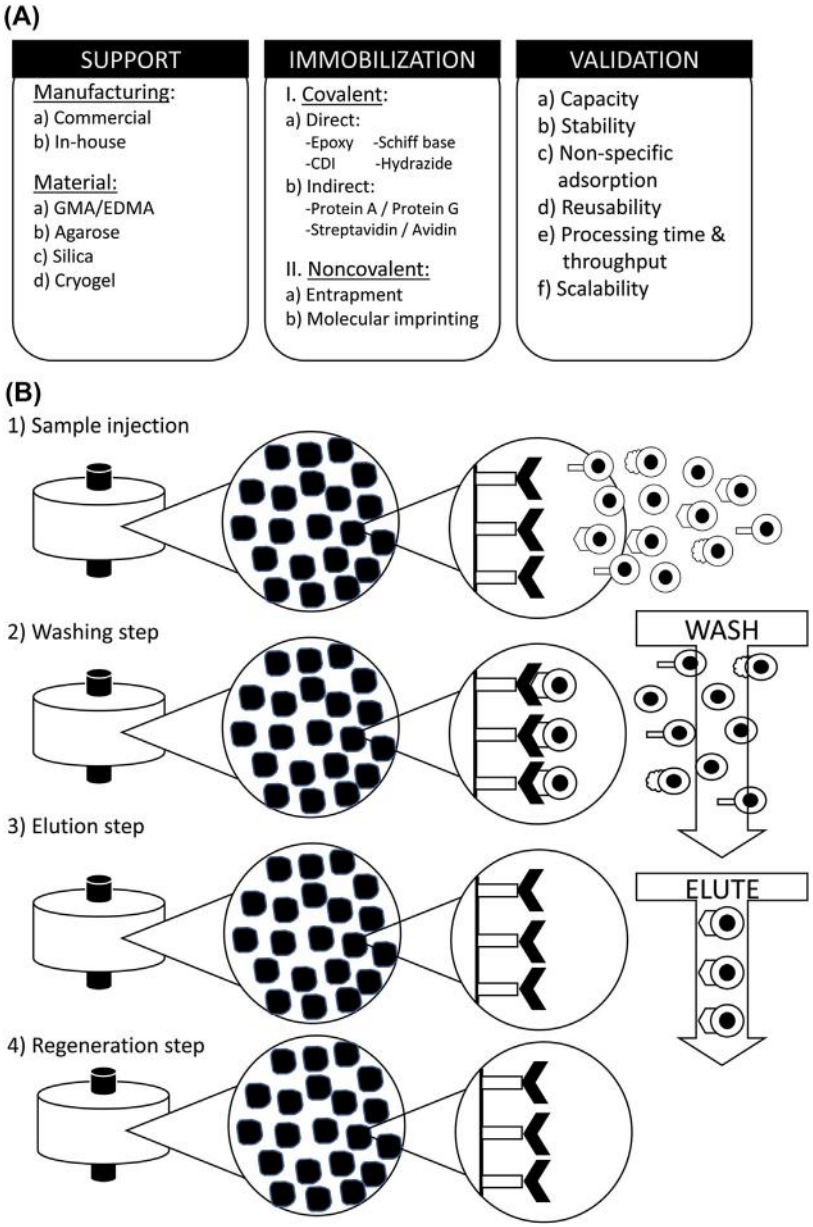

FIG. 5.1 Selection criteria and performance for a separation utilizing antibody-based monolith chromatography. (A) Fundamental elements for the selection of an antibody-based monolith chromatography: (1) type of support, (2) immobilization strategy, and (3) validation parameters. (B) Schematic representation of the implementation of antibody-based monolith chromatography for the purification of stem cells: (1) sample injection, (2) washing step, (3) elution step, and (4) regeneration step.

interest, to the monolithic support. Then, as the cell sample is injected into the affinity monolith (Fig. 5.1B.1) [under conditions that allow strong binding between the surface antigen and the immobilized mAb (Buffer A)], the stem cells that possess the specific cell surface receptor will reversibly bind to the monolith, while the rest of the sample will elute from the monolith in this step. Next, a washing step is performed to completely elute the cells that have little interaction with the immobilized ligand (Fig. 5.1B.2). The following step is to inject the elution buffer to dissociate the target from the affinity ligand (Fig. 5.1B.3). To achieve this, a change in the buffer conditions (Buffer B) is usually required (e.g. change in polarity, pH or ionic strength) and the eluted stem cells are collected for later analysis or use. Finally, after the elution, the monolith must be regenerated prior to the next sample application by injecting Buffer A (Fig. 5.1B.4).

Advantages and limitations

Monoliths possess unique characteristics and features that makes them outstanding for a wide range of preparative and analytical purification applications. For example, their highly interconnected network of channels allows for low backpressures at high eluent flowrates. This results in higher operational speed at a lower pressure drop without compromising the dynamic capacity, permeability, and resolution of the operation. Additionally, a drastic reduction in the operational time is obtained, minimizing product degradation and buffer consumption [2]. Monoliths are also versatile as they can also be manufactured in a variety of sizes (from 0.1 mL to 8 L; suitable for both preparative and analytical processes), shapes (disks, thin membranes, columns, tubes, rods, cryogels, capillaries, microchips), and surface chemistries (suitable for ion-exchange, hydrophobic, hydrophilic, affinity, and reversed-phase chromatography) [2]. They are easy to handle (install, clean, and store), robust, reproducible, do not require packing, can be employed in traditional chromatography systems, allow process integration by assembling in the same housing multiple separation mechanisms (e.g. cation, ionic, and/or hydrophobic), and are compatible for integration with other techniques (mass spectrometry and microanalytical systems) [1,5]. Moreover, the mechanical and chemical stability of the monoliths confer good longevity for the separation system, thus allowing reutilization of the supports for cost-effective bioprocessing. Regarding scalability, the implementation of monoliths at manufacturing scale is possible given the different available commercial capacities. All of these advantages, make monoliths a flexible technology for a wide variety of therapeutic applications that will be further discussed in the next sections. Lastly, monoliths could be in-house produced as cryogels [6], but they are also commercially available from a vast number of manufacturers including BIA Separations, Bio-Rad Laboratories, Dionex, Merck, Phenomenex, Agilent Technologies, and Sartorius.

Although monoliths are an exceptional bioprocessing unit for the purification and concentration of biotherapeutics, there are still limitations that should be addressed in order to establish monoliths as the gold standard purification strategy in the clinical, biomedical, and pharmaceutical sectors. These limitations include the monolith's lack of being a generic downstream solution for any given therapeutic product due to the high degree of specificity of the antibody-antigen interaction. As a result, different antibodies need to be coupled to the monolith, depending on the specific molecule that must be purified, for example, for purification of different strains of a virus [8]. A second limitation for antibody-based monoliths are

the availability and production costs of the coupling antibodies. A third restraint is associated with the strong affinity interaction between the antibody and the antigen, having an association equilibrium constant often greater than $10^6\ M^{-1}$ under physiological conditions [9], requiring extreme elution conditions (usually low pH) to reverse the binding. This could limit the application of this purification strategy if the target stability and functional state are impaired. Another restriction is the fact that, despite the commercial availability of monoliths of up to 8 L volume (e.g. CIMmultus columns from BIA Separation), affinity monoliths greater than 1 L remain unavailable given their manufacturing complexity associated with pore distribution, cracking, and their high production cost [10]. The last but most important obstacle in the implementation of this technique in the market are the technical and economic implications of shifting an existing downstream bioprocess of an ongoing production to one utilizing an antibody-based monolith.

Recent advances in purification of antibodies

The popularity of antibodies has increased given the multiple therapeutic applications that they have demonstrated, either directly for clinical applications (treatment for cancer, autoimmune disorders, infectious and inflammatory disease) [11] or employed as reagents in other techniques (including antibody-based monolith chromatography). Monoclonal antibodies and derivatives of them represent a high proportion of all molecules now tested in clinical trials. Antibodies in their different classifications (monoclonal, polyclonal, recombinant, and fragments) are commonly purified employing immunoaffinity chromatography with the aid of IgG-binding proteins such as protein A, G, L, M, D, and P [12,13]; with protein A and protein G being the most employed ligands. As mentioned earlier, given the outstanding advantages that monoliths have demonstrated, it is not surprising that monoliths have gained popularity for the purification of antibodies. Some examples of the use of monoliths for the quantification and purification of antibodies are presented in Table 5.1.

These examples demonstrate how antibody-based monoliths have gained in popularity for the purification of antibodies. This can be attributed to the multiple advantages they possess

TABLE 5.1 Antibody-based monoliths employed for the purification or quantification of antibodies.

Application	Product/matrix	Antibody-based monolith	References
Purification	hIgG/human serum	CIM®r-protein A	[14]
Purification	hIgG/human serum	Capillary column-protein G	[15]
Quantification	hIgG/human serum [μL]	Capillary column-protein A	[16]
Purification	IgM/rheumatoid arthritis plasma patient	PHEMA cryogel-protein A	[17]
Quantification	IgG/purified samples and crude supernatant from CHO cells	CIM®r-protein A	[18]
Purification	IgG/cell culture supernatant	Spongy-protein A	[19]
Quantification	Single-chain variable fragment immunotoxin	CIM®r-Protein L	[20]

compared with traditional chromatographic supports, highlighting their speed and ease of use. This can be furthermore validated by analyzing the literature, as every review regarding antibody purification includes a section for affinity monoliths [11,13].

Recent advances in immobilization of antibodies

As previously mentioned, two different options are available to immobilize the antibodies in the monoliths: (a) covalent immobilization to the support or (b) noncovalent immobilization methods. The covalent strategy can be further sub-divided depending if the antibody is directly or indirectly bound to the monolith. In direct covalent immobilization, different coupling techniques (e.g. epoxy, hydrazide) have been reported either using static (immersing the monolith in a solution containing the antibody) or dynamic (injecting the ligand solution through the monolith) modes. A brief description of each technique is described in the following. In the epoxy method, the epoxy groups in the monolith can be used directly for immobilization by coupling with the amine groups of the antibody (which are distributed throughout the antibody and are easily accessible) or as precursors for other coupling methods (e.g. diol under acidic conditions) [21,22].

In the Schiff base method, an amine-based coupling chemistry is used, performed by converting the epoxy groups in the monolith into diols, which are then oxidized with periodic acid to form aldehyde groups. These aldehyde groups can then react with the primary amines of the antibodies. Since this is a reversible reaction, the adducts must be reduced with sodium cyanoborohydride to give a secondary amine. The remaining aldehyde groups could later be reduced to alcohols by adding sodium borohydride or could be eliminated by reacting them with a small amine-containing ligand [22,23].

In the carbonyldiimidazole (CDI) method, diol groups present or that have been placed onto the monolith are reacted with 1,1′-carbonyldiimidazole to produce imidazolyl carbamate groups. These groups can be reacted with the primary amines of the antibody through a nucleophilic substitution reaction, resulting in a stable amide linkage [21].

Finally, in the hydrazide method, the antibody is attached via its carbohydrate chains to the monolith support [7]. The first step in this procedure is to produce an aldehyde-activated monolith (described earlier for the Schiff base method). The support is then activated with a dihydrazide, which can react with the aldehyde groups of the previously oxidized antibodies to form stable hydrazine bonds.

Indirect covalent attachment or biospecific adsorption consists of the covalent immobilization of a secondary ligand to the monolith support, followed by the noncovalent immobilization of the ligand of interest. For example, monoliths coupled with protein A [14,16–19] or protein G [15] employ this type of immobilization. Another option is to attach streptavidin or avidin onto the monolith and bind them to biotinylated antibodies [24].

The noncovalent strategies include entrapment and molecular imprinting [22,23]. Entrapment requires to confine the antibody inside the support, which is generally achieved by incorporating the antibody as part of the polymerization mixture. The molecular imprinting strategy requires a template molecule to create cavities in the monolith, which are then going to be occupied by the antibody.

The selection of the immobilization strategy will depend on the nature of the antibody that will be immobilized, the product to be recovered, and the type of support to be employed. The versatility of monoliths have been demonstrated within the previous sections regarding the multiple materials that have been reported for the supports and the different chemical reactions that can be performed to attach the antibody of interest.

Therapeutic applications

The implementation of antibody-based monolith chromatography for applications in biotherapeutics can result in a substantially simplified and faster platform purification process that will eventually reduce the downstream cost, enabling economically feasible business models with affordable market prices. Antibodies can be employed as biological sensors for diagnostics or can be administrated as the therapeutic agent for a specific treatment, and so will continue revolutionizing modern medicine and laboratory-based analysis testing.

Diagnosis and analysis

Antibody-based monolith chromatography can be employed for the isolation, purification, screening, and analysis of drugs, hormones, peptides, proteins, enzymes, viruses, cell components, and other biologically-relevant molecules with surface antigens [22]. Additionally, biological interactions can also be analyzed with these types of monoliths. For example, the interaction between drug binding with serum proteins, enzymes, and receptors, as well as the interaction of biomolecules and antibodies. The resulting information includes the overall extent of binding, the equilibrium constant, as well as the number of and types of binding sites.

Immunoaffinity extraction can be utilized to remove targets from samples in less than a few seconds and can also be employed to measure the free fractions of drugs and hormones in serum or drug/protein mixtures with antibody-based monolith chromatography [25].

Affinity depletion can be employed to remove abundant compounds from a complex sample prior to analysis of the non-retained sample components by a second method. Thus an antibody-based monolith coupled with mAbs against human serum albumin (HSA) could be employed to deplete this highly abundant protein from serum prior to the analysis of the lower abundance proteins in the sample [26].

Competitive or non-competitive flow-based immunoassay can also be performed with antibody-based monolith chromatography. The competitive mode consists of a competition between the target analyte and a labeled analog analyte for a limited number of binding sites in the monolith. Either the retained or non-retained fraction of the labeled analog is then detected via fluorescence, absorbance, chemiluminescent, or by other strategies. This approach will give an indirect measure of the amount of target that was in the original sample. Three different modes of injecting the target analyte and the labeled analog analyte can be performed:

(1) simultaneous injection: both analytes injected at the same time,
(2) sequential injection: first inject the target followed by the labeled analog, and
(3) displacement injection: first inject the labeled analog in excess followed by target analyte, displacing some of the labeled analog.

The non-competitive flow-based immunoassay can be performed in two modes:

(1) One-site immunometric assay: incubate the sample with a known excess of labeled antibodies that bind to the target, this mixture is injected into the monolith containing the immobilized analyte analog. The immobilized analog binds to the free antibodies and that bound to the labeled antibodies are eluted.

(2) Two-site immunometric assay: two different antibodies that bind simultaneously to the target are employed. The first is immobilized in the monolith and the second is labeled to use for detection. After washing the monolith, the elution step is performed and the signal generated by the label is a direct measure of the amount of target in the sample.

Biological interactions can be analyzed by performing: (1) zonal elution experiments, (2) frontal analysis, (3) plate height method, (4) peak profiling, and (5) peak decay method. These strategies have been previously reviewed [5,22,27]. Moreover, these techniques could be then followed by other methods, including: high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), reversed-phase HPLC (RP HPLC), or electrophoresis.

Table 5.2 summarizes major applications of antibody-based monolith chromatography in biotherapeutics. This reports demonstrated the potential of cryogels as a medical device for extracorporeal apheresis [26] and the implementation of antibody-based monoliths (with appropriate antibodies) as a useful tools for general applications in sample preparation in

TABLE 5.2 Biotherapeutic applications of antibody-based monoliths.

Application	Product	Antibody-based monolith	References
Purification	Human serum albumin in whole blood	Cryogel-anti-human albumin antibody	[26]
Extraction	Fluorescein	GMA/EDMA monolith-anti-FITC (epoxy, CDI, Schiff base, hydrazide)	[25]
Purification	Blood group antigens (Knops, JMH, and Scianna) from cell culture supernatant	CIM® epoxy disks- monoclonal anti-V5 antibody	[28]
Purification	HSA, transferrin, fibrinogen, and haptoglobin in blood plasma	Hydrazide- and CDI-modified CIMac™ -IgGs	[29]
Quantification	Testosterone	Capillary column- anti-testosterone	[30]
Purification	Erythropoietin	Monolith-anti-EPO	[31]
Purification	Ialp proteins	CIM® hydrazide-chicken anti-HSA IgY and mAb 69.26	[32]
Quantification	Intracellular galactosidase	Agarose monolith-anti-β-galactosidase	[33]
Extraction	FITC-tagged human serum albumin and FITC-IgG	Microchannel column-anti-FITC	[34]
Extraction	Exosomes from clinical samples	Silica microtips-anti-CD9	[35]
Purification	$CD34^+$ human acute myeloid leukemia KG-1 cells	Protein A cryogel- anti-CD34	[6, 36]

clinical research and prior to *in vitro* diagnostics [31]. Additionally, antibody-based monoliths could be employed for high-throughput screening of new drug targets, to evaluate drug candidates or to investigate drug effects *in vivo* [37].

Treatment

Antibody-based monolith supports are expected to radically improve the purification of viruses, toxins, and proteins for the development of vaccines. Despite such remarkable advantages, reports on the use of this type of support for the purification of biomolecules for the production of vaccines are scarce in the scientific literature. One successful report presented the use of a CDI-monolithic support coupled with CB. Hep-1 mAb for the purification of particles with the hepatitis B surface antigen [38].

Other treatments that have been elucidated from the purification of reported antibody-based monoliths include:

- Sepsis utilizing the antibody-based monolith coupled with mAb 69.26, which is directed toward human IaIp, a protein found in human serum [32].
- *Bacillus anthracis* infection employing a cryogel with immobilized protein A coupled with a glycosylated human monoclonal antibody for the removal of anthrax toxin protective antigen [39].

A clear niche exists for the employment of antibody-based monoliths for medical applications. We are certain that the benefits of using monoliths are clear and in the near future more literature will be forthcoming. Thus, the next section highlights the future trends we can glimpse in the production of monoliths.

Future trends and concluding remarks

Antibody-based monolith chromatography can truly lead a revolutionary paradigm shift in the biomedical and pharmaceutical industries when a cost-effective ligand for any given antigen is successfully manufactured. The availability of such a ligand can then enable a robust downstream process for these industries. Another area of opportunity is the development of a new generation of ligands that possess reversible interaction affinity under mild elution conditions to preserve viability, structure, and functionality of the product. Antibody mimics such as aptamers could be another option when trying to lower the investment costs [24,40,41]. An additional trend in the manufacturing of monoliths includes the development of "immortal" monoliths that could defy expiration dates. The addition of a preservation storage buffer that could maximize the lifetime of the monolith would be a very promising option to accomplish this goal. Another approach that would transform the antibody-based market would be the employment of nanomaterials or novel polymers with "super" physiochemical properties. For example, this will aid in the development of flexible supports that could adapt their capacity depending on the requirements of the process or that they could "intelligently" free the immobilized product when desired.

This chapter has explored the topic of affinity-based monoliths and has examined the principles, advantages, challenges, as well as examples and possible applications of this immunoaffinity chromatography approach in biotherapeutics. Likewise, the most commonly employed materials and immobilization methods for antibodies have been addressed.

Although affinity chromatography has been a highly exploited technique in clinical, biomedical and pharmaceutical analysis, the use of antibody-based monoliths has not been explored in all the multiple applications that this chapter has addressed. In this respect, the advantages that monoliths possess over traditional chromatography have not yet been enough to motivate clinicians and researchers to make broad and routine use of them for biotherapeutics research and development. Years to come will reveal if it was just matter of time for more research to be performed in this field or if the development of the next generation of ligands (cheaper, universal and/or "smart" to dissociate without requiring harsh conditions) would trigger the exponential use of antibody-based monoliths.

References

[1] Przybycien TM, Pujar NS, Steele LM. Alternative bioseparation operations: life beyond packed-bed chromatography. Curr Opin Biotechnol 2004;15(5):469–78.
[2] González-González M, González-Valdez J, Mayolo-Deloisa K, Rito-Palomares M. Monolithic chromatography: insights and practical perspectives. J Chem Technol Biotechnol 2017;92(1):9–13.
[3] Hill SJ, Handbook of HPLC, Katz E, Eksteen R, Schoenmakers P, Miller N. 1998 xi + 1008 pages. US $225. ISBN 0-8247-9444-3. In: Applied Organometallic Chemistry, vol. 14. New York: Marcel Dekker; 2000. p. 130–1 (2).
[4] Vignesh R, Christoph H, Oliver S. Monoliths in bioprocess technology. Chromatography 2015;2(2):195–212.
[5] Pfaunmiller EL, Paulemond ML, Dupper CM, Hage DS. Affinity monolith chromatography: a review of principles and recent analytical applications. Anal Bioanal Chem 2012;405(7):2133–45.
[6] Kumar A, Bhardwaj A. Methods in cell separation for biomedical application: cryogels as a new tool. Biomed Mater 2008;3(3):1.
[7] Sproß J, Sinz A. Monolithic media for applications in affinity chromatography. J Separ Sci 2011;34(16–17):1958–73.
[8] Zhao M, Vandersluis M, Stout J, Haupts U, Sanders M, Jacquemart R. Affinity chromatography for vaccines manufacturing: finally ready for prime time? Vaccine 2019;37(36):5491–503.
[9] Handbook of affinity chromatography (University of Nebraska). In: Hage DS, editor. Journal of the American chemical society. 2nd ed., vol. 128. Boca Raton, FL: CRC Press/Taylor and Francis Group; 2006. p. 6521. 2006. xx + 944 pp. $176.36. ISBN 0-8247-4057-2 (19).
[10] Ongkudon CM, Kansil T, Wong C. Challenges and strategies in the preparation of large-volume polymer-based monolithic chromatography adsorbents. J Separ Sci 2014;37(5):455–64.
[11] Barroso T, Hussain A, Roque ACA, Aguiar-Ricardo A. Functional monolithic platforms: chromatographic tools for antibody purification. Biotechnol J 2013;8(6):671–81.
[12] Denizli A. Purification of antibodies by affinity chromatography. Hacettepe J Biol Chem 2011;39(1):1–18.
[13] Murphy C, Devine T, O'Kennedy R. Technology advancements in antibody purification. Antib Technol J 2016;6:17–32.
[14] Leblebici P, Leblebici ME, Ferreira-da-Silva F, Rodrigues AE, Pais LS. Separation of human immunoglobulin G subclasses on a protein A monolith column. J Chromatogr B 2014;962:89–93.
[15] Armenta JM, Gu B, Humble PH, Thulin CD, Lee ML. Design and evaluation of a coupled monolithic preconcentrator-capillary zone electrophoresis system for the extraction of immunoglobulin G from human serum. J Chromatogr A 2005;1097(1):171–8.
[16] Pan Z, Zou H, Mo W, Huang X, Wu R. Protein A immobilized monolithic capillary column for affinity chromatography. Anal Chim Acta 2002;466(1):141–50.
[17] Alkan H, Bereli N, Baysal Z, Denizli A. Selective removal of the autoantibodies from rheumatoid arthritis patient plasma using protein A carrying affinity cryogels. Biochem Eng J 2010;51(3):153–9.

[18] Tscheliessnig A, Jungbauer A. High-performance monolith affinity chromatography for fast quantitation of immunoglobulin G. J Chromatogr A 2009;1216(13):2676–82.
[19] Kubota K, Kubo T, Tanigawa T, Naito T, Otsuka K. New platform for simple and rapid protein-based affinity reactions. Sci Rep 2017;7(1):178.
[20] Satzer P, Sommer R, Paulsson J, Rodler A, Zehetner R, Hofstädter K, Klade C, Jungbauer A. Monolith affinity chromatography for the rapid quantification of a single-chain variable fragment immunotoxin. J Separ Sci 2018;41(15):3051–9.
[21] Mallik R, Jiang T, Hage DS. High-performance affinity monolith Chromatography: development and evaluation of human serum albumin columns. Anal Chem 2004;76(23):7013–22.
[22] Mallik R, Hage DS. Affinity monolith chromatography. J Separ Sci 2006;29(12):1686–704.
[23] Li Z, Rodriguez E, Azaria S, Pekarek A, Hage DS. Affinity monolith chromatography: a review of general principles and applications. Electrophoresis 2017;38(22–23):2837–50.
[24] Zhao Q, Li X-F, Le XC. Aptamer-modified monolithic capillary chromatography for protein separation and detection. Anal Chem 2008;80(10):3915–20.
[25] Jiang T, Mallik R, Hage DS. Affinity monoliths for ultrafast immunoextraction. Anal Chem 2005;77(8):2362–72.
[26] Akande W, Mikhalovska L, James S, Mikhalovsky S. Affinity binding macroporous monolithic cryogel as a matrix for extracorporeal apheresis medical devices. Int J Biomed Mater Res 2015;3(5):56.
[27] Hage DS, Anguizola JA, Bi C, Li R, Matsuda R, Papastavros E, Pfaunmiller E, Vargas J, Zheng X. Pharmaceutical and biomedical applications of affinity chromatography: recent trends and developments. J Pharmaceut Biomed Anal 2012;69:93–105.
[28] Mönster A, Hiller O, Grüger D, Blasczyk R, Kasper C. Isolation and purification of blood group antigens using immuno-affinity chromatography on short monolithic columns. J Chromatogr A 2011;1218(5):706–10.
[29] Černigoj U, Vidic U, Nemec B, Gašperšič J, Vidič J, Lendero Krajnc N, Štrancar A, Podgornik A. Characterization of methacrylate chromatographic monoliths bearing affinity ligands. J Chromatogr A 2016;1464:72–8.
[30] Chen H-X, Huang T, Zhang X-X. Immunoaffinity extraction of testosterone by antibody immobilized monolithic capillary with on-line laser-induced fluorescence detection. Talanta 2009;78(1):259–64.
[31] Lönnberg M, Dehnes Y, Drevin M, Garle M, Lamon S, Leuenberger N, Quach T, Carlsson J. Rapid affinity purification of erythropoietin from biological samples using disposable monoliths. J Chromatogr A 2010;1217(45):7031–7.
[32] Brne P, Lim YP, Podgornik A, Barut M, Pihlar B, Štrancar A. Development and characterization of methacrylate-based hydrazide monoliths for oriented immobilization of antibodies. J Chromatogr A 2009;1216(13):2658–63.
[33] Nandakumar MP, Pålsson E, Gustavsson P-E, Larsson P-O, Mattiasson B. Superporous agarose monoliths as mini-reactors in flow injection systems. Bioseparation 2000;9(4):193–202.
[34] Sun X, Yang W, Pan T, Woolley AT. Affinity monolith-integrated poly (methyl methacrylate) microchips for on-line protein extraction and capillary electrophoresis. Anal Chem 2008;80(13):5126–30.
[35] Ueda K, Ishikawa N, Tatsuguchi A, Saichi N, Fujii R, Nakagawa H. Antibody-coupled monolithic silica microtips for highthroughput molecular profiling of circulating exosomes. Sci Rep 2014;4:6232.
[36] Kumar A, Rodríguez-Caballero A, Plieva FM, Galaev IY, Nandakumar KS, Kamihira M, Holmdahl R, Orfao A, Mattiasson B. Affinity binding of cells to cryogel adsorbents with immobilized specific ligands: effect of ligand coupling and matrix architecture. J Mol Recogn 2005;18(1):84–93.
[37] Bantscheff M, Scholten A, Heck AJR. Revealing promiscuous drug–target interactions by chemical proteomics. Drug Discov Today 2009;14(21):1021–9.
[38] Valdés R, Černigoj U, Sánchez E, Hernández D, Nemec B, Limonta M, Castillo M, Pérez B, Vidic U, Vidič J, Žorž M, Tamayo A, Gómez L, Ferro W, Aldama Y, Krajnc NL, Marc D, Štrancar A. Hepatitis B surface antigen particle purification by immunoaffinity chromatography based on CDI-CB.Hep-1 mAb monolithic supports. BioProcess J 2018;17.
[39] Ingavle GC, Baillie LWJ, Zheng Y, Lis EK, Savina IN, Howell CA, Mikhalovsky SV, Sandeman SR. Affinity binding of antibodies to supermacroporous cryogel adsorbents with immobilized protein A for removal of anthrax toxin protective antigen. Biomaterials 2015;50:140–53.
[40] Wang Z, Zhao J-C, Lian H-Z, Chen H-Y. Aptamer-based organic-silica hybrid affinity monolith prepared via "thiol-ene" click reaction for extraction of thrombin. Talanta 2015;138:52–8.
[41] Chen Y, Ding X, Zhu D, Lin X, Xie Z. Preparation and evaluation of highly hydrophilic aptamer-based hybrid affinity monolith for on-column specific discrimination of ochratoxin A. Talanta 2019;200:193–202.

CHAPTER

6

Recent advances in harvest clarification for antibodies and related products

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Introduction

Methods for harvest clarification in monoclonal antibody (mAb) production processes have evolved significantly over the last decade. While some of these improvements were incremental advancements, others have been truly disruptive in nature. Three motivating factors driving these advancements can be broadly classified as: fundamental changes to the monoclonal antibody production process, increased bioreactor productivity, and changing regulatory requirements [70]. A trend within the biopharmaceutical industry toward the adoption of single use technologies has likewise influenced the development of encapsulated depth filter technologies that provide reduced cleaning costs, fewer hardware requirements, and faster equipment turnaround times [3]. Improvements in high titer cell culture processes are providing significantly higher cell densities and impurity profiles which may result in the development of bottlenecks within the clarification process. To overcome these challenges, novel centrifuge bowl designs, pretreatment technologies, and improved depth filtration media have been developed [12,75].

These advancements can also be categorized according to the core clarification technologies such as centrifugation and depth filtration. Adaptations of traditional clarification technologies, such as microfiltration, have also been used to better enable perfusion processes [15]. Body feed technologies are also being investigated [94a].

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Perhaps the largest impact on harvest clarification processes can be attributed to advancements in depth filtration technologies. Depth filters have evolved to become the mainstay of almost all clarification processes. Major advancements have been made both in device format and the filtration media. Traditional lenticular filters can now be replaced with encapsulated formats and improved depth filtration media grades are available for various applications including flocculated feed streams, secondary polishing, and soluble impurity clearance. Synthetic depth filter media with reduced organic extractables are also available. These options reduce the concerns around process-related impurities and may enable the use of depth filters in applications further downstream.

In addition to the core clarification technologies described above, significant advancements have also been made in the analytical methods for the characterization of particulate matter, for monitoring cell culture flocculation processes, and for the inline measurement of filtrate turbidity [11,12]. These capabilities help to further the understanding of the clarification process and could enable real time process monitoring and control.

This chapter focuses on advancements in clarification technologies and process development enabling the efficient harvest and purification of monoclonal antibodies and antibody-based therapeutics.

Advances in mechanical separation methods

Mechanical separation methods have been employed for primary clarification applications, targeting removal of large particulates including whole cells and cell debris. The technologies which are used include centrifugation, hydrocyclones and enhanced settling. Of these mechanical separation technologies, centrifugation is most prevalent. Disc stack centrifuges were first evaluated for primary clarification applications in the 1990s [68]. This was mainly due to advent of cell culture processes with higher cell densities and increased particulate loading that limited the ability of tangential flow microfiltration for harvesting [69]. Initial prototype testing demonstrated the feasibility of mechanical separation methods and helped to identify the gaps which needed to be addressed in order to mature these technologies into robust unit operations. There have been significant further advancements in mechanical designs as well as process understanding which has maintained the usefulness of these technologies for harvest clarification [2,12].

There are various types of centrifuges which are used in bioprocessing. These include tubular bowl, chamber bowl, disc stack and decanters. For mammalian cell culture applications, disc stack centrifuges and tubular bowl centrifuges are the most widely adopted designs. Disc stack centrifuges offer high throughput and high solids handling capacity but may demonstrate higher yield losses due to poor dewatering capability with higher density cell cultures. Cleaning can also be a challenging for disc stack centrifuges. In contrast, tubular bowl centrifuges offer a more simplistic, easy to clean design and good dewatering capabilities, but this design provides only a limited throughput [49].

In 1995, a prototype disc stack centrifuge for mammalian cell culture harvest clarification was evaluated and efficient cell removal with decent yield was achieved. The results were promising but some limitations were identified during the testing. These include inefficient solid ejection at high cell densities, susceptibility to pressure fluctuations and increase in temperature over extended residence times. Additionally, the smaller particles and fines

were not efficiently removed, and a secondary clarification step was proposed to address the fines [68]. Modern centrifuges used for mammalian cell culture harvesting have seen significant design changes to address these aspects. Full hermetic and hydro-hermetic feed zone designs reduce the air-liquid interface and offers a more controlled feed acceleration which reduces shear due to mismatched tangential velocities of the feed and pool liquid. Temperatures are controlled by incorporating cooling jackets [2]. Optimal back pressure can be experimentally determined to ensure that the hydro hermetic centrifuge remains flooded and, in case of hermetic centrifuge back pressure, helps mitigate air entrainment in the outlet to improve the accuracy of the inline turbidity measurement [49].

Historically, centrifugation processes have been scaled based on the Sigma theory for sedimentation type centrifuges proposed by Ambler in 1959. Sigma, the index of centrifuge size is defined as the equivalent settling area of a settling tank theoretically capable of delivering same amount of clarification in a unit gravitational field. The scaling methodology recommended by the theory is to keep the ratio of feed flowrate and the sigma constant when scaling up [9]. Although the theory provides an effective mathematical basis for scaling centrifugation processes, there are three main limitations. First, the equation contains a separation efficiency term which needs to be experimentally determined for each centrifuge. Second, a limitation in the theory is that it does not account for the shear susceptibility of cells and cell debris. Scale down batch centrifuges cannot reproduce the shear exposure of cell cultures in industrial centrifuges. This results in a significantly different centrate quality obtained from a bench-top, batch centrifuge and from an industrial centrifuge, even after accounting for the separation efficiency [90]. Third, the theory was originally developed in terms of unhindered settling and with increasing cell densities, the distance between the particles decreases and inter-particle interactions can reduce the settling velocities. The increase in solids concentration can also result in an increase in the viscosity [35].

These limitations make it difficult to generate a representative centrate during process development using bench-top bucket centrifuges for the subsequent sizing of the depth filters that are used for secondary clarification. In recent years, significant efforts have been made in creating scale-down and ultra scale-down models to overcome these scaling challenges.

The smallest disc stack centrifuge requires in excess of 10 L of cell culture, which limits its utility for process development applications [70]. To further scale down the system, Maybury et al. investigated an approach to replace a defined number of active discs with blank discs to reduce the separation area. A ten-fold reduction in active area could be achieved, but since the solid holding capacity of the bowl remained unchanged, there was no impact on feed material requirement [44]. In subsequent work, the bowl holdup volume was reduced by adding stainless steel conical inserts. An approximately four-fold scale down was achieved with this approach with comparable clarification performance [40].

These approaches were able to reduce the feed requirement from tens of liters to a few liters, but this feed volume requirement is still considered a bottleneck. A suitable ultra-scale down (USD) method is required to be able to predict the industrial centrifuge clarification efficiency using milliliter quantities of feed material. Maybury et al. have investigated use of a laboratory centrifuge to predict the performance of industrial centrifuges for both shear-sensitive and shear-insensitive materials. Sigma theory was adapted to accommodate the acceleration and deceleration times of laboratory centrifuges. Accurate predictions for clarification performance was achieved for shear-insensitive materials, whereas for shear-sensitive feeds at the laboratory scale, the efficiency was still significantly over predicted. This may be due to the inability to

mimic the shear of the disc stack centrifuges [41]. Boychyn et al. also observed similar discrepancies in clarification performance between continuous flow centrifuge and laboratory scale down centrifuges due to the breakage of precipitates under higher shear stress [53].

Levy et al. developed a rotating shear device (RSD) which was used to study the effect of shear on plasmid DNA [54]. Boychyn et al. have investigated the pre-shearing of the feed material in a similar RSD prior to using a laboratory centrifuge for clarification. High flow stresses in the feed zone of the centrifuge were modeled using computational fluid dynamics (CFD) and were reproduced in a rotating shear device similar in design to the one described by Levy et al. Pre-sheared feed material was centrifuged in a laboratory centrifuge at pilot equivalent Q/Σ and corrected for acceleration and deceleration time. Using this approach, the production centrifuge clarification efficiency was estimated for both a disc stack centrifuge and a CARR Powerfuge [51,52]. Tunisian et al. adapted the ultra-scale down approach for predicting the centrifugation for high cell density cultures by incorporating a correction for hindered settling [10]. Westoby et al. opted for a capillary based shear system for generating pre-sheared feed. A simple design of the shear system made it easy to build with minimal custom parts. A 1000 mL syringe pump offered the flexibility to work with both low volumes as well as to generate enough material to carry out depth filter sizing [57]. Joseph et al. compared the performance of the rotating shear device and a preparative capillary system. Depth and sterile filtration studies were performed to compare the centrate generated using the preparative capillary shear system to centrate from a pilot scale centrifuge. Similar turbidity and pressure profiles suggest the applicability of this platform to generate a representative centrate for bench scale filter sizing studies [5]. In subsequent work, the use of a fast protein liquid chromatography system was used instead of the syringe pump that was used in the previous work to automate the platform [6].

In addition to developing scale down systems, researchers have continued to optimize operating parameters and to investigate new designs to enhance the performance and keep up with changing upstream requirements. Zhao et al. have investigated the feed flow regulation and solid discharge strategy to improve the product recovery and overall process yield. The partial bowl discharge method was also evaluated where only a fraction of solids are discharged. The method enables a quick reset and re-initiation of feed, but the bowl is momentarily partially empty. This impacts the performance due to an air-liquid interface in the partially empty bowl, which increases shear on the cells [91]. Further Work identified a full shot coupled with a buffer flush was helpful in managing the issue of air liquid interface and offered a better clarification efficiency as compared to the partial discharge method [70,91]. For processing high cell density cultures, an intermittent discharge disc stack centrifuge can run into limitations of the inability to manage clarification performance and yield due to a high solid discharge frequency. A novel tubular bowl centrifuge design with a piston type discharge was evaluated, which can offer better dewatering capabilities to mitigate the yield loss [71]. Ardarion et al. evaluated a continuous discharge centrifuge for high cell density mammalian cell culture harvesting. A turbidity-based discharge control strategy was also implemented on a continuous discharge centrifuge. The cumulative impact of implementing continuous discharge over intermittent discharge and the turbidity-based discharge control was greater than a 10% increase in yield, a reduced turbidity and a decrease in secondary depth filter fouling [12].

The biopharmaceutical industry's progression toward single use manufacturing limits the use of traditional stainless-steel centrifuges for primary clarification. Two single use

TABLE 6.1 Summary of commercially available centrifuge.

	Vendor			
Centrifuge type	**GEA Westfalia**	**Carr® Centrifuge**	**Celeros**	**Sartorius**
Multi-use stainless steel	CSA-1 CSC-6 (periodic discharge) CSD-130 (periodic discharge) CFD-130 (continuous discharge)	Powerfuge® 12, P18, P6, pilot Viafuge® pilot, V12	APD (Automatic piston discharge), ADP 0.5,1.0,5.0,10,50	N/A
Single use	N/A	Centritech® cell II, Unifuge® pilot	N/A	KSep® 400, KSep® 6000s

centrifuge options for clarification operations have been reviewed by A Shukla et al. These are a single use, fluidized bed centrifuge KSep® Systems and a rotating tubular bowl design from Pneumatic Scale Angelus's CARR UniFuge® [4]. To date, there have been limited reports of single use centrifuges in the clarification of mammalian cell culture batch processes and their performance has been evaluated for perfusion and cell harvesting applications [3,38,56]. A summary of commercially available centrifuges is provided in Table 6.1.

In addition to centrifugation, other mechanical separation technologies have been evaluated for clarification operations in mammalian cell culture. These include hydro cyclones and settlers [13,27]. As most of the technologies do not match the solid stream dewatering capabilities of the centrifuge, the product yields remain limited or may require a buffer wash of the solids in order to increase the product recovery.

Encapsulated depth filters

The use of charge-modified depth filters for the large scale filtration of biopharmaceutical process fluids containing concentrated colloidal particles began in the mid-to-late 1970's in parenteral blood fraction manufacturing [89]. Depth filter media made of cellulose and diatomaceous earth (DE) have been used in plate and frame configurations that enable a low-cost deployment of large filter areas. Plate and frame filtration is still used in plasma fractionation where recovery of the filter cake containing the plasma-derived product is required [36]. The use of the same type of depth filter media in a lenticular disk format enabled process containment within large stainless-steel housings and this arrangement was suitable for early adoption in biopharmaceutical manufacturing processes. To avoid both the large infrastructure requirements (e.g. cranes to deal with bell housings) and the equipment cleaning requirements, disposable, modular self-contained depth filters were developed and these have been widely adopted within the biopharmaceutical industry.

Lenticular disk filters often undergo pre-use steam-in-place (SIP) after assembly into a holder. The post-use removal of lenticular filters wetted with the product-containing feed stream is not a desirable task and presents multiple risks from an ergonomic and accident

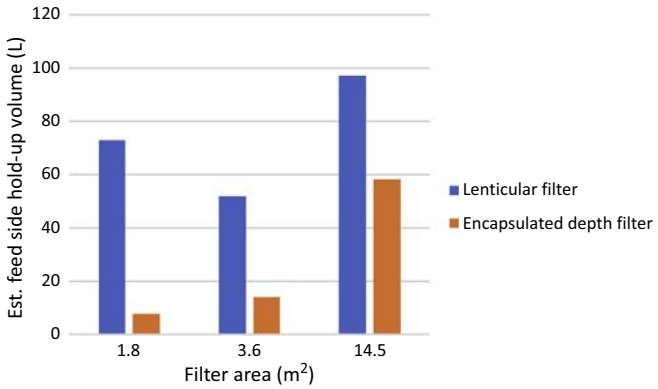

FIG. 6.1 Comparison of depth filtration device fill volumes [61].

risk perspective. After filter removal, clean-in-place (CIP) is needed to prepare the equipment hardware for the next cycle. As shown in Fig. 6.1 below, conventional stacked-disc lenticular filters typically present larger device fill volumes than encapsulated depth filters [61]. Increased fill volumes may contribute to longer processing times, product dilution, and decreased product recovery. Encapsulated depth filters address many of these operational challenges.

Depending on application requirements, the options for both pre- or post-use sterilization are scale-dependent. Fully self-contained capsules, such as Supracap™ 100 capsules, are available with filtration areas up to 0.15 m^2 for double layer depth filter devices or 0.30 m^2 for single layer devices [65]. These devices can also be sterilized by autoclave. For larger installations requiring assemblies of multiple devices within a filter holder, the use of an autoclave can be more difficult, but caustic and hot water sanitization processes may also be considered.

Adsorptive depth filters provide a higher capacity for colloidal particles than can be achieved with size-based retention in normal flow filtration. They achieve this increased capacity by binding through multiple adsorption mechanisms (e.g. anion exchange, hydrophobic interactions) [76]. The depth filter media often contains a diatomaceous earth-based filter aid which is especially important for these interactions [16]. The diatomaceous earth provides the majority of the internal surface area for the depth filters [93]. Depth filtration media also may contain a positively-charged binder resin that is used to increase the wet-strength of the filtration layers. The charged binder resin may also contribute to the binding of soluble impurities (such as host cell proteins or DNA) from the process stream [17]. Most depth filter media manufacturers offer filtration devices containing multiple grades of filter media that are layered in series to maximize capacity and retention across broad particle size distributions. Activated carbon is another filter aid that is blended with cellulosic supports and utilizes its micro and mesoporous structure to adsorb small to large molecular entities such as host cell proteins, or components that would contribute to odor and color [81].

Encapsulated depth filters containing depth filter media comprised of cellulose and DE or activated carbon filter aids are available from multiple vendors within the biopharmaceutical industry. Due to a combination of adsorptive and particle sieving mechanisms, these filters

TABLE 6.2 Summary of commercial encapsulated depth filters.

Application	Vendor			
	MilliporeSigma	Pall	Sartorius	3 M
Single Layer-No Filter Aid	Millistak+®Pod Depth Filter CE-Series	STAX™ Bio-Series	NA	NA
Single Layer-DE	Millistak+® Pod Depth Filter DE-Series	STAX™ P-Series	Sartoclear® S-Series	Zeta Plus™ EZP ZA, SP and HP Series
Multiple Layer-DE	Millistak+® HC Pod Depth Filter	STAX™ HP-Series	Sartoclear® DL-Series	Zeta Plus™ EZP ZA, SP, and HP Series
Activated Carbon	Millistak+® Pod Depth Filter CR40-Series	STAX™ Seitz AKS-Series	NA	Zeta Plus™ EZP ZC Series

can retain particles over a very wide range of particle sizes. Filtration performance is dependent on feed properties, process parameters, and filter loading. Some common examples are listed below (Table 6.2).

Non-woven depth filters

Filtration cartridges containing melt-blown polypropylene microfiber or glass fiber filtration media are available from many manufacturers. The device formats may include string wound, wrapped filters, pleated, or hybrid wrapped-over-pleat filtration media configurations [37]. Filter retention is provided by a combination of particle sieving and adsorption. Layered, gradient density prefilters are also available that provide a coarse filtration zone for the removal of large particles, followed by a tighter layer for the reduction of colloids and bioburden [26].

In upstream processing applications, these prefilters are largely used to extend the life of downstream sterilizing grade membrane filters in media filtration and in cell harvest clarification applications. In downstream processing applications, the filters can be used for bioburden reduction and particulate removal prior to chromatography steps.

Polypropylene depth filters demonstrate good chemical compatibility over a wide pH range [39]. Both polypropylene and glass microfiber-containing depth filters may be sterilized using autoclave, hot water sanitization, or steam-in-place processes.

Wrapped polypropylene depth filters have also been used in low-volume clarification applications for low cell density, high viability feed streams [92]. Due to the absence of positively-charged components in the filtration media, polypropylene prefilters typically demonstrate a low retention of negatively-charged colloidal particles in process streams. These filter cartridges also demonstrate low effective filtration areas. As a result, polypropylene depth filters have limited utility in large-volume cell culture harvest applications. A selection of commercial non-woven prefilter and depth filter options are provided in the summary table below (Table 6.3).

TABLE 6.3 Summary of commercial polypropylene and glass microfiber pre-filters.

Material	Millipore Sigma	Pall	Sartorius	3 M Cuno	Meissner	GE/ Domnick Hunter
Poly-propylene	Clarigard® Polygard® CR, CN, CT	Profile® (II, II+, star) Poly-fine II, Polypure, HDC II	Sartopure® PP3, PP2	Betafine® DP/PEG/ PPG/PTG	Deltadepth®, Deltamax®, Duraclear®, Vangard®, ALpHA BW1	ULTA® prime PP
Glass microfiber	Polygard® CE Polysep® II Lifegard	StarClear®	Sartoclean® GF Sartopure® GF+ Sartogard® GF	*BioLIFE*®	Protec®	ULTA® prime GF

Synthetic depth filters

Traditional depth filters typically comprise naturally-derived materials of construction, including cellulose and diatomaceous earth filter aids [93]. These components may contribute low levels of unwanted soluble and particulate extractibles to the process stream and there are some concerns that these extractibles can contaminate the process fluid [30,67]. Also, due to the increased adoption of single-use systems (SUS) in biopharmaceutical production processes, there have been recent efforts to standardize extractibles testing and to provide a common set of expectations for SUS systems and filters used in these processes [25]. As a result, new depth filters have been developed that replace the cellulose and diatomaceous earth filter components with all-synthetic materials of construction [64]. Synthetic depth filters can demonstrate a decreased extractibles profile and reduced pre-use flushing requirements.

Poly (propylene)-based synthetic depth filters have been investigated for use with polymer-flocculated high-density mammalian cell culture harvests [78]. New synthetic adsorptive hybrid filters have been investigated in post-protein A / low pH viral inactivation steps as a means to remove process-related impurities including HCP, DNA, and product aggregates and have been evaluated for viral clearance applications [77]. Increased performance of synthetic depth filters versus conventional DE-based filters in the clearance of soluble down-stream process impurities has been investigated [64]. The performance of several types of synthetic depth filters in various applications modes has been previously reviewed [76].

Synthetic depth filter compatibility with pre-use caustic sanitization and autoclave sterilization processes are dependent on the materials of construction. The impact of the sanitization or sterilization processes on filter performance and extractibles profile should be assessed prior to use.

Body feed

There is renewed interest in the use of body feed filtration methods as another approach for the primary clarification of cell culture harvests with high solids loading [86]. In a body

feed filtration process, the feed stream is contacted with a quantity of an adsorptive filter aid such as diatomaceous earth (DE). The filter aid greatly increases the permeability of the resulting filter cake, and the resulting combined mass can be more easily separated using a filter septum. In biopharmaceutical processing applications, the filter septum can be a membrane filter, a lenticular depth filter, or an encapsulated depth filter. These processes typically require additional accessory equipment and mixing tanks in order to introduce the powdered filter aid into the feed stream without contaminating the controlled production environment.

Similar to cell culture flocculation approaches, a body feed filtration process requires the determination of the optimum quantity and grade of DE to achieve a good supernatant clarity and to minimize the encapsulated filter area requirements. Higher loadings of DE filter aids typically improve the cake permeability and supernatant clarity but also increases the filter cake depth and the filter areas required.

The compatibility of the filters and equipment used to capture the filter cake produced by body feed filtration is comparable to direct depth filtration depending on the housing or encapsulated filters chosen.

Body feed filtration systems can effectively utilize the adsorptive characteristics of the diatomaceous earth filter aids [8]. The performance of body-feed filtration methods versus conventional cell culture harvest processes including centrifugation has been previously evaluated [33]. No significant difference on turbidity depletion, mAb product recovery, or charge heterogeneity was reported. The impact of DE filter aid loading levels and pH on mAb product recovery and soluble impurity clearance is presented in Table 6.4. The filter aid loading requirements can be reduced at low application pH values.

TABLE 6.4 Impact of DE body feed loading level and pH on mAb product recovery and soluble impurity clearance.[a]

pH	DE loading (g Cellpure® P300/500 mL cell culture)	HCP (mg/mL)		DNA (μg/mL)		mAb02 (mg/mL)	
		Value	Removal	Value	Removal	Value	Yield
4.5	10 g	0.23	81%	0.24	100%	1.39	114%
	20 g	0.21	82%	0.23	100%	1.28	105%
	Feed	0.22	81%	2.65	95%	1.10	90%
5.5	10 g	1.26	−8%	7.04	86%	1.19	98%
	20 g	1.12	4%	6.02	88%	0.96	79%
	Feed	1.34	−15%	4.77	90%	1.05	86%
7.0	10 g	1.17	0%	52.07	−6%	1.19	98%
	20 g	1.14	2%	37.63	23%	1.10	90%
	Feed	1.37	−18%	42.04	14%	1.35	111%
Untreated	Feed	1.17		49.00		1.22	

[a]*EMD Millipore Corporation. Unpublished internal test data.*

TABLE 6.5 Summary of commercial filter aid suitable for body feed applications and compatible filtration systems.

	Vendor	
System	Sartorius	FILTROX
Filter Aid	Celpure® 300	Celpure® Series (25–1000)
Septum	Sartoclear® Dynamics Filter (7–12 μm)	FILTRODISC™ BIO SD Series (>35 to <0.6 μm)

Encapsulated filters and systems suitable for body feed filtration using DE filter aid in biopharmaceutical harvests are available from multiple vendors. Suitable grades of DE filter aid and receiving septum filters are available. Some common examples are listed below (Table 6.5).

Pretreatment technologies

High cell density cultures with increased impurities and cellular debris can present challenges for harvest clarification processes. This challenge may include low filtration capacities for depth filters and premature fouling for tangential flow microfiltration devices. Harvest pretreatment methods such as precipitation or flocculation can significantly improve the impurity clearance and enhance the clarification efficiency. Precipitation relies on the alteration of the solubility of impurities in the harvest whereas a flocculation process produces agglomerates (flocs) of smaller particles that are easier to remove by filtration processes. Various mechanisms have been proposed for flocculation and these include charge neutralization, polymer bridging, electrostatic patch, hydrophobic interactions, and enmeshment within sweep flocs [22,31]. A variety of pretreatment methods have been evaluated for harvest clarification, these include acid precipitation, co-precipitation with calcium phosphate and polymeric flocculation. Acid precipitation involves reducing the pH of the cell culture to between 4.5 and 5. This method can be a simple and effective method of inducing precipitates of fine particulates and other impurities that are present in cell culture [50]. Westoby et al., investigated effect of acid precipitation on settling and clarification with tangential flow microfiltration (MF). The acid precipitated supernatant obtained after settling showed a significant reduction in DNA and turbidity compared to the untreated settled harvest. An improved microfiltration flux and throughput was also observed when processing the acid precipitated harvest relative to the native harvest [90]. Co-precipitation with salts is another pretreatment method that can be used for impurity removal and improving the clarification step. This method involves the addition of two soluble solutions that form a precipitate which assists in impurity removal. Alternatively, the solution can consist of cations which can interact with impurities forming insoluble precipitates [43]. Chen et al. investigated calcium phosphate coprecipitation to harvest high cell density cell cultures using an alternating tangential flow (ATF) filtration method [18].

Flocculation with organic polymers is another pretreatment technology that has been evaluated for impurity clearance and enhanced clarification. Flocculants can be classified on the basis of their functional groups that impart an ionic character to the polymer, and these categories include anionic, cationic and mixed mode functionalities. Polymer flocculation processes can also be designed with the objective of precipitation of the target molecule or alternatively, the precipitation of soluble impurities such as DNA and host cell proteins. Anionic flocculants are primarily used for precipitating the target molecule while most of the impurities remain in the supernatant. The precipitates are recovered and resolubilized to allow a further purification during the subsequent unit operations. McDonald et al., investigated the effect of solution pH, solution ionic strength and the molecular weight of flocculants on the precipitation of an antibody by using three anionic polyelectrolytes, polyvinyl sulfonic acid (PVS), polyacrylic acid (PAA), and polystyrene sulfonic acid (PSS) [59].

Cationic and mixed mode polyelectrolytes are typically used for the flocculation of cells, cellular debris and other impurities while the soluble target molecule remains in the supernatant. The flocs are removed and discarded, and supernatant containing the target molecule can then be further purified over the subsequent unit operations. Cationic polymers like chitosan [29], poly diallyl dimethyl ammonium chloride (pDADMAC) [60,85], and polyamines [66] have been evaluated for the pretreatment of cell culture fluids for impurity removal and for improving clarification efficiency. A multimodal flocculant can offer a broader range of impurity clearance as compared to anionic and cationic flocculants [75]. Kang et al., evaluated a partially benzylated poly (allylamine) salt-tolerant cationic polymer (Clarisolve® mPAA, EMD Millipore) for the flocculation of cell culture. The performance was compared with two cationic polymers, Poly (ethyleneimine) and pDADMAC. The multimodal flocculant achieved a higher HCP reduction and also increased the aggregate removal. Filtration studies were performed on Clarisolve® Depth filters. In this study, a three to five-fold increase in the filter capacity with equivalent filtrate turbidity was observed versus an untreated harvest filtered through a conventional two-stage depth filtration train consisting of Millistak+® D0HC and Millistak+® X0HC depth filters [45]. Polymeric flocculants can be cytotoxic, hence their clearance in the drug product below an acceptable exposure limit needs to be demonstrated. The acceptable exposure limit needs to be established based on the method of administration, and this process can entail in vitro and in vivo toxicity studies. Robust analytical procedures are also required for the quantification of the flocculant concentrations and for establishing the polymer clearance level during the subsequent purification steps in the biopharmaceutical production process [60,75].

Cell retention devices

The growing trend of continuous processing in the biopharmaceutical industry is driven by multiple factors. The key factors include the expansion of biosimilars, the needs for high volumetric productivity, operational flexibility, improved product quality, and lower production costs.

Due to advancements in mammalian cell culture production, current fed-batch bioreactor processes can routinely express mAbs at titers above 5 g/L [1]. Continuous processing of

perfusion cell cultures can result in further increases in the overall productivity of bioreactor operations.

The upstream portion of a continuous process typically utilizes a perfusion bioreactor. Perfusion processes can enable cell densities higher than 20 million cells/mL. The operation of a perfusion bioreactor may involve the continuous or semi-continuous addition of fresh cell culture medium with simultaneous removal of the target product along with metabolic waste, while the cells remain in the bioreactor. Such operations are enabled via the use of cell retention technologies.

A wide variety of technologies and devices have been historically applied in the industry for the purpose of cell retention [80,82]. These technologies include density based retention like gravity settlers, centrifuges and hydrocylcones, acoustic separators; Sieving based retention using membranes like spin filters, dynamic filters and tangential flow filters, and some unique cell characteristics likes dielectrophoretic separators or by employing modified upstream processes like cell immobilization.

Regardless of the type of cell retention device, an efficient retention of cells and a robust operation must be maintained throughout the duration of a perfusion process. For a robust operation, a desired volumetric flow rate, high cell integrity and viability, and acceptable product yield should be maintained without the need of the device replacement or maintenance, thus minimizing the risk of bioreactor contamination [48]. Cell retention devices must also be scalable across the laboratory ($\leq$5 L), pilot, and the final production scales ($\geq$200 L).

More recently, perfusion-based intensification of fed-batch mammalian cell production has enabled significantly higher cell densities (up to and above 100 million cells/mL). Such intensification is accomplished by increasing the cell density in the N-1 seed or N-2 seed bioreactors prior to the production bioreactor (N). These processes are carried out with the use of a cell retention device attached to the seed bioreactor. This allows the production bioreactor to be inoculated at a much higher cell density. As a result, the overall productivity is increased due to a significantly shortened run time that is achieved via the elimination of several small-scale expansion stages and accelerating the time to steady-state cell density [14,58].

The limitations in perfusion rates, longer residence times, and limited scalability associated with some types of cell retention devices, such as gravity settlers, make them difficult to employ in intensification applications. Membrane-based cell retention devices are more adaptable for that purpose, as their scale-up is nearly linear and they are more easily integrated within a continuous operation. Two technologies that are commonly applied in current perfusion operations are traditional TFF (Tangential Flow Filtration) and ATF (Alternating Tangential Flow) systems [15,46].

Both TFF and ATF cell retention systems utilize microfiltration (MF) membranes typically made of modified polyether sulfone (PES) or polysulfone (PS). Traditional TFF devices are also available with hydrophilized polyvinylidene fluoride (PVDF) [19] and mixed cellulose ester (ME) microfiltration membranes. Membrane-based devices are offered in pre-sterilized or SIP/autoclavable device formats (Table 6.6).

Concentrated fed-batch perfusion processes are also accomplished with the use of TFF devices containing ultrafiltration (UF) membranes. In such applications, the product molecule

TABLE 6.6 Summary of commercial microfiltration membrane-based cell retention devices.

Vendor	MilliporeSigma	Repligen		GE	Artemis Biosystems
Brand	Prostak™	XCell™	KrosFlo®	ReadyToProcess	Virus Harvest Units (VHU™)[a]
Format	Flat Sheet	HF	HF	HF	HF
Flow	TFF	ATF	TFF	TFF/ATF	TFF
Pore size (μm)	1.1−0.65	0.2	1.1−0.65	1.1−0.65	nd
Material	PVDF	PS	mPES, PS, ME	PS	nd

[a]*Used in viral harvest applications.*

is retained and accumulated in a bioreactor while the lower molecular weight components and toxic byproducts are removed in the permeate. UF perfusion is suitable for stable biomolecules where there is no concern about degradation during its accumulation in the bioreactor. These processes can offer a simple approach for improving the space/time yield of a facility where an antibody is produced [88].

The main difference between ATF and TFF operated systems is the way that the cell culture feed flow is supplied. In ATF operation, the feed flow is moved in alternating directions (back and forth) by a diaphragm pump. In TFF systems, a unidirectional cross-flow is commonly supplied with the use of a peristaltic pump. Both the two-directional flow and the one-direction recirculated feed flow are optimized to minimize membrane polarization and to prevent membrane fouling during the lengthy perfusion process. Selecting a low-shear pump for operating a perfusion system has been shown to be one of the key factors for maintaining cell integrity and minimizing cell lysis [73].

In membrane operations, cell lysis can aggravate polarization and eventually lead to membrane fouling which gets more profound at high cell density, low cell viability, and at increased permeate flow rates. Polarization and fouling can diminish the recovery of the product molecule into the permeate, consequently resulting in an overall low yield and reduced productivity [32].

Membrane-less cell retention devices based on spiral inertial focusing microfluidic devices utilize a combination of hydrodynamic forces. These devices are dependent on the particle size and density for an inertial sorting of cells in order to focus and to separate the particles laterally into a continuous flow within a spiral microchannel with trapezoidal cross-section. The control of the motion of particles only requires the hydrodynamic forces that are derived from the channel structure and particles, without the need for active force fields, such as electric fields or acoustic waves [84]. Microfluidic devices have been shown to be feasible for use in continuous perfusion culture of suspended mammalian cells [87]. The cell retention efficiency in microfluidic devices depends on the input cell concentration. Increased input cell concentrations result in increased cell-to-cell interactions because the cells are competing for the same equilibrium position along the channel. This leads to a broadening of the focused band and a decreased efficiency of inertial focusing. The retention efficiencies of microfluidic spiral devices used with CHO perfusion cultures at 15.4×10^6 cells/mL and 22.2×10^6 cells/mL

are 99.2% and 92.2%, respectively. These values dropped to below 83% with cell concentrations above 26.5 x 10^6 cells/mL, due to cell loss at the outer outlet of the device. At 4.2 x 10^6 cells/mL (1.2 million cells/mL) (mean (range), $n = 3$) the perfusion bioreactor was continuously processed through a microfluidic cell retention device for 145 h with no impact on cell viability and with a retention efficiency that was maintained at 98%. The retention efficiency can be further improved with a modified channel dimension and flow rates for use with higher cell densities. For larger volumes, perfusion microfluidic devices could be scaled-up via parallel stacking of several spiral microchannels, with 600 connected channels capable of supporting perfusion rates close to 1000 L/day) [83].

While inertial focusing microfluidics technology shows a great potential for continuous cell culture operations, there are presently no commercial devices offered in the industry suitable for the GMP production of biotherapeutics at large scale.

Membrane-free acoustic settler retention devices utilize acoustic waves to enhance the settling velocity of suspended cells.

The technology of the BioSep® cell retention system is based on high frequency resonant ultrasonic waves. Acoustic resonance generates radiation and Bernoulli forces that can separate cells by inducing a loose aggregation followed by sedimentation without affecting the cell viability [28]. The separation of cells from the medium is based on the difference in size, density and compressibility, and the viable cells tend to be retained more selectively and more efficiently [47]. The separation efficiency of the BioSep® system is controlled by adjusting the acoustic power input to the resonator. The typical separation efficiency ranges from 90% to 99% with cell densities of 20–25 x 10^6 cell/mL.

Another commercially available acoustic separator technology is based on an acoustophoretic separation with low frequency waves. Acoustic wave separators (AWS) utilize acoustic forces that can generate a 3-dimensional standing wave across a countercurrent flow of a cell culture fluid in the device channel. As a result, the cells are trapped by the acoustic forces and they migrate to the nodes of the standing wave. The cells start clumping together until they settle out of the suspension by gravity. The system is optimized for CHO cells at cell densities of 20–50 $\times$ 10^6 cells/mL. However, the system can also be applied to a range of biologic products regardless of variability in particulate concentrate or cell culture density [74].

The noted drawbacks of acoustic systems are related to a high power consumption and scalability issues associated with differences in acoustic patterns among different size devices [94].

In centrifugation-based devices, the solid-liquid separation is achieved not with a physical barrier but through density differences between cellular solid particles and the culture fluid. Centrifuges have been scaled up successfully for several perfusion processes and they can be operated at relatively high flow rates.

For use in perfusion operations, a centrifugation device has to be aseptically and continuously operated through the required duration of time with no detrimental effect on the integrity and viability of the cells. Single-use kSep® systems are bowl-type centrifuges that do not stop rotating during solids discharge. This system can concentrate high density cells (10^8 cells/mL) with full product recovery (>95%) and no significant viability loss or cell lysis [62] (Table 6.7).

TABLE 6.7 Summary of commercial membrane-less cell retention devices.

Vendor	Pall	Applikon	Sartorius
Technology	Acoustic	Acoustic	Continuous centrifugation
Brand	Cadence™ AWS	BioSep	kSep®
Flow Rate	0–350 L/h [34]	0.1–1000 L/day	<720 L/h

Analytical technologies for clarification

Harvest clarification operations target the removal of insoluble particulates and soluble impurities without a detrimental impact on the product quality and yield. Analytical methods are required to characterize these aspects of purification and to assess the product titer, product quality and to support the development and monitoring of the production process. An array of standard analytical tools and techniques are used for the characterization of impurities and product quality through the entire monoclonal antibody purification process [79]. The particulate characterization is a relatively unique aspect of the harvest clarification and this requires specific analytical methods for process development and monitoring.

The most commonly used method for the assessment of the clarification efficiency is the determination of the solution turbidity or optical density. Turbidity is a measure of the haze or cloudiness caused by presence of suspended particulates present in the sample. It is measured using Nephelometric turbidimeters where the intensity of scattered light is correlated to the sample turbidity on the basis of a Formazin calibration curve. In their original design, nephelometers located a detector at an angle of 90 degree to the incident beam. However, modern nephelometers can utilize multiple detectors [72]. Turbidimeters are available for both offline and inline turbidity measurements. Researchers have also used the optical density for estimating the clarification efficiency [10]. The major limitations of turbidity and optical density measurements are the inability to provide any information about the particle size distribution.

The determination of the particle size distribution and concentration is necessary for the assessment of the effectiveness of any mechanical separation or filtration method used in clarification. The major challenges for particle distribution characterization methods are the lack of a single method to accurately and reliably measure the particle size and concentration over the entire size range relevant for clarification. This range spans from approximately 100 nm–100 μm in diameter.

For larger particle sizes ranging between 1 μm and 1 mm, focused beam reflectance measurement (FBRM) has been used by various researchers for tracking the change in the particle size distribution during flocculation. Senczuk et al. evaluated the use of FBRM to track the shift in the particle size over time in pDADMAC flocculated feeds [11]. Burgstaller et al. used a FBRM probe with a customized flow cell to monitor the particle counts of two different particle size ranges for inline flocculation [21]. The chord length distribution (CLD) obtained from FBRM is related to the particle size distribution (PSD), but does not

provide an actual PSD. To obtain the particle size distribution, a calibration with image analysis is typically required. Pandit et al. have proposed an algorithm to convert the CLD to PSD and this showed a good agreement when confirmed with image analysis [7]. Another approach is dynamic imaging analysis, where information such as size distribution, count and shape of subvisible particulates is extracted directly from an image taken by micro imaging systems [23].

For smaller particle size measurements, three methods are commonly used: laser diffraction, dynamic light scattering (DLS) and coulter counter. Laser diffraction particle size analyzers estimate the particle size based on angular variation in the intensity of scattered light. Particle size measurements can be made over a broad range from 10 nm to 2000 μm, although identifying accurate optical parameters can be challenging [20]. Hutchinson et al. have used a Mastersizer (Malvern Instruments) for particle size analysis [63]. Dynamic light scattering (DLS) is another popular method for the particle size measurement of biomaterials. Under Brownian motion, the particles show very short time scale fluctuations in the intensity of scattered light. The rate of these fluctuations is dependent on size of the particles. DLS estimates the hydrodynamic radius of the particles in suspension based on these rates of variations in the intensity of the light scatter [55]. The range is limited compared to laser diffraction and, depending on the instrument, this range can vary over 0.3 nm−10 μm [23]. Coulter counters on other hand, measure particle size based on the change in impedance across an aperture when a non-conducting particle passes through it. This technology can make robust particle size distribution measurements, but the range is limited for a given tube aperture size. Particle measurements from different aperture tubes can be combined to obtain an overall range from 0.4 μm to 1600 μm [23]. Westoby et al. used a Beckman Coulter Multisizer III equipped with a 30 μm aperture for the analysis of a centrate particle size distribution with a capillary shear device based on the ultra scale down method [90].

The filterability on depth filters and sterile filters have also been used as a method to compare the clarification efficiency. Joseph et al. compared the centrate quality obtained from a pilot scale centrifuge to one obtained from a scale down system by assessing the filterability through a Millistak®+ X0HC depth filter with a 0.1−2 nominal micron rating [5].

In addition to these conventional methods, certain indirect methods of characterization have been investigated by researchers. These include monitoring lactate dehydrogenase (LDH) as an indicator of cell lysis during shear studies. LDH is a soluble cytoplasmic enzyme which is released when the cells rupture. Petersen et al. used LDH levels to quantify the extent of cell lysis when investigating the shear sensitivity of hybridoma cells and their dependence on the mode of growth, cell culture age and the metabolite concentration [42]. Joseph et al. used LDH levels to calibrate the preparative capillary shear device to a rotating shear device [5]. Although LDH measurement is effective in tracking the cell lysis, it is not a useful for the assessment of flocculation or precipitation processes due to its soluble nature. Senczuk et al. investigated cholesterol as a surrogate for estimating the concentration of fine particulates in cell culture centrate. These particles predominantly consist of cellular debris. Cholesterol, a component of the animal cell membrane lipid bilayer can be relatively easily analyzed and the levels of cholesterol in the centrate were found to correlate well with the depth filter throughputs [11].

References

[1] Shukla AA, Wolfe LS, Mostafa SS, Norman C. Evolving trends in mAb production processes. Bioeng Transl Med 2017:58–69.

[2] Shukla AA, Gadam S, Etzel MR. Process scale bioseparations for the biopharmaceutical industry. Boca Raton: CRC Press; 2006.

[3] Shukla AA, Mostafa S, Wilson M, Lange D. Vertical integration of disposables in biopharmaceutical drug substance manufacturing. BioProcess Intern Tech 2012:34–47.

[4] Shukla AA, Gottschalk U. Single-use disposable technologies for biopharmaceutical manufacturing. Trends Biotechnol 2013:147–54.

[5] Joseph A, Kenty B, Mollet M, Hwang K, Rose S, Goldrick S, Bender J, Farid SS, Titchener-Hooker N. A scale-down mimic for mapping the process performance of centrifugation, depth and sterile filtration. Biotechnol Bioeng 2016:1934–41.

[6] Joseph A, Goldrick S, Mollet M, Turner R, Bender J, Gruber D, Farid SS, Titchener-Hooker N. An automated laboratory-scale methodology for the generation of sheared mammalian cell culture samples. Biotechnol J 2017:1–8.

[7] Pandit AV, Ranade VV. Chord length distribution to particle size distribution. Am Ins Chem Eng J 2016:4215–28.

[8] Al-Ghouti MA, Khraisheh MAM, Allen SJ, Ahmad MN. The removal of dyes from textile wastewater: a study of the physical characteristics and adsorption mechanisms of diatomaceous earth. J Environ Manag 2003;69:229–38.

[9] Ambler CM. The theory of scaling up laboratory data for the sedimentation type centrifuge. J Biochem Microbiol Technol Eng 1959:185–205.

[10] Tustian AD, Salte H, Willoughby NA, Hassan I, Rose MH, Baganz F, Hoare M, Titchener-Hooker NJ. Adapted ultra scale-down approach for predicting the centrifugal separation behavior of high cell density cultures. Biotechnol Prog 2007:1404–10.

[11] Senczuk A, Petty K, Thomas A, McNerney T, Moscariello J, Yigzaw Y. Evaluation of predictive tools for cell culture clarification performance. Biotechnol Bioeng 2015:568–75.

[12] Richardson A, Walker J. Continuous solids-discharging centrifugation A solution to the challenges of clarifying high–cell-density mammalian cell cultures. BioProcess Intern Tech 2018:38–47.

[13] Batt BC, Davis RH, Kompala DS. Inclined sedimentation for selective retention of viable hybridomas in a continuous suspension bioreactor. Biotechnol Prog 1990:458–64.

[14] Wright B, Bruninghaus M, Vrabel M, Walther J, Shah N, Bae S-A, Johnson T, Yin J, Zhou W, Konstantinov K. A novel seed-train process using high-density cell banking, a disposable bioreactor, and perfusion technologies. BioProcess Intern 2015;13.

[15] Bielser JM, Wolf M, Souquet J, Broly H, Morbidelli M. Perfusion mammalian cell culture for recombinant protein manufacturing – A critical review. Biotechnol Adv 2018:1328–40.

[16] Buyel JF, Gruchow HM, Fischer R. Depth filters containing diatomite achieve more efficient particle retention than filters solely containing cellulose fibers. Front Plant Sci 2015;6:1134.

[17] Charlton HR, Relton JM, Slater NK. Characterisation of a generic monoclonal antibody harvesting system for adsorption of DNA by depth filters and various membranes. Bioseparation 1999;8:281–91.

[18] Chen G, Su Z, Li F, Liu HF. Application of calcium phosphate flocculation in high-density cell culture fluid with high product titer of monoclonal antibody. Bioproc Biosyst Eng 2017:703–14.

[19] Martin C, Padilla-Zamudio J, Rank, D, McInnis P, Kozlov M, Reynolds S, Parella J, Madrid L. Novel small scale TFF cell retention device for perfusion cell culture systems. BMC Proceedings. 2015;9:25. https://doi.org/10.1186/1753-6561-9-S9-P25.

[20] Cornelia M, Keck RH. Size analysis of submicron particles by laser diffractometry—90% of the published measurements are false. Int J Pharm 2008:150–63.

[21] Burgstaller D, Krepper W, Haas J, Maszelin M, Mohoric J, Pajnic K, Jungbauer A, Satzer P. Continuous cell flocculation for recombinant antibody harvesting. J Chem Technol Biotechnol 2018:1881–90.

[22] Dao VH, Cameron NR, Saito K. Synthesis, properties and performance of organic polymers employed in flocculation applications. Polym Chem 2016:11–25.

[23] Das TK. Protein particulate detection issues in biotherapeutics development–current status. AAPS PharmSciTech 2012:732–46.

[24] Deleted in review.
[25] Ding W, Madsen G, Mahajan E, O'Connor S, Wong K. Standardized extractibles testing protocol for single-use systems in biomanufacturing. Pharmaceut Eng 2014;34(6):1–11.
[26] Dixit M, Braeutigam U. Biopharmaceutical industry: the importance of prefiltration. Filtrat Separ 2007:24–6.
[27] Elsayed EA, Wadaan AM. The potential of hydrocyclone application for mammalian cell separation in perfusion cultivation bioreactors. Int J Biotechnol Wellness Ind 2013:153–63.
[28] Trampler F, Sonderhoff SA, Pui PWS, Kilburn DJ, Piret JM. Acoustic cell filter for high density perfusion culture of hybridoma cells. Bio Technol 1994:281–4.
[29] Frank R, Schroeder J, Belliveau J, Kang X, Kutzko J, Menon MK. The use of chitosan as a flocculant in mammalian cell culture dramatically improves clarification throughput without adversely impacting monoclonal antibody recovery. J Biotechnol 2007:813–23.
[30] Gao Y, Allison N. Extractibles and leachables issues with the application of single use technology in the biopharmaceutical industry. J Chem Technol Biotechnol 2016;91:289–95.
[31] Ghernaouta D, Ghernaout B. Sweep flocculation as a second form of charge neutralisation-A review. Desalin Water Treat 2012:15–28.
[32] Bolton GR, Apostolidis AJ. Mechanistic modeling of the loss of protein sieving due to internal and external fouling of microfilters. Biotechnol Prog 2017:1323–33.
[33] Grauf M, Lagrange B, Schone K. Simplified small-scale harvest of CHO cells for mAb analytics. Genet EngBiotechnol News 2018;38. https://doi.org/10.1089/gen.38.05.07.
[34] Harvesting technology guide for mAb processes. Pall Corporation; 2018.
[35] Salte H, King JM, Baganzi F, Hoare M, Titchener-Hooker NJ. A methodology for centrifuge selection for the separation of high solids density cell broths by visualisation of performance using windows of operation. Biotechnol Bioeng 2006:1218–27.
[36] Hilgers U, Thiemann J. Depth filtration in plasma challenges. Filtrat Separ May/June 2019:50–1.
[37] Honig E, Schwartz P. Impact of design and selection of prefilters on operating cost. Filtrat Separ 1997:73–8.
[38] Ko HF, Ravi Bhatia R. Evaluation of single-use fluidized bed centrifuge system for mammalian cell harvesting. Biopharm Int 2012:1–5.
[39] Hutten IM. Handbook of nonwoven filter media. Oxford: Elsevier Ltd; 2007.
[40] Maybury JP, Mannweiler K. The performance of a scaled down industrial disc stack centrifuge with a reduced feed material requirement. Bioporcess Eng 1998:191–9.
[41] Maybury JP, Hoare M. The use of laboratory centrifugation studies to predict performance of industrial machines: studies of shear-insensitive and shear-sensitive materials. Biotechnol Bioeng 2000:265–73.
[42] Jonathan F, Petersen LV. Shear sensitivity of cultured hybridoma cells (CRL-8018) depends on mode of growth, culture age and metabolite concentration. J Biotechnol 1988:229–46.
[43] Coffman JL, Russell I, Shpritzer RI, Vicik SM. USA patent No. US 7,855,280 B2. 2010.
[44] Mannweiler K, Hoare MH. The scale-down of an industrial disc stack centrifuge. Bioprocess Eng 1992:19–25.
[45] Kang YK, Hamzik J, Felo M, Qi B, Lee J, Ng S, Liebisch G, Shanehsaz B, Singh N, Persaud K, Ludwig DL, Balderes P. Development of a novel and efficient cell culture flocculation process using a stimulus responsive polymer to streamline antibody purification processes. Biotechnol Bioeng 2013:2928–37.
[46] Karst DJ, Serra E, Villiger TK, Soos M, Morbidelli M. Characterization and comparison of ATF and TFF in stirred bioreactors for continuous mammalian cell culture processes. Biochem Eng J 2016:17–26.
[47] Kilburn DG, Clarke D. Enhanced sedimentation of mammalian cells following acoustic aggregation. Biotechnol Bioeng 1989;34:559–62. https://doi.org/10.1002/bit.260340415.
[48] Leda R, Castilho RA. Cell retention devices for suspended-cell perfusion cultures. Adv Biochem Eng Biotechnol 2002;74:129–69. https://doi.org/10.1007/3-540-45736-4_7.
[49] Leung W. Centrifugal separations in biotechnology. Oxford: Elsevier; 2007.
[50] Lydersen BK, Gibson TB, Murel A. Acid precipitation of mammalian cell fermentation broth. Ann N Y Acad Sci 1994:222–31.
[51] Boychyn M, Yim SSS, Shamlou PA, Bulmer M, More J, Hoare M. Characterization of flow intensity in continuous centrifuges for the development of laboratory mimics. Chem Eng Sci 2001:4759–70.
[52] Boychyn M, Yim SS, Bulmer M, More J, Bracewell DG, Hoare M. Performance prediction of industrial centrifuges using scale-down models. Bioproc Biosyst Eng 2004:385–91.
[53] Boychyn M, Doyle W, Bulmer M, More J, Hoare M. Laboratory scaledown of protein purification processes involving fractional precipitation and centrifugal recovery. Biotechnol Bioeng 2000:1–10.

[54] Levy MS, Ciccolini LAS, Yim SSS, Tsai JT, Titchener-Hooker N, Shamlou PA, Dunnill P. The effects of material properties and fluid flow intensity on plasmid DNA recovery during cell lysis. Chem Eng Sci 1999:1371–8.
[55] Malvern. A basic guide to particle characterization. Malvern Instruments Limited; 2015.
[56] Johnson M, Lanthier S, Massie B, Lefebvre G, Kamen AA. Use of the centritech lab centrifuge for perfusion culture of hybridoma cells in protein-free medium. Biotechnol Prog 1996:855–64.
[57] Westoby M, Rogers JK, Haverstock R, Romero J, Pieracci J. Modeling industrial centrifugation of mammalian cell culture using a capillary based scale-down system. Biotechnol Bioeng 2010:989–98.
[58] Stettler M, Monchois V, Jordan M. Intensification of large-scale mammalian fed batch Processes. Pharmaceut Bioprocess 2018.
[59] McDonald P, Victa C, Carter-Franklin JN, Fahrner R. Selective antibody precipitation using polyelectrolytes: a novel approach to the purification of monoclonal antibodies. Biotechnol Bioeng 2008:1142–51.
[60] McNerney T, Thomas A, Senczuk A, Petty K, Zhao X, Piper R, Carvalho J, Hammond M, Sawant S, Bussiere J. PDADMAC flocculation of Chinese hamster ovary cells: enabling a centrifuge-less harvest process for monoclonal antibodies. mAbs 2015:413–27.
[61] Corporation Millipore. Innovative pod platform delivers disposable option for depth filtration. Retrieved from: http://www.iptonline.com; 2005. http://www.iptonline.com/articles/public/IPT_18_pg12&13_2005.pdf.
[62] Morrow KJ. Sartorius takes a single-use approach to derive value from biomanufacturing. Genet Eng Biotechnol News 2019.
[63] Hutchinson N, Bingham N, Murrell N, Farid S, Hoare M. Shear stress analysis of mammalian cell suspensions for prediction of industrial centrifugation and its verification. Biotechnol Bioeng 2006:483–91.
[64] Nguyen HC, Langland AL, Amara JP, Dullen M, Kahn DS, Costanzo JA. Improved HCP reduction using a new, all-synthetic depth filtration media within an antibody purification process. Biotechnol J 2018:1700771.
[65] Pall Corporation. Stax™ disposable depth filter systems. (n.d.). Retrieved from: www.pall.com: https://shop.pall.com/us/en/biotech/depth-filtration/depth-filter-housings/stax-disposable-depth-filter-systems-zidgri78m6c.
[66] Peram T, McDonald P, Carter-Franklin J, Fahrner R. Monoclonal antibody purification using cationic polyelectrolytes: an alternative to column chromatography. Biotechnol Prog 2010:1322–31.
[67] Pillai SA, Chobisa D, Urimi D, Ravindra N. Filters and filtration: a review of mechanisms that impact cost, product quality, and patient safety. J Pharmaceut Sci Res 2016;8(5):271–8.
[68] Kempken R, Preissmann A, Berthold W. Assessment of a disc stack centrifuge for use in mammalian cell separation. Biotechnol Bioeng 1995:132–8.
[69] van Reis R, Zydney A. Membrane separations in biotechnology. Curr Opin Biotechnol 2001:208–11.
[70] Roush DJ, Yuefeng L. Advances in primary recovery: centrifugation and membrane technology. Biotechnol Prog 2008:488–95.
[71] Lander R, Daniels C, Meacle F. Efficient, scalable clarification of diverse bioprocess streams using a novel pilot-scale tubular bowl centrifuge. Bioprocess Int Tech 2005:32–40.
[72] Sadar M.J. Turbidity science technical information series—booklet No. 11 (n.d.). Hach Company.
[73] Wang S, Godfrey S, Ravikrishnan J, Lin H, Vogel J, Coffman J. Shear contributions to cell culture performance and product recovery in ATF and TFF perfusion systems. J Biotechnol 2017:52–60.
[74] Sargent B. Clarification using acoustic wave separation offers advantages including – continuous process solution. Retrieved from: https://downstreamcolumn.com; 2016.
[75] Singh N, Chollangi S. Next generation clarification technologies for downstream processing of antibodies. In: Gottschalk U, editor. Process scale purification of antibodies; 2017. p. 81–112.
[76] Singh N, Arunkumar A, Chollangi S, Tan ZG, Borys M, Li ZJ. Clarification technologies for monoclonal antibody manufacturing processes: current state and future perspectives. Biotechnol Bioeng 2016;113:698–716.
[77] Singh N, Arunkumar A, Peck M, Voloshin AM, Moreno AM, Tan Z, Hester J, Borys MC, Li ZJ. Development of adsorptive hybrid filters to enable two-step purification of biologics. mAbs 2017;9(2):350–64.
[78] Singh N, Pizzelli K, Romero JK, Chrostowski J, Evangelist G, Hamzik J, Soice N, Cheng KS. Clarification of recombinant proteins from high cell density mammalian cell culture systems using new improved depth filters. Biotechnol Bioeng 2013;110(7):1964–72.
[79] Flatman S, Alam I, Gerard J, Mussa N. Process analytics for purification of monoclonal antibodies. J Chromatogr B 2007:79–87.

[80] Woodside SM, Bowen BD, Piret JM. Mammalian cell retention devices for stirred perfusion bioreactors. Cytotechnology 1998:163–75.
[81] Stone MT, Kozlov M. Separating proteins with activated carbon. Langmuir 2014;30(27):8046–55.
[82] Subramanian G. Continuous biomanufacturing: innovative technologies and methods. John Wiley & Sons; 2017. Science.
[83] Taehong K, Holly P, Jonas O, Nyasha M, Majid EW, François HJ, Jongyoon H. Microfluidic cell retention device for perfusion of mammalian suspension culture. Sci Rep 2017;7:6703. https://doi.org/10.1038/s41598-017-06949-8.
[84] Applications of microfluidic systems in biology and medicine. In: Ros A, Manabu Tokeshi, editors. Bioanalysis, advanced materials, methods, and devices. Springer; 2019.
[85] Tomic S, Besnard L, Fürst B, Reithmeier R, Wichmann R, Schelling P, Hakemeyer C. Complete clarification solution for processing high density cell culture harvests. Separ Purif Technol 2015:269–75.
[86] Meer TVD, Minow B, Lagrange B, Krumbein F, Rolin F. Diatomaceous earth filtration: innovative single-use concepts for clarification of high-density mammalian cell cultures. BioProcess Int 2014, September 23. Retrieved from: https://bioprocessintl.com/downstream-processing/filtration/diatomaceous-earth-filtration-innovative-single-use-concepts-clarification-high-density-mammalian-cell-cultures/.
[87] Voisard D, Meuwly F, Ruffieux PA, Baer G, Kadouri A. Potential of cell retention techniques for largescale high-density perfusion culture of suspended mammalian cells. Biotechnol Bioeng 2013:751–65.
[88] Yang WC, Minkler DF, Kshirsagar R, Ryll T, Huang YM. Concentrated fed-batch cell culture increases manufacturing capacity without additional volumetric capacity. J Biotechnol 2016:1–11.
[89] Waterson RW. Novel depth filtration technologies: strategies for process development. Pharmaceut Eng 1990;10(5):22–9.
[90] Westoby M, Chrostowski J, de Vilmorin P, Smelko JP, Romero JK. Effects of solution environment on mammalian cell fermentation broth properties: enhanced impurity removal and clarification performance. Biotechnol Bioeng 2010:50–8.
[91] Zhao Z, Zhou J. Effect of solid ejection size on continuous centrifugation during mammalian cell culture product recovery. In: 234th ACS National Meeting, BIOT Division, Boston; 2007.
[92] Yavorsky D, Blank R, Lambalot C, Brunkow R. The clarification of bioreactor cell cultures for biopharmaceuticals. Pharmaceut Technol 2003:62–76.
[93] Yigzaw Y, Piper R, Tran M, Shukla AA. Exploitation of the adsorptive properties of depth filters for host cell protein removal during monoclonal antibody purification. Biotechnol Prog 2006;22:288–96.
[94] Yoshida T. Applied bioengineering: innovations and future directions. In: Advanced biotechnology. John Wiley & Sons; 2017.
[94a] Meer VDT. Single-Use Clarification System for high cell density cell cultures. Contract Pharma 2015:65–7.

CHAPTER

7

Recent advances in ultrafiltration and virus filtration for production of antibodies and related biotherapeutics

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Introduction

Virus filtration and ultrafiltration are two key technologies in biopharmaceutical manufacturing which typically comprise the final two steps of downstream processing for antibodies. While neither specifically purifies the drug substance in the same way that, for instance, a chromatography operation is considered to, they each play a critical role in the overall process.

The need to remove potential harmful virus contaminants from biopharmaceutical drugs has always been a concern, both for early plasma-derived and newer animal cell culture derived drug products. Virus safety in the biopharmaceutical industry has evolved over the years. Virus filtration has and continues to play a key role in the mission of process safety for biopharmaceutical drug products. These filters are added into the process to robustly remove both endogenous and adventitious viruses via size exclusion (viruses are retained and the drug product passes through). They provide additional value over added steps like virus inactivation (solvent/detergent treatment or low pH hold) because they do not leave viral material behind in the process stream. Virus filters also are considered more robust compared to adsorption-based virus removal steps because their viral removal capability is less impacted by operating parameters such as feed concentration, flow rate, pH, or conductivity.

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Virus filters have evolved to better suit the needs of the biopharma industry, including current trends such as continuous processing and higher monoclonal antibody (mAb) concentrations. New techniques in filter manufacturing have improved virus removal capability, process robustness, and integrity testing sensitivity. Additionally, new feed prefiltration methods have improved achievable virus filter volumetric throughputs, reducing virus filter costs for biopharmaceutical manufacturers. Because virus filtration is tied to mandatory safety regulation, virus filter validation strategies have also had to keep pace with new process trends.

Tangential-flow ultrafiltration (UF) and diafiltration (DF) provide effective and efficient means to concentrate protein, reduce volume, and exchange buffer, as drug product is retained by the filter while water and buffer salts pass through. Traditional antibody purification templates have been streamlined to require a single UF/DF step - at the end of the bulk drug substance production prior to final fill - to concentrate the protein and exchange it into the formulation buffer appropriate for patient dosing. Screened-channel cassette filters, used in recirculating tangential-flow filtration (TFF) mode and cleaned/re-used multiple times between production batches, have worked very well for this application for many years. However, several new industry drivers are causing manufacturers to re-optimize how UF/DF is implemented.

UF/DF has historically followed virus filtration in most processes, but there is a trend to use ultrafiltration more frequently prior to various chromatography steps to improve the efficiency of the chromatography. This creates an impact on the virus filtration process performance due to higher feed concentrations. In addition, trends around high concentration formulations, single-use, and continuous processing are impacting the design and operation of UF filters, systems, and applications.

Virus filtration

Introduction

Virus filtration is one of the key unit operations commonly used to assure the viral safety of biopharmaceutical products. These filters feature specially-designed membranes with pores small enough to retain viruses while still allowing passage of the mAb product. This size-exclusion retention mechanism is typically considered to be a robust virus removal step which is complementary to other removal steps, such as chromatography, or to viral inactivation steps, such as a low pH hold [1]. Virus removal filtration is typically used together with these other steps to achieve an overall level of safety which meets regulatory guidance (e.g. one viral particle or less per 1,000,000 doses) [2]. This approach for clearing potential viral contaminants from process intermediates during the downstream purification process is part of an overall virus safety strategy for biopharmaceutical manufacturing which also includes careful screening of raw materials and testing the drug product and intermediates for the presence of viruses. This strategy for virus safety was developed in response to earlier cases of virus transmission to patients from plasma-derived drug products [3–7]. There have not been any reports of similar transmissions to patients receiving treatments of mAbs, which are produced using recombinant animal culture.

Although virus filtration membranes have been in use as part of biopharmaceutical manufacturing for more than 30 years, a number of advances have occurred in recent years. A thorough examination of many of the factors related to virus filtration in the biopharmaceutical industry is given in the Parenteral Drug Association (PDA) Technical Report 41 [8] and several other older reviews are available in the literature [9,10]. In the following sections of this chapter, more recent advancements in this field have been summarized in three general areas:

- Recent regulatory trends
- Relevant bioprocessing trends
- Filtration technology development

While these topics are discussed separately in this book, in practice, developments in each area tend to be closely related to the others. Advancements in one section may reflect reactions to opportunities or challenges created by developments in the other areas.

Recent regulatory trends

Virus filter classification and use

Historically, virus filtration membranes have been characterized in two general classifications based on their performance: retrovirus-retentive filters, which retain larger viruses (e.g. 50 nm and larger) while allowing smaller particles to pass through [11]; and parvovirus-retentive filters, which retain smaller viruses as well as larger viruses (e.g. 20 nm and larger) while allowing mAbs and other smaller species to pass downstream. Naming and testing conventions of these filters traditionally have varied among different filter manufacturers, without common designations such as molecular weight cutoffs or nominal pore size ratings that are used with ultrafiltration and microfiltration membranes, respectively. However, consensus rating methods for both small virus-retentive filters [12,13] and large virus-retentive filters [14] have been developed and evaluated by the PDA in order to better standardize how these filters are classified. These methods rely on testing the ability of the filters to retain an appropriately-sized model virus to a specified level under defined conditions.

Current development of new mAb processes utilize parvovirus-retentive filters almost exclusively. In the past, use of retrovirus-retentive filters had been more common because they are effective in removing the retrovirus-like particles produced in CHO expression systems and because in some cases use of parvovirus-retentive filters was not feasible due to severe membrane fouling. However, multiple cases have been reported of processes becoming contaminated by smaller, adventitious viruses such as minute virus of mice (MVM) [15] which are not retained by the larger pore size retrovirus-retentive filters [11]. Also, the introduction of newer generations of parvovirus-retentive filters and adsorptive prefilters, as well improvements in the purity of the streams from upstream purification steps that feed into virus filters, has led to increased filter capacity. For these reasons, the justifications used in the past for use of retrovirus-retentive filters in mAb processes may no longer be valid compared to the benefits of using parvovirus-retentive filters.

Modular virus clearance validation

The history of successful use of virus-retentive filters [1] has allowed for potential increased flexibility in the regulatory expectations for validation of their viral clearance performance. Some parvovirus-retentive filter users proposed the use of a more modular approach for virus filter validation at early phase [16]. This approach utilizes the extensive historical data some biopharmaceutical manufacturers may have using specific filters within a templated process for mAb production. Similar process conditions were used for many different mAb products, and results from the accompanying viral clearance studies demonstrated consistent removal levels despite any differences between the therapeutic molecules. For the 10 different drug products tested, the retention of MVM was in the range of 5.5 to >6 while all samples for xenotropic murine leukemia virus-related virus (xMuLV) had no virus detected in the filtrate. These results justify the submission of only MVM data to regulators for future molecules in early phase, with the rational that the larger virus was never observed to break through the parvovirus-retentive filters. Note that such a modular approach relies on the future processes to remain within the parameters used in the original studies and only applies to molecules of a similar structure (e.g. only mAbs).

Quality by design

Another approach enabling regulatory flexibility is the use of quality by design (QbD) methodologies to define the design space for the virus filtration process [16,17]. This science-based and risk-based approach to process validation considers process parameters in a multivariant manner to assess how they impact critical quality attributes of the drug product. In the case of virus filtration, viral clearance is the sole critical quality attribute affected by the unit operation. Other elements of filter performance, such as filter capacity, flow rates, and product yield, may be important in determining the manufacturing costs, but do not directly affect product quality and are out of scope of QbD methods for virus filtration. The integrity of virus filtration devices—the assurance that they are free of defects—has been identified by some authors as a critical process parameter affecting viral clearance.

Virus filter clearance validation using only small viruses

Due to the size exclusion removal mechanism employed by virus-retentive membrane filters, it is reasonable to assume that removal of larger viruses such as retroviruses will always be at a level as high or higher than the removal of smaller viruses such as parvoviruses. Although small virus removal represents a worst-case for filtration, the traditional approach to validation of viral clearance has been to experimentally demonstrate clearance of small viruses as well as larger viruses. For nearly all parvovirus retentive filters, removal levels demonstrated with larger viruses are bound not by the retention capabilities of the filter, but by the limit of detection of the assays and the virus titer in the challenge feed [1]. The sensitivity of these assays is a function of the amount of the virus which is used in the clearance study—in many cases this amount is limited by filter fouling caused by the addition of the virus preparation, which is an experimental artifact not present in actual manufacturing operations [18–21]. Some mAb manufacturers have provided a rationale for using only a small virus (e.g. MVM) as a worst-case for removal in viral filter validation studies [22]. The benefits of this approach are not only to simplify and reduce the time and cost of these

studies, but there is also the potential to enable higher claims of retrovirus removal. Since MVM preparations are often less fouling of virus filters than xMuLV preparations, higher levels of virus can be used in these studies without fouling the filter, leading to the ability to demonstrate higher levels of viral clearance.

Relevant process trends

Increased mAb concentration

Virus filtration is usually implemented near the end of the purification process, after the chromatography steps and prior to the ultrafiltration and diafiltration steps. This placement is important because the relatively small size of the membrane pores can easily become clogged by impurities which are more prevalent prior to the chromatographic purification steps. Also, the high protein concentrations present after ultrafiltration can potentially limit virus filter performance due to increased viscosity, osmotic pressure, membrane fouling, or other effects. However, typical mAb concentrations being fed to the virus filters have increased in recent years from 1–5 g/L to concentrations of 6–20 g/L, or even higher. This increase in concentration is in part related to higher loadings being realized on bind/elute chromatography steps further upstream or the use of salt-tolerant flow-through chromatography steps which require less dilution. These concentrations are likely to continue to increase as additional emphasis is placed on intensifying mAb manufacturing processes (e.g. use of single-pass TFF steps to concentrate intermediates prior to polishing chromatography [23]). Virus filtration of feeds with increased concentration can potentially lead to both positive and negative effects on the unit operation. For example, increases and decreases in the filter capacity with increasing concentration have been observed [24,25] experimentally, confirming the challenges in generalizing behavior. Specific molecule and process properties likely have important effects. Among the relevant process properties which might affect filterability at increased concentrations are the use and relative filtration area of adsorptive prefiltration steps and solution conditions such as pH and conductivity [24,26].

Operating the virus filtration step at higher concentrations can offer several benefits. When this filtration step is limited by time, i.e. for processes in which filter fouling is minimal and the flux is primarily limited by the membrane permeability, operating at increased concentration can improve the productivity of the filtration step in terms of the mass of the mAb product filtered per unit area of the filter for a given period of time. This productivity improvement is most likely under conditions such as relatively short filtration times, low membrane permeability, low filter fouling, and/or low mAb concentration. Productivity improvements result when the proportional increase in the concentration is greater than any potential decrease in the flux. Operating the virus filtration at higher concentration can also offer more holistic benefits to the overall process. For example, the size of required hold tanks decreases due to the reduced volumes associated with higher concentrations. Also, for those processes in which the mAb concentration is relatively high in the feed to the virus filter, avoiding a dilution step is beneficial because of the reduced demand for both the dilution buffer and on the subsequent ultrafiltration step.

Increasing mAb concentrations can negatively affect the virus filtration step in several ways. In some cases, mAb aggregation increases at higher concentrations. Aggregates of

approximately hexamer-size or larger can approach the size of the viruses these filters are designed to remove, leading to higher rates of filter fouling and reduced capacity. Additionally, as previously mentioned, the increased mAb concentration may lead to higher solution viscosity or osmotic pressure, which both can decrease the filtrate flux. It should be noted that the relevant mAb concentration for these phenomena is not necessarily the bulk concentration of the feed to the filtration device, but the localized protein concentration at the most retentive portion of the membrane. Concentration polarization, whether above the membrane surface or within the substructure of an asymmetric membrane, can lead to higher local concentrations which can negatively affect performance. Decreases in flux due to effects related to concentration polarization tend to be reversible—the effects may decrease if the flow rate or mAb concentration decrease—in contrast to flux decreases due to membrane fouling, which tend to be irreversible.

Connected and continuous processing

Significant changes in mAb manufacturing approaches, including the use of connected, and continuous processes, are being explored as a means of reducing costs [27,28]. These approaches allow the use of smaller, more efficient unit operations throughout the process train. Compared to more traditional processes where the virus filtration step is operated as a distinct batch step and the feed is constant and generally represents a well-mixed pool, these next generation manufacturing strategies present several challenges. For example, the feed coming into the virus filters can vary over time. Also, different ways of operating the virus filters are needed to meet the requirements of constant flow of material into and/or out of this step.

For connected and/or continuous purification processes, the distinct process steps present in the chromatography steps further upstream may result in changes during the process in the properties of the feed stream to the virus filters. For example, bind and elute chromatography steps may result in peaks or gradients in the concentration, pH, conductivity, and impurity levels within the eluate. Superimposed on these profiles are effects from any subsequent flow-through chromatography steps, which typically feature a wash step during which the product concentration approaches zero. Both the flow-through chromatography and the bind and elute chromatography steps may feature the use of multiple cycles, with different cycling periods for each step, potentially resulting in complex profiles over time of the feed properties.

For continuous mAb manufacturing processes, the flow of material into the virus filtration step is constant and can occur over many days. Although for traditional batch processes, the entire batch is fed to all of the required virus filters in one step, this approach may not be optimal for continuous processes where the duration can be one or two orders of magnitude longer. There are several benefits to periodically replacing the filters throughout the process (e.g. once every 1−7 days), such as minimizing the risk of bioburden growth, better flexibility to handle unexpected processing events, and enabling the use of smaller manufacturing systems and device formats. Also, the period of replacement for the virus filter could be selected to mirror the time or volume used to define lots or sub-lots of the drug substance, for which there may be risk-mitigation considerations for selecting shorter durations (on the order of days, as opposed to weeks) in case of process upsets and deviations.

At least two approaches can be taken to allow periodic filter replacement in a continuous process. One approach would be the use of a system featuring two parallel filtration trains. While one filter is being used for processing the incoming product intermediate stream, the second filtration train can be used for removing old filters, system sanitization, and the installation, flushing, and integrity testing of new filters. When the capacity of the first filter is reached, flow into and out of the virus filtration step can be switched to the second filter and the first filter can be replaced. Alternatively, filtration steps can handle a continuous feed in a manner more similar to batch processes by utilizing surge tanks or bags to temporarily hold material upstream during the downtime when the filter is being replaced. Although this approach does require an additional, dynamic hold step, it does offer benefits such as increased operational flexibility, a simpler filtration manufacturing system, and it may dampen out feed variation from upstream steps. While the use of a surge step prior to virus filtration with a single filtration train results in a discontinuous flow of material out of the filter, in many cases this discontinuity may not be problematic, depending on the strategy used in the subsequent ultrafiltration step.

Virus clearance validation for connected and continuous processes

One of the challenges in implementing connected and continuous virus filtration operations is developing appropriate scale-down methods for evaluating viral clearance [29]. For traditional batch processes, a model virus preparation is spiked into a single pool of the mAb intermediate, which is then filtered. For batch processes using adsorptive prefiltration upstream of the virus-retentive filter, often the mAb intermediate feed is first filtered through the prefilter, then the virus preparation is added before the spiked intermediate is used to challenge the virus filter [30]. This approach is discussed in greater detail later in this chapter. These approaches may not be appropriate for connected and/or continuous processes for two reasons: when the properties of the feed solution coming from the upstream purification steps are not constant over time, a consistent single pool is not representative; when the filtration times are relatively long, the mAb and/or virus may not be stable, potentially leading to decreases in either the achievable throughput or the measurable log retention value (LRV) of the filter for viral clearance. One solution under consideration for these challenges is to continuously inject the virus preparation into the stream being filtered inline immediately upstream of the virus retentive filter [31], as shown in Fig. 7.1. Even when the feed coming into the virus filtration operation is variable, a constant virus titer in the challenge to the filter is created. For studies using absorptive prefiltration upstream of the virus filter, as shown in Fig. 7.1, the inline virus injection can be used after the prefilter to avoid any apparent viral clearance due to the prefilter, which would not be considered part of the robust, size-based removal step being evaluated. In cases where the mAb solution and/or virus are not stable, the use of this inline injection method offers several benefits. Each individual solution—the mAb intermediate feed, the virus-containing solution being injected, the virus-containing solution being sample upstream of the filter, and the filtrate solution—can be removed and/or replaced and assayed at appropriate time intervals. Another benefit of the inline injection method is that the prefilter remains inline with the virus-retentive filter, avoiding potential reaggregation of the mAb after being prefiltered. For methods for which the prefilter and virus-retentive filter are decoupled, increased protein reaggregation may be

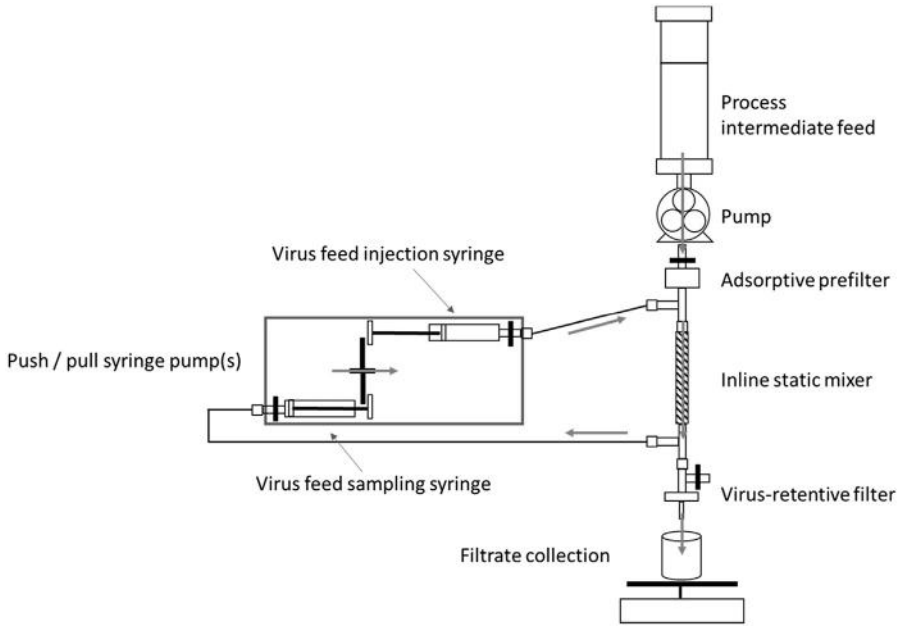

FIG. 7.1 Schematic representation of the inline virus injection method used in viral clearance studies on virus-retentive filters with adsorptive prefiltration. *Figure used with permission from EMD Millipore Corporation.*

more likely for continuous processes due to the extended filtration time and accompanying increased hold time between steps.

Process pauses

During virus filtration operations, temporary pauses in the flow into and out of the filters are common. In some cases, these pauses may be unexpected, due to causes such as interruptions in the power supply or equipment failure. But more often, these pauses are a planned part of the procedures, with the flow being stopped when the feed to the filter needs to be switched, such as when the mAb intermediate feed is stored in multiple tanks or when buffer is used to flush the filters to recover material in the filter and system holdup volume at the end of the filtration process.

Several studies have demonstrated that under some conditions, additional virus can pass through the filter for processes with pauses, compared to processes without pauses [32–35]. This phenomenon of increased viral passage has been observed to occur under specific conditions. For example, a decrease in LRV associated with process pauses has been observed with some filters but not with others, even when operated under similar conditions. Solution properties such as the pH, conductivity, and the specific protein product being filtered also have been observed to affect the decrease in LRV. In some cases, the decrease in LRV has been greater than 1 log, while in other cases, there has been no observable effect.

In order to mitigate the risk associated with reduced viral clearance due to process pauses, three strategies can be considered in parallel. The first consideration is selecting a specific

virus filter for which the LRV has not been shown to be compromised by flow pauses. The second consideration is how the risk can be mitigated by process control and during process development. For example, a filtration system might be designed to enable switching feed solutions without stopping flow. It also is important to consider ahead of time what procedures will be followed during manufacturing if there is an unplanned interruption—will the batch be filtered again or discarded? The third method for mitigating the risk associated with process pauses is to explicitly quantify their impact during viral clearance studies. These studies will help determine what level of viral clearance can be claimed by the virus filtration step. They may also help inform which process parameters might need particular attention during manufacturing-scale operation. The parameters may include characteristics of the feed solution, filter properties, or the specific properties of the flow pause (e.g. the duration, at what point in the process it occurred, how many pauses occurred, etc.).

Filtration as an upstream viral barrier

While virus filtration is typically relied on in the downstream process to help assure product safety, virus-retentive filters also can be used in the upstream process as a viral barrier to reduce the chance of contamination by adventitious viruses [15]. These filters can be complimentary to other risk mitigation strategies associated with upstream materials, such as high-temperature short-time treatments and sourcing of materials without any animal-derived components. Several vendors have developed parvovirus-retentive filters specifically designed for the filtration of cell culture media, which represents one of the higher risk materials. Past investigations into contaminations caused by adventitious MVM have concluded that the most likely contamination source was cell culture media [15]. In contrast to the use of downstream viral clearance steps, which are a regulatory expectation, the use of upstream viral barriers can be viewed in some ways as a risk mitigation strategy, with the costs and likelihood of a contamination event being weighed against the costs of implementing additional treatments to the raw materials.

Filtration technology development

Membranes and filtration devices

A wide range of technical approaches have been implemented historically for the development of virus filtration devices. Commercially available filters feature membranes with several different polymer backbones, including regenerated cellulose, polyvinylidene difluoride, and polyethersulfone, which in some cases are modified with proprietary surface chemistries. A range of membrane morphologies are commercially available, including symmetric, composite, and asymmetric membranes which are used in devices containing either one, two, or three layers of membrane in series. Although historically both tangential flow filtration and normal flow filtration modes had been used for virus filtration, nearly all new processes use normal flow filtration due to its relative operational simplicity [36–38]. Virus filtration devices also exhibit a range of designs, including hollow fiber, pleated, and flat-sheet membrane configurations. Due to these seemingly divergent technical developments and the proprietary nature of many membrane manufacturing processes, it is difficult to generalize more than just a few universal trends in membrane development,

which may not fully account for the significant improvements in performance seen with newer-generation virus filters.

Although asymmetric, composite membranes had originally been used in tangential flow mode with the retentive skin-side of the membrane upstream, reduced filter fouling had been observed in the normal flow mode when the filters were used in the reverse orientation, with the skin-side downstream of the supportive substructure [39]. The more open membrane substructure apparently acts as a prefilter and retains some of the foulants away from the tighter pores of the membrane, and possibly acts to prevent the formation of a protein cake layer. Newer virus filters which employ asymmetric membrane structures are designed with this skin-side downstream orientation to take advantage of this effect.

One key characteristic of virus-retentive filters is the membrane pore size distribution. Other common filtration steps in bioprocessing involve the separation of species approximately an order of magnitude different in size, such as the separation of protein from cells with microfiltration and the separation of protein from salt and water with ultrafiltration. On the other hand, mAbs and small viruses such as parvovirus only differ in size by a factor of approximately 2 or 3. For this reason, the control of both the mean pore size in the filters and the breadth of the pore size distribution is a critical factor. Several studies [40,41] have examined the use of liquid-liquid porosimetry to characterize the pore size distribution in virus-retentive filters and have shown these results correlate with their retention characteristics. In the case of one study [40], it was shown that the LRV for different viruses and membranes with different pore size distributions could be predicted using a mechanistic model based on the principle of retention by size exclusion. In addition to supporting the viral clearance mechanism of the filters, this method also has been used as an at-line test during membrane manufacturing to improve filter consistency.

Beyond using membrane which is retentive of viruses, it is essential that virus filtration devices are free from microscopic defects which could allow some flow to bypass the membrane and allow significant passage of virus into the filtrate. Traditionally, virus filter integrity has been demonstrated both by vendors and filter end-users using either gold-particle retention or air-water diffusion tests. In more recent years, a binary gas integrity test has been developed which can improve the level of retention assurance by an order of magnitude [42].

Adsorptive prefiltration

One key development in improving the capacity of the virus filtration step has been the use of adsorptive prefilters. While parvovirus-retentive filters are typically placed near the end of the mAb manufacturing process after a high level of purification has taken place, aggregated mAb may be present and can significantly foul the filters. Aggregates approximately 20 nm or larger in size can block the membrane pores, even when the concentration of these higher order aggregates is too low to be quantifiable with methods such as size exclusion chromatography. It has been shown that using a dedicated adsorptive prefilter upstream of the virus filter to remove these higher order aggregates and/or other foulants can dramatically increase the achievable throughput for the virus filtration step [43,44]. Costs savings are realized because the costs of the virus-retentive filters are typically much higher than the costs of the prefilters. These savings are weighed against any potential increase in process complexity resulting from introduction of the prefilters.

Multiple vendors offer adsorptive prefilters which are marketed specifically for the protection of virus-retentive filters. These prefilters are available with a variety of media including both membranes and depth filters. Adsorption mechanisms include cation exchange, hydrophobic effects, and mixed-mode interactions. Hence, prefilter effectiveness may be a function of the solution conditions such as pH and conductivity. The use of sterilizing-grade filters (e.g. 0.1 or 0.2 μm nominal ratings) as size-based prefilters for virus filters in mAb processing has often been less successful than the adsorptive prefilters, implying that many of the foulants are smaller than these pore sizes.

While the use of adsorptive prefilters upstream of the virus-retentive filters offers significant costs savings for manufacturing processes, this approach does introduce some complications for the laboratory methods used to evaluate the virus clearance during this step. While in some cases it may be possible to add the virus preparation to the mAb intermediate being fed to the filtration train (both the prefilter and virus filter), this approach is usually not feasible because the prefilters may remove the virus as well as the foulants. Virus removal by the prefilter does not occur on the same basis of size exclusion that the virus filter employs and must be removed from the claimed LRV—potentially necessitating the need for additional viral clearance steps in the process if the overall LRV is too low. The most common solution to this problem is to decouple the prefiltration and virus filtration steps during the viral clearance studies [30], as illustrated in Fig. 7.2. The mAb intermediate is first filtered using the prefilter, without the addition of any virus. The virus preparation is then added to this prefiltered pool, which is subsequently filtered through the virus filter. Although this approach of decoupling the prefiltration step from the virus filter during the viral clearance study is the most common approach and is effective the majority of the time, in some cases the act of decoupling the filters may result in lower filter capacity, potentially due to reaggregation of the mAb in the feed material. Although many specific techniques have been reported to avoid this complication [30], in those cases where the decrease in capacity is unavoidable, the inline spiking methodology [31] can be used, instead.

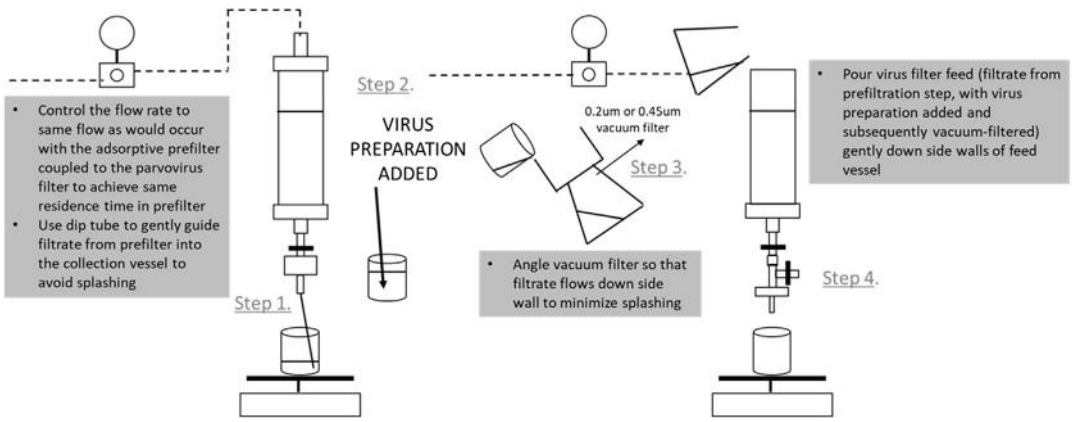

FIG. 7.2 Schematic representation of decoupling prefiltration and virus filtration steps for viral clearance studies. *Figure used with permission from EMD Millipore Corporation.*

This approach was discussed in greater detail earlier in this chapter (see Connected and Continuous Processing section and Fig. 7.1).

Virus retention mechanisms

The mechanisms by which virus filters retain viruses has been a subject of substantial research [34,45–50], even beyond the effects related to process pauses discussed earlier in this chapter. One important observation has been that in some cases, the LRV of virus filters can decrease throughout the filtration operation. This behavior seems to be related to the specific filter being used. In some cases [45], the decrease in LRV during the process has been attributed to preferential fouling of the filter's tighter pores, resulting in a higher fraction of the flow passing through the membrane's non-retentive pores. Models of this effect showed an increased reduction in retention for filters with larger numbers of distinct membrane layers or effective separation layers. In cases with a different filter [48], the decrease in LRV was attributed to the accumulation of virus within the membrane due to internal concentration polarization. In that work, the rate of accumulation was modeled to be a function of the available volume within the membrane structure which was upstream of the retentive portion of the membrane. Studies with yet a third different virus filter [50] have focused more on the effects of conditions such as the operating pressure and the quantity of virus particles challenging the filter which can lead to decreased LRV. The results of these studies emphasize the difficulties in making generalizations in terms of virus filter performance, especially when considered in tandem with the wide range of membrane materials, membrane morphologies, and device formats discussed earlier.

Ultrafiltration

High concentration formulation

In recent years, drug developers have moved toward product formulations for subcutaneous delivery to patients as opposed to traditional intravenous infusion or intravenous large bolus injection formulations. Subcutaneous delivery is generally preferred by patients and can be more cost-effective because it requires less facility, staff, and patient time for drug preparation and delivery [51]. In addition, subcutaneous delivery of a drug that must be taken on an on-going basis allows the potential for self-dosing by patients in a home setting. For a subcutaneous injection to be well-tolerated, the total dose volume must be no larger than 1–2 mL [52], meaning that biotherapeutics requiring high mass dosing must be formulated to higher protein concentrations. This creates a challenge for traditional TFF processes which must accommodate high solution viscosities, low final batch volumes, and increased electrostatic interactions between the protein and the formulation excipients. Adapting control strategies and system designs can help manufacturers achieve some extra processing room, but do not provide full capability. Filter vendors have responded by developing new cassette channel geometries optimized for high viscosities, and manufacturers have developed practices for mitigating formulation offsets resulting from electrostatic interactions.

High solution viscosity

Solution viscosity increases as protein concentration increases. There is a practical viscosity limit of approximately 30 cP [53] that can be readily delivered via a manual needle injection. For this reason, drug formulators have been motivated to investigate solution conditions and excipient additions that result in lower viscosities in the final vial at high concentration of the biotherapeutic. Some approaches that have been successful are modifying pH, adding inorganic salts, and/or adding hydrophobic salts [54–57]. While this can alleviate the problem of drug delivery, product viscosities at the final UF/DF step are still higher than in a traditional process and can exceed the capability of the filters and some system components. Therefore, additional strategies are needed to ensure robust manufacturability of high concentration therapeutics.

The majority of UF/DF operations for antibody purification and formulation utilize screened, flat-sheet filter cassettes. The screens act to increase mass transfer and reduce concentration polarization in the filters, leading to higher permeate flux at lower feed flow rates than would be seen in open channel devices. UF/DF processes are typically controlled via a feed flow rate setpoint to establish the tangential flow across the membrane surface and a transmembrane pressure (TMP) setpoint to establish driving force for filtration [58]. The feed flow rate is optimized to provide efficient permeate flux with the specific screen design, cassette channel geometry, and protein/buffer solution in a given process. At these constant operating conditions, flux is a function of protein concentration, as shown in the simplified Stagnant Film model in Eq. 7.1. As protein concentration increases, flux drops due to the smaller difference between the bulk protein concentration and the concentration in the polarized layer at the membrane surface.

Eq. 7.1 Simplified Stagnant Film model [59]:

$$J_f = k \ln(C_{wall}/C_{bulk}) \tag{7.1}$$

where J_f is permeate flux through the membrane, k is the mass transfer coefficient, and C_{wall} and C_{bulk} are the protein concentrations at the membrane surface and in bulk solution, respectively. (C_{wall} is sometimes also referred to as C_{gel}). The simplified model assumes no passage of product to the permeate. When C_{gel} is used in place of C_{wall}, this model is often referred to as the gel model.

Pressure drop through any given TFF filter cassette is a function of both viscosity and feed flow rate, where pressure drop increases with increasing viscosity and increasing feed flow rate, as shown in Eq. 7.2.

Eq. 7.2 TFF cassette pressure drop [60]:

$$\Delta P = K\mu^m Q_F^n \tag{7.2}$$

where ΔP is the pressure drop along the fluid flow channel from cassette feed inlet to retentate outlet, K, m, and n are constants, μ is the solution viscosity, and Q_F is the feed flow rate into the cassette.

As protein concentration and solution viscosity increase, the resulting increase in pressure drop will first result in an inability to maintain the desired TMP setpoint. Ultimately, since all TFF filters have maximum allowable pressure limitations, the pressure drop may increase to

a point where the process cannot continue without exceeding this pressure threshold unless changes to the operating conditions are made.

Literature exists showing that increasing process temperature to greater than 40 °C during the UF/DF step can be used as one strategy for reducing solution viscosity enough to achieve a high target final protein concentration without exceeding cassette pressure limits [61]. While the authors demonstrated no adverse quality impact for their molecules at the elevated temperature, this is likely to be very protein specific. In addition, a high temperature approach increases system design complexity and operating risk, so it has not been widely pursued as a solution at manufacturing scale.

Many UF/DF processes for antibodies have TMP setpoints in the range of approximately 1−2 bar. For the low viscosities and concentrations at the start of the process, achieving the TMP setpoint will generally require partially closing the retentate control valve to apply additional pressure beyond what is exerted from the feed flow. As concentration and pressure drop increase, the valve will need to be opened to reduce retentate pressure in order to maintain the same TMP setting. This relationship is apparent in the definition of TMP, shown in Eq. 7.3.

Eq. 7.3 Definition of Transmembrane Pressure:

$$\mathrm{TMP} = [(\mathrm{P_{feed}} + \mathrm{P_{retentate}})]/2 - \mathrm{P_{filtrate}} \tag{7.3}$$

While the initial TMP setpoint could be maintained further into the concentration operation if the retentate valve is allowed to open fully, it is advisable to maintain a retentate pressure of at least approximately 0.5−0.7 bar in order to avoid Starling flow—reverse flow from the permeate back into the feed channel—as higher protein concentrations are reached [62,63]. This "reverse flux" is a result of high protein osmotic pressure at the membrane surface acting against the applied TMP, as shown in the Osmotic pressure model in Eq. 7.4.

Eq. 7.4 Osmotic pressure model [64]:

$$\mathrm{J_f} = \mathrm{L_m}(\mathrm{TMP} - \sigma\Delta\Pi) \tag{7.4}$$

where $\mathrm{J_f}$ is permeate flux, $\mathrm{L_m}$ is membrane permeability, TMP is transmembrane pressure, σ is the osmotic reflection coefficient ($\sigma = 1$ for fully retained proteins) and $\Delta\Pi$ is the difference in protein osmotic pressure between the feed and permeate sides of the membrane (Π in the permeate $= 0$ for fully retained proteins).

Reducing feed flow rate as viscosity increases is a simple and effective process control modification to extend UF capability without exceeding a pressure threshold. One drawback is that mass transfer drops as feed flow rate is reduced [63,65], leading to lower permeate flux through the filter at any given protein concentration (per the modified stagnant film model in Eq. 7.1), in addition to the flux decline that comes from the increase in protein concentration itself. The minimum flow rate will be based largely on pump selection, which will determine the maximum turn-down that can be achieved. There will also be a practical endpoint where the permeate flux is so low that extra process time will not result in significantly more concentration.

Flat-sheet filter cassettes have historically been available with a variety of turbulence-promoting screen geometries for a range of application needs [58,63]. Open screen weaves

and/or screens installed with spacers so that they are suspended in relatively open channels provide low pressure drop, low shear, and some limited solids-handling capability. However, they also typically have low mass transfer values and therefore produce lower flux. Conversely, tight screen weaves and thin channels have higher mass transfer values and high flux but come at the cost of higher pressure drop and a low limitation on viscosity. In recent years filter vendors have focused on screen and channel designs specifically adapted to provide an effective compromise between good mass transfer for high permeate flux and low pressure drop to allow higher concentration, higher viscosity endpoints.

One example of a screened TFF device designed specifically to enable efficient high concentration processing is the Millipore Pellicon® 3 cassette with D screen [66–68]. As shown in Fig. 7.3, when compared to the tighter C screen cassette, the D screen cassette demonstrates significantly lower pressure drop at a fixed feed flow rate at high protein

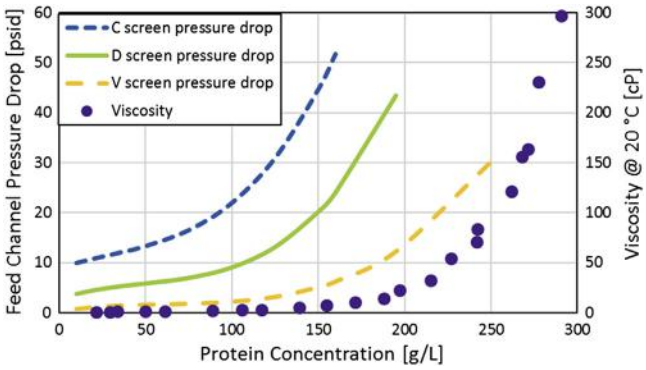

FIG. 7.3 Pressure drop versus polyclonal IgG concentration and viscosity for Pellicon® 2 and Pellicon® 3 cassettes with Millipore Ultracel® membrane and different feed-channel screens running at a feed flux of 6 L/min/m^2. *Figure used with permission from EMD Millipore Corporation.*

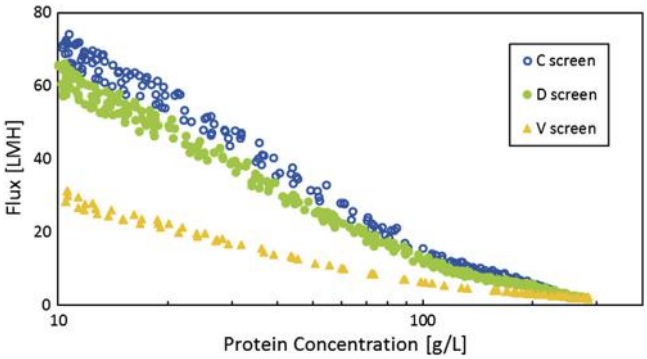

FIG. 7.4 Permeate flux versus polyclonal IgG concentration for Pellicon® 2 and Pellicon® 3 cassettes with Millipore Ultracel® membrane and different feed-channel screens. D screen data represents 16 process runs, C screen data represents 10 process runs, V screen data represents 3 process runs. *Figure used with permission from EMD Millipore Corporation.*

concentrations and viscosities. It shows higher pressure drop than the V screen cassette (only available in Pellicon® 2 format), which is an example of a suspended screen and shows the lowest pressure drop.

However, the tradeoff of the suspended screen format can be seen in Fig. 7.4, where the V-screen cassette has significantly lower permeate flux (lower mass transfer) throughout the protein concentration operation as compared to the other screens. The D-screen cassette, notably, demonstrates only slightly lower permeate flux than the tighter screen and a significantly higher flux than the V-screen cassette.

With the appropriate selection of a cassette designed for high concentration processing, and with the two levers of TMP and feed flow rate, an effective process control solution can be proposed which takes advantage of the optimized process setpoints as long as possible while also achieving the highest possible protein concentration in the UF step. The UF operation will start at low protein concentration with the system controlling at the optimized feed flow rate and TMP setpoints. As concentration increases, feed flow rate is kept constant and the TMP setpoint is maintained by allowing the retentate pressure control valve to open and reduce the retentate pressure while the feed pressure increases. When the retentate pressure reaches a minimum of approximately 0.5-0.7 bar, the control valve is set to maintain this pressure to keep positive driving force to offset osmotic pressure. While still maintaining the original feed flow setpoint, the TMP is then allowed to increase above the starting setpoint as the protein concentration and feed pressure continue to rise. When a maximum feed pressure is reached, based on a vendor-specified limit of the TFF filter cassette and/or any other components of the TFF system, the feed flow rate is reduced to maintain the new high feed pressure setpoint. When the lowest controllable pump speed is reached, or when permeate flux drops to a point where no additional ultrafiltration can be achieved, the process is ended.

Low final batch volume

While better cassette designs can help overcome some of the pressure limitations associated with high concentration processes, the working volume of a recirculating TFF system can also become a limitation. During a UF/DF operation via recirculating TFF, the protein solution is passed across the membranes multiple times while permeate is removed. This requires that the solution volume is large enough to fill the membrane holder and cassette, feed pump chamber, feed and retentate piping with any associated in-line instrumentation, and enough of the recycle tank to ensure that the recirculation flow and agitation does not cause excessive turbulence or air entrainment into the liquid. This volume can be described as the "minimum working volume" of a TFF system.

The minimum working volume of any system will be a function of the membrane area, since this will dictate the required feed flow rate from which the pump size, piping size, and membrane holder size will be specified. Once these key pieces of equipment are selected, careful design engineering can optimize three-dimensional layout so that distances from component to component are minimized as much as possible, effectively reducing the minimum working volume.

The minimum working volume of a given system is also impacted by the flow rate, since the draw of fluid out of the recycle tank and the return of fluid to the recycle tank will be more turbulent at higher flow rates. In order to avoid foaming and/or air entrainment, which can be very damaging to protein, the minimum acceptable solution volume in the tank will

need to be higher at higher flow rates. Therefore, if the process control strategy described above is followed, as protein concentration increases and flow rate is reduced to avoid high pressure limits, the minimum working volume will drop such that it is possible to reach a point where there is very little volume in the recycle vessel and nearly all of the protein is held within the piping, instrumentation, and filters.

Once UF/DF is complete, the product must be recovered from the TFF system into a sterile vessel for final conditioning, quality testing, and, ultimately, transfer to storage or final fill. While draining or pumping fluid out of the bottom port of the recycle tank is simple and straightforward, achieving very high recovery of fluid that is contained within a stretch of piping, instruments, and narrow membrane channels is more complex. The challenge is greater at higher viscosity since the fluid does not flow as well and higher piping slopes may be required to fully empty lines via gravity draining or pumping. At this stage of the production process, every drop of product represents a large investment and has high value, so product recovery goals >95% and up to 99% are common.

After the small fraction of the pool contained within the recycle vessel is pumped to the receiving vessel, a buffer flush or buffer displacement is typically employed to improve product recovery from the lines and membranes [69]. The nuances of the precise valve sequencing and pump or air pressure used for a buffer flush or a buffer displacement will impact the product recovery percentage, however both strategies result in some buffer being added to the final collected product pool, as shown in Fig. 7.5. This causes dilution which needs to be accounted for in the previous UF step by a planned overconcentration to successfully meet the target final protein concentration in the recovery vessel. The dilution is more significant when the fraction of pool in the system piping and membranes is larger than the fraction in the recycle vessel. And overconcentration is more of a challenge when the target final protein concentration is already near the limits of the system or filter capability as described above in the high viscosity section.

While careful system design, optimized filters, and strategic process control can allow attainment of much higher final protein concentration targets than were traditionally possible, there are a growing number of cases where a more significant process change is required to ensure meeting product concentration specifications at acceptable yield. For very high final concentrations and/or very low final batch volumes, it is recommended to

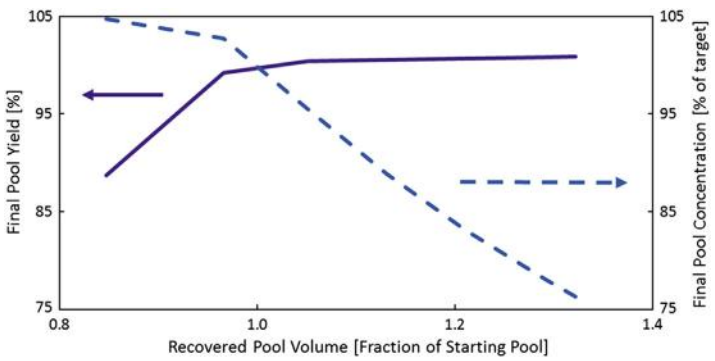

FIG. 7.5 Buffer displacement improves protein recovery from a TFF system but increases dilution. *Figure used with permission from EMD Millipore Corporation.*

perform an initial UF to an intermediate concentration, followed by diafiltration in a standard recirculating TFF system. At this point, the product is recovered, including use of a buffer flush if needed to improve recovery yield. The partially concentrated and buffer-exchanged pool can then be transferred to a much smaller recirculating TFF system for the final concentration. Or, the final concentration can be accomplished using single-pass TFF, a newer mode of operation that maximizes product concentration ability while minimizing system holdup volumes and yield losses. Single-pass TFF will be described more fully later in this chapter.

Excipient offsets

During protein ultrafiltration and diafiltration, buffer components are far smaller than the membrane pore size and are typically expected to freely pass through the membrane to the permeate. Hence, the *expected* buffer exchange as a function of diavolumes can be calculated as shown in Eq. 7.5 and using an assumption that retention (R) is equal to zero.

Eq. 7.5: Fraction of original excipients remaining in retentate during diafiltration

$$C/C_o = e^{(R-1)*N} \tag{7.5}$$

where R is retention of any given excipient and N is the number of diavolumes or wash volumes performed.

As the starting buffer is washed out, it is replaced by the diafiltration buffer to the point where the excipient concentrations and the solution pH are equivalent to that of the diafiltration buffer. For a final formulation operation, 8−10 diavolumes are typically performed to achieve approximately 3-4 logs of buffer exchange with a safety factor to account for any mixing non-idealities in the flowpath. However, when diafiltration (and subsequent final ultrafiltration) is performed at high protein concentration, both volumetric exclusion effects and electrostatic interactions can result in offsets in the excipient concentrations and the solution pH, meaning that the selected number of diavolumes can no longer be relied upon to achieve the formulation targets. And even extended diafiltration (additional diavolumes) will not result in a protein pool that matches the makeup of the diafiltration buffer. These phenomena present a significant challenge to accurate protein formulation at high concentration.

Volume exclusion occurs when the physical volume occupied by the protein on the retentate side of the membrane causes buffer excipients to be displaced to the permeate, resulting in a reduction in the expected concentration. Electrostatic interactions occur due to the accumulation of charged protein on the retentate side of the membrane which can have either an attractive or repulsive interaction with the buffer components. This creates unequal partitioning of components across the membrane in order to balance charge and maintain electroneutrality, a phenomenon known as the Donnan effect. Components with charge opposite to the protein will be preferentially retained (apparent retention will be >0) while those with charge similar to the protein will be preferentially permeated (apparent retention will be <0). As protein concentration increases, the offsets are more pronounced.

In order to successfully formulate proteins at high concentration, these excipient and pH offsets must be mitigated. Since extensive empirical UF/DF iterations at bench-scale require a large investment of time and protein feedstock, there is great interest in using modeling to predict final solution conditions *a priori*. Several authors have described models to determine the offset based on the Poisson-Boltzmann equation [70–72], in some cases combined with protein effects and ion binding [71], and volume exclusion modeling [72]. For these models to be successful, an accurate estimate of protein charge is needed, which has proven to be difficult to obtain. Calculations based on amino acid sequence, and measurements via titration and zeta potential return different values. A more recent approach proposes using very small scale non-TFF experimental work to generate a predictive calibration to model concentration and pH offsets with more accuracy and lower material requirement [73].

While modeling may eventually obviate iterative process development, the end-user will still need to determine how to apply the results to create a manufacturing strategy. There are two primary methods for overcoming buffer offsets once the offset is understood. For both methods, it is important to consider not just the diafiltration step itself, but also any final concentration step where additional offset will occur as protein concentration increases, as well as the product recovery step which generally requires the use of some buffer displacement and protein dilution to achieve high yield. It is the final recovered protein pool that must meet the pH and excipient concentration specifications for proper formulation.

In the first mitigation method, the diafiltration buffer is prepared with pH and excipient concentrations different from the final targets such that during the diafiltration process and subsequent concentration and recovery, the Donnan and volume exclusion offsets act to "adjust" the final protein solution to the desired end state. One challenge with this method is that the volume of buffer used for product recovery must be precisely controlled from run to run in order to reach consistent solution conditions. For large-scale manufacturing, the amount of buffer required for high recovery will usually be determined on the final system, meaning that the manufacturing equipment will need to be characterized before the diafiltration buffer composition can be finalized. In addition, the buffer volume needed will be the same from run to run (as the piping is not changing) while batch sizes typically vary slightly, making a fixed dilution problematic.

The second method involves preparing a diafiltration buffer at the desired targets, allowing the offset to develop during diafiltration and final concentration, and using an "adjustment" buffer to condition the final recovered pool. This strategy works better to enrich depleted excipients than it does to dilute out enriched excipients, unless this dilution can be accounted for by overconcentration in the final UF. And again, batch size variations must be considered such that the adjustment buffer allows hitting all final specifications for pH, excipient concentrations, and protein concentration.

While it does require the preparation of an additional buffer solution, a combination of the two above methods may be most robust and provide manufacturing flexibility. First, the user could diafilter using an offset buffer such that the protein at the end of diafiltration and final concentration meets the solution specifications. And then, a buffer could be prepared at the final solution conditions to use for product recovery.

Single-pass tangential flow filtration

In a traditional downstream processing train, opportunities exist to reduce manufacturing costs while maintaining product quality and safety [63]. Single-pass Tangential Flow Filtration (SPTFF) has become an application of choice to handle such opportunities [74–77]. Due to its efficient use of plant footprint, its small hold-up and processing volumes and the ease of configuration with other unit operations, the Biotech industry is finding more creative ways to fit it into downstream processing.

Single-pass TFF operation

Single-pass TFF differs from traditional batch TFF in several ways. Batch TFF unit operations are traditionally found toward the end of a biomolecule purification train prior to final fill and finish. During batch concentration the purified molecule is fed via a pump from a tank tangentially over the membrane surface of the separation devices. As the feed material passes over the membrane surface the filtrate permeates through the membrane and is sent to waste while the smaller, more valuable particles are retained on the upstream side of the membrane, collected through the retentate port and returned to the feed tank. This recirculation is completed multiple times, typically over many hours, until enough filtrate has been removed to achieve the target final product concentration. In single-pass operation the filtrate removal occurs in one single pass over the membrane surface and the retentate is removed at the end of the step without the need to recirculate. In order to achieve a comparable final concentration, the single-pass operation is accomplished at lower feed flow rates with subsequently smaller pipe and pump sizes and lower hold-up volumes compared to batch TFF [63].

SPTFF is operated by targeting a desired conversion, or amount of feed flow converted to filtrate or permeate flow. The conversion is achieved in a single pass over the membrane surface by increasing the residence time of the fluid in the feed channel. Residence time can be increased by elongating the length of the feed channel, decreasing the process flow rate or, ideally, both. While operating in batch mode TFF the optimal process settings (TMP and feed flow rate) are chosen such that the flux is between the non-polarized and fully polarized operating regime, while single pass operation works best in the fully polarized regime [78].

TFF mass transfer can be modeled rather simply while operating in the polarized regime by the use of the gel model referenced in the section above (Eq. 7.1). The gel model indicates that as the concentration in the bulk increases to that of C_{gel}, or the maximum achievable concentration, the flux approaches zero. Inversely, flux can be changed by adjusting the feed flow rate, which changes the mass transfer coefficient. By decreasing the feed flow rate, the concentration in the bulk will increase toward the gel concentration C_{gel} along the feed channel, so as permeate is removed and the protein continues to progress through the feed channel there is a subsequent drop in flow rate. There is a non-linear relationship between the mass transfer and volumetric flow rate such that we see diminishing returns in flux as the feed channel becomes infinitely long. Ideally, one should choose a suitable feed channel length and decrease feed flow rate to achieve the desired conversion or concentration.

Feed channel length can be customized with the use of diverter plates or simply arranging filter devices serially in multiple holders [79] as shown in Fig. 7.6 below. In this configuration one can customize the device assembly for a given process target. The diverter plates are

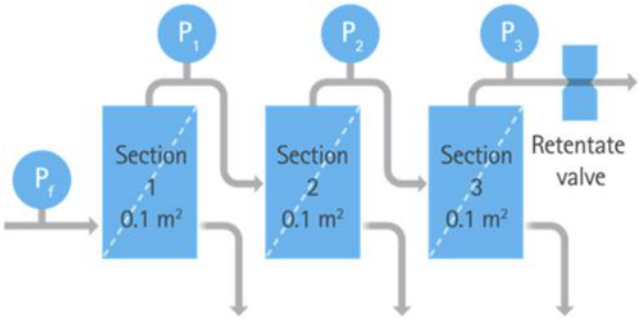

FIG. 7.6 Single-pass TFF assembly in series. *Figure used with permission from EMD Millipore Corporation.*

placed between the TFF cassettes to guide the flow in a serial path creating a longer feed path length. The number of cassettes in series, hence the number of diverter plates needed, is based on process optimization and factors such as pressure drop, process flow rates, volume and product concentration.

Proper operating set-points will vary based on feed conditions. In general, the more dilute a feedstream, the higher the conversion potential [78]. Conversion is also affected by membrane permeability so higher conversions are expected with more open membrane structures. Feed flow rates for SPTFF operations are typically between 0.2 and 1.2 L/min/m^2, with lower flow rates leading to higher final retentate concentrations. The data in Fig. 7.7 below demonstrates the relationship between feed flow rate and the resultant retentate concentration for a given mAb with feed concentration of 11 g/L.

Applications for single-pass TFF

Single-pass TFF is a universal tool which can be used for multiple antibody molecules throughout the downstream bioprocessing train. In many cases it is used for shear sensitive molecules where multiple pump passes present conditions that may increase the risk of

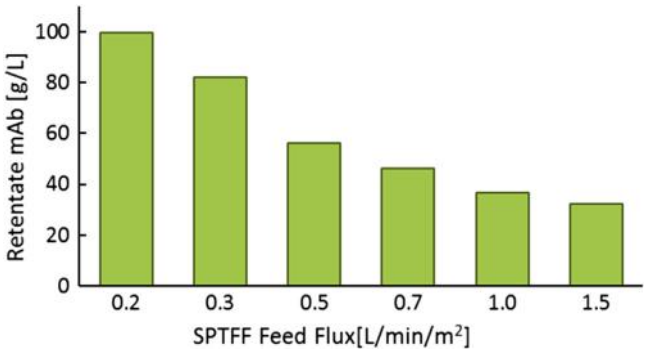

FIG. 7.7 Effect of feed flux on mAb retentate concentration in single-pass TFF operating mode with mAb feed concentration of 11 g/L *Figure used with permission from EMD Millipore Corporation.*

protein damage. This is particularly true at the higher product concentrations often seen toward the end of the purification process.

Volume reduction is another common application for which SPTFF is being employed [74,76]. To meet ever stringent quality and cost demands for biotherapeutics, the industry is forced to adapt their manufacturing space to accommodate multiple product lines. This becomes a difficult task when process volumes interfere with the ability to use the same tankage over multiple processes [74]. With facility fit constraints in mind, SPTFF offers a solution to reduce in-process volumes without the need for a large, recirculating TFF skid and, often times, an additional pump when used in-line with a previous process step. Another benefit of the lower process flow rates and tank reduction associated with single-pass TFF is the resultant low hold-up volume. Pipe and pump sizes are reduced and certain flow paths are eliminated when the flow rates are decreased and recirculation loops are removed. This eliminates a large portion of hold-up volume and reduces the amount of dilution required when recovering molecules at the end of the final UF unit operation, as described in the high-concentration section above. Additionally, plug flow recovery with buffer, performed at the process feed flow rate, enables high product yields. For these reasons, manufacturers are able to achieve higher final concentrations [79].

Recent trends are moving biomanufacturing closer to continuous and connected processing where closed system processing provides economic benefits [74,79]. Much like having a facility fit constraint, SPTFF is a tool which can be used for volume reduction in a connected processing scheme. In many cases, feed material must be pooled and conditioned between unit operations such as before and after capture and ion exchange chromatography steps. To make adjustments to conductivity and pH, large water or buffer volumes must often be added to the pool. By using SPTFF to first reduce volume prior to conditioning, a continuous system designer has more flexibility for optimizing the chromatography steps and can reduce resin utilization and manufacturing costs [63]. Additionally, when using equal area membrane sections to achieve the single-pass path length, there is minimal pressure drop across the module. This makes it ideal to integrate into chromatography steps where process pressures can be a concern.

Single-pass TFF is ideal in a continuous state when the feed conditions are not changing over time, but it can also be used for feed streams with changing concentrations. In instances where SPTFF is used on a dynamic feed stream, such as post bind and elute chromatography, a change in feed concentration will create a self-adjusting situation where conversion will drift over time based on the incoming concentration of the feed. As earlier stated, conversion increases with a decrease in product concentration. As the elution peak exits the column, the conversion will start out high and pressures will be low. As the concentration in the peak reaches its maximum, the conversion will decrease and the pressure will increase then eventually return to its beginning state as the entire product peak is captured. Although the resultant concentration profile of the SPTFF retentate will not be perfectly flat during product elution, the system will self-regulate to normalize its characteristics. The product concentration range should be considered when optimizing this type of application. Process flow rate and retentate pressure should be chosen to avoid fouling conditions (i.e. high pressures with the highest expected concentration from the column elution).

Reducing the amount of chromatography resin without reducing the amount of product produced or increasing processing time will save manufacturing costs [23]. The more

TABLE 7.1 2000 L fed-batch Bioreactor case study @ 3 g/L feed concentration, intensified versus standard process.

AEX polishing step	Units	Intensified process as compared to standard process
Dilution buffer volume	L	3.5× lower
Column loading	g mAb/L resin	2.7× higher
Column productivity	g/L/h	2.5× higher
Process cost (buffer, resin, sterile filter and TFF filter)	$	23% lower

productive one can make the resin, the greater the cost benefit. Increasing product concentration prior to AEX chromatography has been shown to increase resin productivity [23]. Table 7.1 below shows the benefits of an intensified process compared to a traditional AEX unit operation. By using SPTFF to increase the feed concentration prior to flow-through polishing, the dynamic binding capacity of the AEX resin can be increased as much as a factor of 4 without changing the mass transfer through the column, while still maintaining appropriate levels of viral and HCP clearance [23]. As previously discussed in the virus filtration section, it is important to consider the impact of protein concentration on the performance of the virus filtration operation.

Pre-concentrating before Protein A chromatography has also shown to result in a large reduction in load time while maintaining mass transfer. This enables more cycles per batch over a given Protein A column operation, which is useful for not only process intensification but in a continuous, multi-column cycling operation as well [80]. Depending on the cleanliness of the feed harvest material, SPTFF may be used alone or in conjunction with a clarification step prior to the Protein A step [77].

SPTFF system considerations

Many considerations must be factored in when designing a SPTFF system. In some cases, it is desirable to add a recirculation loop and tank. As previously discussed, SPTFF operations work best once the gel layer has formed on the membrane surface and a steady pressure profile has been achieved. In this state one can expect a consistent conversion over time when the incoming feed is unchanged. For dilute feed solutions, steady state may take more time to establish. In this case it may be more beneficial to return the retentate to the tank while the system is stabilizing before capturing the final product stream, to ensure that only product that meets the concentration target is collected. Keep in mind the feed tank concentration will decrease during recirculation as protein is incorporated into the developing gel layer. Single-pass conversion is based on the starting concentration in the tank after gel layer formation, so this needs to be considered when designing a process. If a recirculation loop is not used, the gel layer will form as the feed stream is introduced. A higher bulk concentration will take less time to form a gel layer than a more dilute bulk feed stream. In either case the operation will not achieve its steady-state, optimum conversion until the gel layer is

formed and may initially produce a more dilute product stream from the retentate. Once steady-state is achieved, the conversion will remain consistent until the membrane capacity is reached. For single-pass operation without a recirculation loop, an initial dilution factor should be accounted for if final product concentration is critical.

Often, a Single-pass system must work within specific pressure and flow rate processing constraints. SPTFF is optimized when operating under polarizing conditions at a feed flow rate which corresponds to the desired conversion. Polarization is achieved by increasing the retentate pressure until a large decrease in retentate flow is observed [78]. Working above the optimal retentate pressure may create fouling conditions over time and should be avoided. Working below the optimal retentate pressure will give sub-optimal conversion and will require more area for the given volume. If the process flow rate cannot be altered, for instance in the case of a continuous process delivering feedstream to the SPTFF system at a constant rate, the total required membrane area to accommodate that flow is determined by dividing the flow rate (L/min) by the feed flux ($L/min/m^2$, or LMM) setpoint that was determined in process development to achieve the desired conversion. This total area is then divided equally into the number of required sections identified during process development. SPTFF is scaled simply by maintaining retentate pressure, number of sections and feed flux (LMM) to achieve a given process volume in a given time.

Cleaning is a large aspect of the SPTFF system design and, similar to batch TFF, there are many factors to consider. Depending on validation strategy and system and material compatibility, there are several options for the SPTFF system. In a non-recirculating path, cleaning can either be accomplished off-line or statically by allowing the cleaning solution to soak for a given amount of time within the system membranes and piping [81]. Time, temperature and concentration of the cleaning solution will all effect the efficiency of the static soak. Off-line systems as well as dedicated cleaning pumps can be used to clean the system in traditional recirculating mode operation for quicker cleaning cycles. As with all CIP systems, the chosen approach must be validated for the given molecule.

Single-Pass TFF is a powerful tool to eliminate many downstream processing constraints or to enable continuous and connected processing. It applies to a wide range of biomolecules and can easily fit between many unit operations. When designed correctly, a SPTFF system can operate continuously under steady state conditions for extended periods of time. Systems should be designed to include cleaning as well as be optimized to fit within existing processing constraints.

Single-use tangential flow filtration

As biomolecule manufacturers seek opportunities to reduce cost and increase both product quality and safety they are turning toward disposable, single-use product alternatives [82,83]. From the bioreactor to final fill, there are many opportunities to integrate single-use tubing, collection vessels, connectors, sensors and separation devices into a downstream processing train. In a multi-use facility many separation devices, such as those used for clarification, sterile filtration and virus removal, are used once and discarded. These devices are often offered pre-sterilized or are capable of being sterilized in place and can therefore be easily integrated into single-use systems and flow paths. Single-use systems are commercially available from

multiple suppliers for UF/DF operations, however TFF filter cassettes are traditionally re-used multiple times for sequential product batches and are cleaned in place between runs to maintain system cleanliness and product quality. While TFF devices could be used once and then discarded, most require flushing, sanitization and pre-conditioning prior to use, which adds a time and resource investment to the step that make re-use attractive. Recent advancements in single-use TFF devices offer new options for overcoming the hurdles associated with integrating traditional multi-use TFF devices into a single-use system.

Single-use assemblies are ideally suited for continuous and connected processing systems, controlled non-classified space operations, bioreactor volumes of 2000 L or less [82] and for processing toxic components such as those associated with Antibody drug conjugate (ADC) manufacturing [84–86]. With these systems a fully closed flow path is desirable so that bioburden levels can be controlled and contact between the process fluid and the operator and process space can be avoided. One option for maintaining sterility is the use of pre-sterilized devices, which are often gamma irradiated in closed packaging prior to shipment from the supplier. When using TFF cassettes, which must be removed from the packaging and compressed in a holder that provides sealing force, special single-use liners are installed in the holder with the devices to create a disposable flow path. For devices that don't come pre-sterilized, a clean in place step must be performed prior to use. In all cases the liners and devices are intended to be discarded at the end of processing, eliminating the need for post-use cleaning. These options are ideal for closed processing where the product does not pose a risk to operator safety.

ADC manufacturing presents a unique situation where product contact with the operator must be limited, if not entirely eliminated, due to the cytotoxic nature of the product [84–86]. Pre-sterilized devices and the use of liners are excellent for ensuring containment during the UF/DF process, however they still present a risk to the operator during disassembly at the end of a production cycle. While the liners are integrally connected with the single-use assemblies which comprise the complete flow path, the cassettes themselves are held in place by the compression on the holder and therefore, care must be taken to avoid fluid release when they are uninstalled and disposed. Holderless TFF devices, which are self-contained and pre-sterilized, are an ideal, novel option for this type of manufacturing where both product and operator safety are paramount.

The advancement of holderless and pre-sterile TFF devices offers flexibility in all types of continuous and closed flow paths. Not only do they eliminate the need for pre-cleaning, but also reduce the amount of hardware and subsequent manufacturing footprint, providing even more economic benefit. Holderless devices can also be easily integrated throughout the downstream process in single-pass operations for volume reduction, process intensification and high concentration applications [81]. For single-pass operation, these devices can be connected in series without the need for additional equipment as shown below in Fig. 7.8.

Advances in single-use TFF devices have provided both economic benefit as well as product and operator safety in biomolecule production. Many options are available including pre-sterilized and holderless configurations to allow flexibility for a wide range of applications.

FIG. 7.8 Holderless single-use TFF devices connected in SPTFF configuration. *Figure used with permission from EMD Millipore Corporation.*

References

[1] Miesegaes G, Lute S, Brorson K. Analysis of viral clearance unit operations for monoclonal antibodies. Biotechnol Bioeng 2010;106:238–46. https://doi.org/10.1002/bit.22662.
[2] Guidance for industry Q5A viral safety evaluation of biotechnology products derived from cell lines of human or animal origin, US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) september 1998 IC.
[3] Burnouf T. An overview of plasma fractionation. Ann Blood 2018;3. https://doi.org/10.21037/aob.2018.05.03. 33-33.
[4] Morfini M, Coppola A, Franchini M, Minno G. Clinical use of factor VIII and factor IX concentrates. Blood Transfus (Trasfusione del sangue) 2013;11(Suppl. 4):s55–63. https://doi.org/10.2450/2013.010s.
[5] Munnucci PM. AIDS, hepatitis and hemophilia in the 1980s: memoirs from an insider. J Thromb Haemostasis 2003;1:2065–9.
[6] EMA/CHMP/BWP/706271/2010, Committee for medicinal products for human use (CHMP), Guideline on plasma-derived medicinal products. July 21, 2011.
[7] EMA/CPMP/BWP/268/95. Published 14/02/1996 Effective from 14/08/1996, virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses.
[8] Sofer G, Brorson K, Abujoub A, Aranha H, Burnouf T, Carter J, et al. PDA technical report No. 41: virus filtration. PDA J Pharm Sci Technol 2005;59:1–42.
[9] Meltzer TH, Jornitz MW. Filtration in the biopharmaceutical industry. New York: Marcel Dekker; 1998.
[10] Miesegaes G, Lute S, Aranha H, Brorson K. Virus retentive filters. 2010. https://doi.org/10.1002/9780470054581.eib319.
[11] Brough H, Antoniou C, Carter J, Jakubik J, Xu Y, Lutz H. Performance of a novel viresolve NFR virus filter. Biotechnol Prog 2002;18:782–95. https://doi.org/10.1021/bp010193.

[12] Lute S, Riordan W, Pease L, Tsai DH, Levy R, Haque M, et al. A consensus rating method for small virus-retentive filters. I. Method development. PDA J Pharm Sc Technol 2008;62:318–33.
[13] Brorson K, Lute S, Haque M, Martin J, Sato T, Moroe I, et al. A consensus rating method for small virus-retentive filters. II. Method evaluation. PDA J Pharm Sc Technol 2008;62:334–43.
[14] Brorson K, Sofer G, Aranha H. Nomenclature standardization for 'large pore size' virus-retentive filters. PDA J Pharm Sc Technol 2005;59:341–5.
[15] Kiss RD. Practicing safe cell culture: applied process designs for minimizing virus contamination risk. PDA J Pharm Sc Technol 2011;65:715–29. https://doi.org/10.5731/pdajpst.2011.00852.
[16] Specht R, Bakhshayeshi M. Session 4.1 case studies of application of generic claims and QbD for viral clearance authors: Rachel Specht and Meisam Bakhshayeshi. PDA J Pharm Sci Technol 2016;70. https://doi.org/10.5731/pdajpst.2016.006957.
[17] EMD Millipore Corporation. Virus retention performance of Viresolve® pro devices under a range of processing conditions. Literature No. MK_WP3374EN Ver. 1.0.2019-19591.08/2019.
[18] Asher DR, Slocum A, Bergmann KF, Genest P, Katz AB, Morais JJ, et al. Predicting virus filtration performance with virus spike characterization. BioProcess Int 2011;9:26–36.
[19] Slocum A, Burnham M, Genest P, Venkiteshwaran A, Chen D, Hughes J. Impact of virus preparation quality on parvovirus filter performance. Biotechnol Bioeng 2013:110. https://doi.org/10.1002/bit.24600.
[20] de Vilmorin P, Slocum A, Jaber T, Schaefer O, Ruppach H, Genest P. Achieving a successful scale-down model and optimized economics through parvovirus filter validation using purified TrueSpikeTM viruses. PDA J Pharm Sci Technol 2015;69:440–9. https://doi.org/10.5731/pdajpst.2015.01054.
[21] PDA Journal of Pharmaceutical Science and Technology. Technical report, No. 47, Preparation of virus spikes used for virus clearance studies. Iowa City (Iowa): University of Iowa; 2010.
[22] Gefroh E, Dehghani H, McClure M, Connell-Crowley L, Vedantham G. Use of MMV as a single worst-case model virus in viral filter validation studies. PDA J Pharm Sci Technol 2014;68:297–311. https://doi.org/10.5731/pdajpst.2014.00978.
[23] Elich T, Goodrich E, Lutz H, Mehta U. Investigating the combination of single-pass tangential flow filtration and anion exchange chromatography for intensified mAb polishing. Biotechnol Prog 2019. https://doi.org/10.1002/btpr.2862.
[24] Bolton G, Basha J, Lacasse D. Achieving high mass-throughput of therapeutic proteins through parvovirus retentive filters. Biotechnol Prog 2010;26:1671–7. https://doi.org/10.1002/btpr.494.
[25] Marques B, Roush D, Göklen K. Virus filtration of high–concentration monoclonal antibody solutions. Biotechnol Prog 2009;25:483–91. https://doi.org/10.1002/btpr.177.
[26] De Souza J, Scott K, Genest P. Virus-filtration process development optimization: the key to a more efficient and cost-effective step. BioProcess Int 2016;14(4):62–74.
[27] Zydney A. Continuous downstream processing for high value biological products: a review. Biotechnol Bioeng 2015;113. https://doi.org/10.1002/bit.25695.
[28] Gillespie C, Kozlov M, Phillips M, Potty A, Skudas R, Stone M, et al. Integrating continuous and single-use methods to establish a new downstream processing platform for monoclonal antibodies. 2015. https://doi.org/10.1002/9783527673681.ch04.
[29] Johnson SA, Brown MR, Lute SC, Brorson KA. Adapting viral safety assurance strategies to continuous processing of biological products. Biotechnol Bioeng 2017;114:1362. https://doi.org/10.1002/bit.26245.
[30] Genest P, Ruppach H, Geyer C, Asper M, Parrella J, Evans B, Slocum A. Artifacts of virus filter validation. BioProcess Int 2013;11:54–61.
[31] Lutz H, Chang W, Blandl T, Ramsey G, Parella J, Fisher J, Gefroh E. Qualification of a novel inline spiking method for virus filter validation. Biotechnol Prog 2011;27:121–8. https://doi.org/10.1002/btpr.500.
[32] Lacasse D, Genest P, Pizzelli K, Greenhalgh T, Mullin L, Slocum A. Impact of process interruption on virus retention of small-virus filters. BioProcess Int 2013;11:34–44.
[33] Lacasse D, Lute S, Fiadeiro M, Basha J, Stork M, Brorson K, et al. Mechanistic failure mode investigation and resolution of parvovirus retentive filters. Biotechnol Prog 2016;32. https://doi.org/10.1002/btpr.2298.
[34] Dishari S, Venkiteshwaran A, Zydney A. Probing effects of pressure release on virus capture during virus filtration using confocal microscopy: probing virus retention with confocal microscopy. Biotechnol Bioeng 2015. https://doi.org/10.1002/bit.25614.

[35] Woods M, Zydney A. Effects of a pressure release on virus retention with the ultipor DV20 membrane. Biotechnol Bioeng 2014;111. https://doi.org/10.1002/bit.25112.
[36] Dileo A, Allegrezza AE, Builder SE. High resolution removal of virus from protein solutions using a membrane of unique structure. Biotechnology 1992;10:182–8. https://doi.org/10.1038/nbt0292-182.
[37] Dileo A, Vacante AD, Deane EF. Size exclusion removal of model mammalian viruses using a unique membrane system, Part I: membrane qualification. Biologicals 1993;21:275–86. https://doi.org/10.1006/biol.1993.1085.
[38] Dileo A, Vacante DA, Deane EF. Size exclusion removal of model mammalian viruses using a unique membrane system, Part II: module qualification and process simulation. Biologicals 1993;21:287–96. https://doi.org/10.1006/biol.1993.1086.
[39] Syedain Z, Bohonak D, Zydney A. Protein fouling of virus filtration membranes: effects of membrane orientation and operating conditions. Biotechnol Prog 2006;22:1163–9. https://doi.org/10.1021/bp050350v.
[40] Giglia S, Bohonak D, Greenhalgh P, Leahy A. Measurement of pore size distribution and prediction of membrane filter virus retention using liquid–liquid porometry. J Membr Sci 2015;476. https://doi.org/10.1016/j.memsci.2014.11.053.
[41] Dávila R, Calvo J, ToVinh K, Thom V, Prádanos P, Hernández A. Liquid-liquid displacement porosimetry for the characterization of virus retentive membranes. J Membr Sci 2011;372:366–72. https://doi.org/10.1016/j.memsci.2011.02.022.
[42] Giglia S, Krishnan M. High sensitivity binary gas integrity test for membrane filters. J Membr Sci 2008;323:60–6. https://doi.org/10.1016/j.memsci.2008.06.017.
[43] Bolton G, Spector S, Lacasse D. Increasing the capacity of parvovirus-retentive membranes: performance of the Viresolve™ prefilter. Biotechnol Appl Biochem 2006;43:55–63. https://doi.org/10.1042/BA20050108.
[44] Brown A, Bechtel C, Bill J, Liu H, Liu J, McDonald D, et al. Increasing parvovirus filter throughput of monoclonal antibodies using ion exchange membrane adsorptive pre-filtration. Biotechnol Bioeng 2010;106:627–37. https://doi.org/10.1002/bit.22729.
[45] Bolton G, Cabatingan M, Rubino M, Lute S, Brorson K, Bailey M. Normal-flow virus filtration: detection and assessment of the endpoint in bio-processing. Biotechnol Appl Biochem 2005;42:133–42. https://doi.org/10.1042/BA20050056.
[46] Genest P, Campbell J, Crump S, Cabatingan M, Miao F. An improved method for virus filter qualification and implementation: using flow decay to determine processing endpoint. Bioprocess Int 2006;4:44–50.
[47] Bakhshayeshi M, Jackson N, Kuriyel R, Amit M, van Reis Robert, Zydney A. Use of confocal scanning laser microscopy to study virus retention during virus filtration. J Membr Sci 2011;379:260–7. https://doi.org/10.1016/j.memsci.2011.05.069.
[48] Jackson N, Bakhshayeshi M, Zydney A, Mehta A, van Reis R, Kuriyel R. Internal virus polarization model for virus retention by the Ultipor (R) VF Grade DV20 membrane. Biotechnol Prog 2014;30. https://doi.org/10.1002/btpr.1897.
[49] Rayfield W, Roush D, Barakat S, Cheung J, Chmielowski R, Tugcu N. Prediction of viral filtration performance of monoclonal antibodies based on biophysical properties of feed. Biotechnol Prog 2015;31. https://doi.org/10.1002/btpr.2094.
[50] Strauss D, Goldstein J, Hirasaki T, Yokoyama Y, Hirotomi N, Miyabayashi T, Vacante D. Characterizing the impact of pressure on virus filtration processes and establishing design spaces to ensure effective parvovirus removal. Biotechnol Prog 2017;33. https://doi.org/10.1002/btpr.2506.
[51] Anderson KC, Landgren O, Arend RC, Chou J, Jacobs IA. Humanistic and economic impact of subcutaneous versus intravenous administration of oncology biologics. Future Oncol 2019. https://doi.org/10.2217/fon-2019-0368.
[52] Haller MF. Converting intravenous dosing to subcutaneous dosing with recombinant human hyaluronidase. Pharmaceut Technol 2007;31(10).
[53] Lutz H, Arias J, Zou Y. High concentration biotherapeutic formulation and ultrafiltration: Part 1 pressure limits. Biotechnol Prog 2017;33:113–24.
[54] Larson AM. Liquid protein formulations containing viscosity lowering agents, US Patent Application #20150071925 A1.
[55] Warne NW, Nichols PEL, Loureiro PJ. Protein formulations with reduced viscosity and uses thereof. US Pat #7390786. 2008.

[56] Kanai S, Liu J, Patapoff TW, Shire SJ. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab−Fab interaction that impacts solution viscosity. J Pharm Sci 2008;97:4219−27.
[57] Guo Z, Chen A, Nassar RA, Helk B, Mueller C, Tang Y, et al. Pharm Res (N Y) 2012;29:3102. https://doi.org/10.1007/s11095-012-0802-9.
[58] EMD Millipore Corporation. Protein concentration and diafiltration by tangential flow filtration. Literature No. TB032 Rev. C, 06/2003.
[59] Blatt WF, Dravid A, Michaels AS, Nelsen L. Solute polarization and cake formation in membrane ultrafiltration: causes, consequences, and control techniques. In: Flinn JE, editor. Membrane science and technology. New York, NY: Plenum Press; 1970. p. 47−97.
[60] Denn MM. Process fluid mechanics. Englewood Cliffs: Prentice-Hall; 1980.
[61] Liu HF, Ma J, Winter C, Bayer R. Recovery and purification process development for monoclonal antibody production. mAbs 2010;2(5):480−99. https://doi.org/10.4161/mabs.2.5.12645.
[62] Baek Y, Singh N, Arunkumar A, Borys M, Zheng JL, Zydney A. Ultrafiltration of mAbs and Fc-fusion protein. Biotechnol Bioeng 2017;114(9):2057−65.
[63] Lutz H, editor. Ultrafiltration for bioprocessing. Woodhead Publishing; 2015. p. 7−30. p. 45−75, pp. 103−130, ISBN 9781907568466.
[64] Kedem O, Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. Biochim Biophys Acta 1958;27:229−46.
[65] Bird RB, Warren ES, Lightfoot EN. Transport phenomena. 2nd ed. New York, NY: John Wiley; 2002.
[66] Kinzlmaier D, Goodrich EM. High viscosity tangential flow filtration applications: the impact of feed channel screen on process performance, high concentration capability, and membrane re-use. In: Division of Biochemical Technology, American Chemical Society 253rd Annual Meeting. San Francisco, CA; April 2017.
[67] EMD Millipore Corporation. High viscosity TFF cassette design and application in ultrafiltration process. 2016. Literature No. PS2076EN00 Ver. 1.0.
[68] Arunkumar A, Singh N, Schutsky EG, Peck M, Swanson RK, Borys MC, Zheng JL. Effect of channel-induced shear on biologics during ultrafiltration/diafiltration (UF/DF). J Membr Sci 2016;514:671−83.
[69] EMD Millipore Corporation. Recovery optimization of process scale ultrafiltration/diafiltration systems. 2016. Literature No. TB5882EN00 Ver. 2.0.
[70] Miao F, Velayudhan A, DiBella E, Shervin J, Felo M, Teeters M, Alred P. Theoretical analysis of excipient concentrations during the final ultrafiltration/diafiltration step of therapeutic antibody. Biotechnol Prog 2009;25(4):964−72.
[71] Bolton GR, Boesch AW, Basha J, LaCasse DP, Kelley BD, Acharya H. Effect of protein and solution properties on the donnan effect during the ultrafiltration of proteins. Biotechnol Prog 2011;27(1):140−52.
[72] Teeters M, Bezila D, Benner T, Alfonso P, Alred P. Predicting diafiltration solution compositions for final ultrafiltration/diafiltration steps of monoclonal antibodies. Biotechnol Bioeng 2011;108(6):1338−46.
[73] Abel J, Kosky A, Ball N, Bacon H, Kaushik R, Kleemann G. A small-scale process for predicting donnan and volume exclusion effects during ultrafiltration/diafiltration process development. J Pharmaceut Sci 2018;107(5):1296−303. https://doi.org/10.1016/j.xphs.2018.01.010.
[74] Brinkmann A, Elouafiq S, Pieracci J, Westoby M. Leveraging single-pass tangential flow filtration to enable decoupling of upstream and downstream monoclonal antibody processing. Biotechnol Prog 2018;34:405−11. https://doi.org/10.1002/btpr.2601.
[75] Arunkumar A, Zhang J, Singh N, Ghose S, Li ZJ. Ultrafiltration behavior of partially retained proteins and completely retained proteins using equally-staged single pass tangential flow filtration membranes. Biotechnol Prog 2018;34(5):1137−48. https://doi.org/10.1002/btpr.2671.
[76] Dizon-Maspat J, Bourret J, D'Agostini A, Li F. Single pass tangential flow filtration to debottleneck downstream processing for therapeutic antibody production. Biotechnol Bioeng 2012;109(4):962−70. https://doi.org/10.1002/bit.24377.
[77] Arunkumar A, Singh N, Peck M, Borys MC, Zheng JL. Investigation of single-pass tangential flow filtration (SPTFF) as an inline concentration step for cell culture harvest. J Membr Sci 2017;524:20−32.
[78] Peterson E, et al. Utilizing single-use technology to enable single-pass tangential flow filtration. In: Division of Biochemical Technology, American Chemical Society Annual Meeting. New Orleans, LA; 2018.

[79] Clutterbuck A, Beckett P, Lorenzi R, Sengler F, Bisschop T, Haas J. Single-pass tangential flow filtration (SPTFF) in continuous biomanufacturing. In: Ganapathy S, editor. Continuous biomanufacturing −innovative technologies and methods. John Wiley & Sons, Ltd.; 2017. p. 423−56. https://doi.org/10.1002/9783527699902.ch15.
[80] Elich T, Lutz H. Inline concentration of mAb feed to increase the productivity of a continuous multi-column chromatography capture step. In: Division of Biochemical Technology, American Chemical Society Annual Meeting. Orlando, FL; 2019.
[81] EMD Millipore Corporation. Single-pass tangential flow filtration, a versatile application to streamline biomanufacturing. Literature No. WP2787EN Ver. 1.0.10/2018.
[82] LaBreck M, Perreault M. Single-use TFF offers the greatest savings in clinical and contract manufacturing, where the scale is low and changeovers are frequent. Biopharm Int 2010;2010(Suppl. 9).
[83] País-Chanfrau JM, Zorrilla K, Chico E. The impact of disposables on project economics in a new antibody plant: a case study. Biopharm Int 2009;22(12):62−74.
[84] Czapkowski B, Steen J. Trial of high-efficiency TFF capsule prototype for ADC purification. ADC Rev April,2017. https://doi.org/10.14229/jadc.2017.11.04.001.
[85] Scanlan C, Zhao J, Rogers M, Waghmare R, Xu S, Cross S, et al. Antibody-drug conjugates: manufacturing challenges and trends. ADC Rev March,2017. https://doi.org/10.14229/jadc.2017.21.03.001.
[86] Calkins T. Containment of high-potency products in a GMP environment. BioProcess Int September,2010:14−8.

CHAPTER

8

A roadmap for IgG-like bispecific antibody purification

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List of abbreviations

bsAb bispecific antibody
HC heavy chain
LC light chain
KiH knobs-into-holes
PEG polyethylene glycol
CEX cation exchange
TCR T cell receptor
pI isoelectric point
FT flow-through
AEX anion exchange
IEX ion exchange
scFv single-chain variable fragment
HA hydroxyapatite
HIC hydrophobic interaction chromatography

Introduction

Bispecific antibodies (bsAbs) are antibodies that can bind two epitopes on the same or a distinct target. Their capability to simultaneously engage two targets enables novel mechanisms of action (e.g., dual inhibition of signaling circuits and recruitment of effector cells). Thus, bsAbs have emerged as powerful tools for the treatment of cancers and other diseases [1–4]. The number of therapeutic bsAbs in development has grown considerably over the past several years. Currently, bsAbs are mainly generated through recombinant approaches and they have been developed in a variety of different formats [5,6]. Based on their formats,

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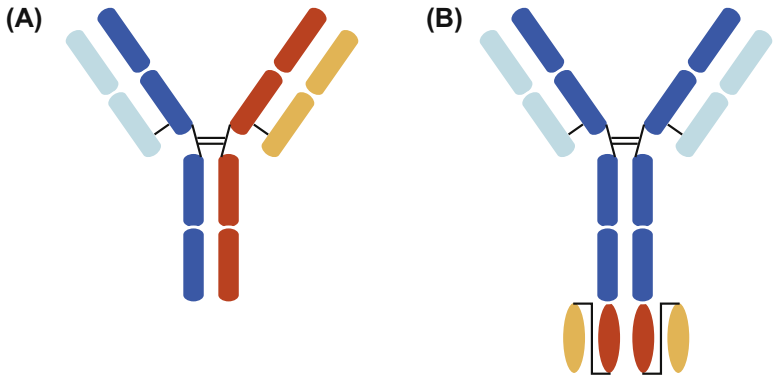

FIG. 8.1 Schematic representation of IgG-like bsAbs in typical (A) asymmetric and (B) symmetric format. For the symmetric format, a scFv is fused to the C-terminus of the HC of a full-length IgG. Chain mispairing and aggregation are major issues for asymmetric and symmetric bsAbs, respectively.

bsAbs can be broadly divided into two main categories: IgG-like molecules comprising an Fc region and smaller non-IgG-like molecules lacking the Fc region. In general, production of IgG-like bsAbs is technically more challenging than that of the Fc-free counterparts. IgG-like bsAbs can be further divided into two main subgroups in accordance with their structural symmetry: asymmetric bsAbs and symmetric bsAbs (Fig. 8.1). Purification of each subgroup of bsAbs usually represents unique challenges. In this work, we introduce a roadmap to guide IgG-like bsAb purification in general. Before launching the purification roadmap, common by-products associated with each subgroup of bsAbs and methods for their removal will be briefly reviewed.

Asymmetric bsAb

Asymmetric bsAbs are normally derived from two parental antibodies with different binding specificities and consequently contain four distinct chains: two different heavy chains (HCs) and two different light chains (LCs). When these chains are coexpressed in the producing cell, heterodimerization of HCs specific for different antigens is required. In addition, the LC specific for one antigen must pair with the cognate HC specific for the same antigen. However, if the four chains are allowed to pair randomly, misassembled species (e.g., homodimers, molecules containing mispaired LCs) could account for about 90% of the total mass [7]. Consequently, a variety of protein engineering approaches have been developed to enforce correct chain pairing [8]. Among them, knobs-into-holes (KiH) and CrossMab technologies are the most widely used ones for overcoming the obstacle of HC homodimerization and LC mispairing, respectively [9,10]. However, despite elegant design of these approaches, incorrect chain pairing cannot be completely avoided. In addition, unbalanced chain expression and suboptimal culture conditions can result in other by-products (e.g., half antibody) [11]. Methods for removing homodimers and half antibody are briefly introduced in the following sections.

Homodimer

In general, homodimers are difficult to remove as they often exhibit physicochemical properties similar to those of the target bsAb. Effective clearance of homodimer requires the purification procedure taking full advantages of the uniqueness of each bsAb design. There are special designs which make separation of homodimer from heterodimeric bsAb relatively straightforward. As homodimers from various designs exhibit different characteristics, their removal will be separately discussed.

bsAb constructed using the KiH technology

Many asymmetric IgG-like bsAbs have used the KiH technology to promote HC heterodimerization (Fig. 8.2A). However, whereas this strategy significantly improves heterodimer formation, homodimerization can still occur at a low level (e.g., 5%) [12,13]. In particular, hole-hole homodimer is observed more often than knob-knob homodimer, whose formation

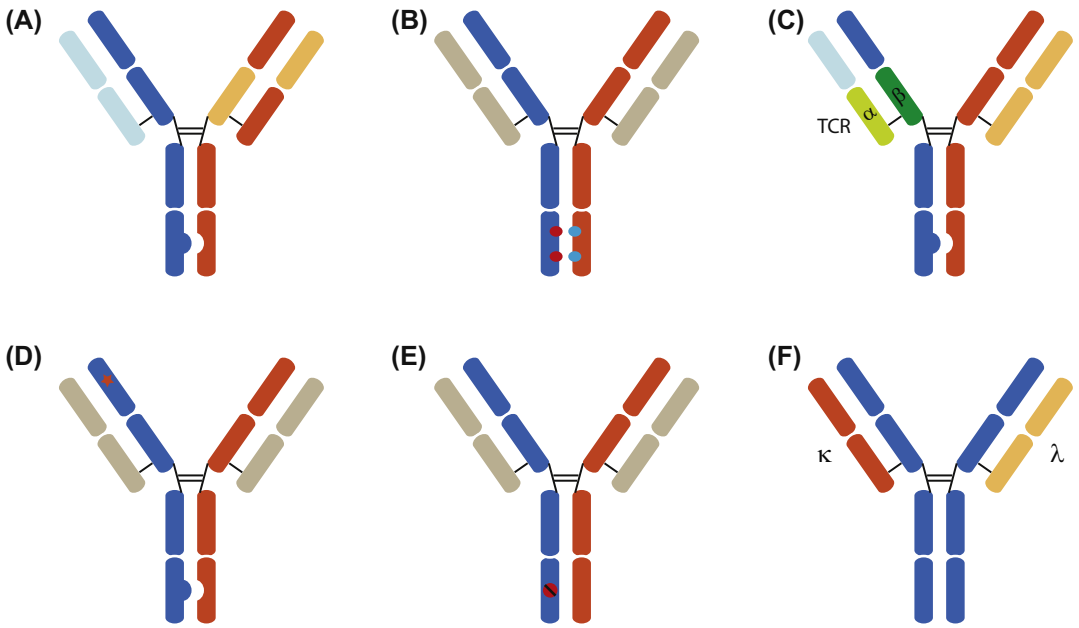

FIG. 8.2 Schematic representation of asymmetric IgG-like bsAbs based on different strategies. (A) KiH based design. HC-LC mispairing can be avoided by using CrossMab or other strategies. (B) bsAb with electrostatic interaction driven heterodimerization. HC-LC mispairing can be avoided by using a common LC or other strategies. (C) bsAb in WuXiBody format. WuXiBody is a novel design that promotes correct HC-LC pairing by replacing one arm's CH1/CL region with the TCR constant domain. HC homodimerization can be avoided by using KiH or other strategies. (D) bsAb with pI engineered HC. HC homodimerization and HC-LC mispairing can be avoided by using KiH and a common LC, respectively. pI engineering is implemented into one of the heavy chain variable regions to increase the pI difference between the bsAb and the homodimeric by-products. (E) bsAb with Protein A-binding abolished HC. HC-LC mispairing can be avoided by using a common LC or other strategies. HC homodimerization can occur, but the formed homodimers can be easily removed. (F) κλ-body. A common HC is used in this design. The HC can bind either of the two LCs, and consequently mono λ and mono κ homodimers can be generated, but they can be effectively removed by using a sequential affinity chromatography.

is somehow hindered by the knob. Thus, homodimers can be a major by-product if the "hole" HC is expressed in excess.

Zhang et al. recently showed that the hole-hole homodimer is less stable than the heterodimer, which is stabilized by the KiH design, when exposed to low pH [14]. Under low pH conditions, the hole-hole homodimer experiences a conformational change and becomes more hydrophobic. A hole-hole homodimer's high sensitivity to low pH and the consequent conformation/hydrophobicity change can be utilized to facilitate its separation. In a recent study, we showed that when loaded at a pH slightly lower (e.g., pH 4.5) than that is normally used (i.e., pH 5.5), Capto MMC ImpRes provides improved resolution between hole-hole homodimer and the KiH heterodimer. In addition, the resolution can be further enhanced by adding 5% (v/v) polyethylene glycol (PEG) 3350 to wash and elution buffers (authors' unpublished work). This approach should have a general value in removing the hole-hole homodimer by-product in purifying bsAbs with the KiH design.

bsAb with electrostatic interaction driven heterodimerization

Charge-introduction substitution at different positions within the CH3 interface is an alternative strategy for promoting HC heterodimerization [15,16] (Fig. 8.2B). In general, residues with opposite charges are introduced into the complementary CH3 regions. Heterodimerization is favored by attractive electrostatic interactions whereas homodimer formation is disfavored by repulsive charged interactions. In contrast to the KiH design where the two types of homodimer are not equally suppressed, in the case of electrostatic steering suppression is equally effective for both kinds of homodimer.

In one study, the authors showed that heterodimer levels ranged from 87 to 100% for designs with different charge substitutions, suggesting that this is an effective approach for promoting heterodimer formation [16]. With a selected design, the best result is often achieved when the two HCs are expressed at a 1:1 ratio. Nevertheless, the two homodimers can still be generated in minor amounts, but they can be effectively removed by cation exchange (CEX) chromatography as homodimers and the heterodimer usually exhibit quite different charge characteristics as a result of charged substitutions [16].

bsAb based on WuXiBody platform

WuXi Biologics recently developed a novel bsAb platform: WuXiBody [17] (Fig. 8.2C). In one Fab arm, the constant region (CH1/CL) was replaced by the T cell receptor (TCR) constant domain. In addition, interaction between TCR α and β regions was reinforced by a non-native interchain disulfide bond. This design ensures cognate HC-LC pairing, and when combined with KiH technology can largely solve the chain mispairing problems in bsAb construction.

The TCR constant domain has a relatively low isoelectric point (pI) and this feature can be used to facilitate homodimer removal. When designing bsAbs based on the WuXiBody format and KiH strategy, it is preferable to have the TCR domain included in the "knob" half antibody. In addition, the "hole" half antibody should be expressed in slight excess. This way, the main by-products will be the "hole" half antibody and hole-hole homodimer. These two by-products do not contain the TCR domain and thus have pIs higher than that of the bsAb, which contains the low-pI TCR domain. This pI difference facilitates hole-hole homodimer removal by anion exchange (AEX) chromatography. For example, under

appropriate conditions, the low-pI bsAb binds to an AEX column whereas the high-pI hole-hole homodimer does not bind and is left in the flow-through (FT).

bsAb with pI engineered HC

pI engineering is an effective means to facilitate bsAb purification. In this approach, the pI value of one HC is altered through mutations to increase the pI difference between the bsAb and homodimeric by-products [18–21]. pI engineering can be implemented into the HC variable region or the Fc region [19,21] (Fig. 8.2D). Alternatively, constant regions of different subclasses (IgG1, IgG2, IgG3 and IgG4) originally having distinct pIs can be used as the constant regions of the two HCs that constitute the bsAb (e.g., constant region of one parental mAb HC is substituted with the constant region from a different subclass to generate the subclass hybrid bsAb) [18]. The increased pI difference greatly facilitates purification of the target bsAb by ion exchange (IEX) chromatography. In a recent study, IEX chromatography exhibited higher resolution using a pH gradient than using a salt gradient. The authors showed that adequate separation of heterodimer from homodimer can be achieved using a highly linear pH gradient even if the pI difference between the two parental antibodies is very small (i.e., 0.1 pH unit) [22].

bsAb with Protein A-binding abolished HC

A strategy similar to pI engineering that also effectively simplifies homodimer removal is modifying the Protein A binding avidity of one HC Fc [23] (Fig. 8.2E). In such a design, one HC Fc domain's Protein A binding capacity is abolished (referred to as Fc*). Then the bsAb and two homodimers will contain FcFc* and FcFc/Fc*Fc*, respectively. As a consequence of the Protein A-binding-ablating substitution, the bsAb (FcFc*) shall possess intermediate binding affinity as compared with FcFc and Fc*Fc* homodimers, which exhibit normal and severely diminished binding avidity, respectively. It is known that human IgG3 isotype does not bind to Protein A, and thus Fc chain originates from human IgG3 is a naturally-occurring Fc*. Alternatively, Fc* can be generated by substituting residues within the wide-type Fc CH3 domain critical for Protein A binding with corresponding ones from IgG3.

The resulting engineered affinity hierarchy among heterodimer and homodimers facilitates separation of the desired bsAb from homodimer impurities using Protein A chromatography [23,24]. Fc*Fc* homodimer shall not bind and therefore appear in the FT. The bsAb (Fc*Fc) and FcFc homodimer both bind but with different strength: the former binds less tightly than the latter. The weakly bound bsAb can be selectively eluted at a less stringent pH, under which condition the strongly bound FcFc homodimer is retained. Separation between bsAb and FcFc homodimer can be further improved by adding $CaCl_2$ or $MgCl_2$ to the elution buffer [23]. Improved resolution is believed to be achieved through modulation of hydrophobic interactions between the antibody and Protein A ligand.

Common HC bsAb - κλ-body

A bsAb design using a common HC completely avoids the HC pairing problem and bispecificity is conferred by two different LCs: one kappa and one lambda [25] (Fig. 8.2F). In this design, no mutation or foreign sequence is introduced. In addition to the desired κλ-body, mono κ and mono λ homodimers will also be generated when the three chains (HC, κ LC and λ LC) are coexpressed. Nevertheless, the unique property of κλ-body allows

these two impurities (κκ and λλ) to be easily removed by a sequential affinity purification composing KappaSelect and LambdaFabSelect resins, which bind the constant domain of the κ and λ LC, respectively. Whereas the bispecific κλ-body can bind both resins, mono λ and mono κ homodimers are eliminated in the column FT of KappaSelect and LambdaFabSelect, respectively, leaving κλ-body as the final purified product. Although the order of the two affinity chromatography is interchangeable, it is preferable to have the predominant monospecific species removed first (e.g., KappaSelect should be placed ahead of LambdaFabSelect if there is more mono λ than mono κ) so that most of the column's loading capacity can be saved for the target κλ-body.

Single-chain variable fragment (scFv)-Fc

A scFv is constructed by connecting the variable regions of the HC and LC via a linker peptide. As a strategy to avoid HC-LC mispairing, some bsAbs adopt the Fab × scFv format, in which one Fab arm is replaced by a scFv [26]. In such a design, coexpression of three chains (HC, LC and scFv-Fc) is required for bsAb assembly. In certain cases, the scFv-Fc is expressed in excess and becomes a major contaminant. As the scFv-Fc contains the Fc region, it cannot be separated from the target bsAb using Protein A affinity chromatography. Nevertheless, a difference between the bsAb and scFv-Fc is the presence and absence, respectively, of a LC constant region. This difference facilitates separation of these two species using an alternative affinity resin, KappaSelect, which specifically binds the constant region of kappa LC [27]. Whereas the bsAb can bind to KappaSelect through its Fab arm, the scFv-Fc, which lacks the LC constant region, does not bind. This approach provides an effective means for removing scFv-Fc monomer and homodimer. It is worth noting that Protein L resin, another kappa LC-binding affinity medium, binds to the variable region instead of the constant region and therefore cannot differentiate between scFv-Fc and the bsAb [28]. As scFv (monomer and dimer) can be readily removed by KappaSelect, it is desirable to keep the expression of scFv in slight excess during the production of bsAbs adopting the Fab × scFv format to minimize the formation of homodimers containing normal Fab arms, which could be difficult to remove.

Half antibody

Half antibody is a common impurity in bsAb production. In certain case, it exists as a result of intended overexpression of the heavy chain that cannot form a homodimer [29]. As half antibody contains only one Fc-domain, it binds Protein A resin weaker than the bsAb, which contains the full Fc-region. Thus, under linear pH gradient elution, half antibody will elute earlier from the Protein A column. Resolution between half antibody and whole antibody can be further improved by adding chaotropic salts to the mobile phase [23]. Based on linear pH gradient elution results, a process consisting of stepwise pH elution and an intermediate pH wash can be developed. The pH for the intermediate wash needs to be low enough to wash off half antibody while still able to retain the bsAb bound to the column. The optimal wash pH depends on the load density. A relatively low and high pH should be used when the column is loaded at low and high density, respectively, to achieve effective

half antibody clearance and reasonable yield. In general, this approach is less robust due to its load-sensitive characteristic.

Hydroxyapatite (HA), a mixed-mode resin that mediates both CEX and metal-affinity interactions, is another medium that can provide a certain degree of resolution between half antibody and the intact bsAb [30]. In general, half antibody will elute earlier than bsAb under linear salt gradient elution conditions. In one case study, the purity of a bsAb was improved from 33% (in the load) to 80% (in the eluate) [30]. In another example, bsAb purity was improved from 77% to 97% [30]. Gagnon et al. previously showed that including 5%−10% PEG in both the gradient-start and gradient-end buffers significantly improves HA resolution [31,32]. Therefore, more complete separation between half antibody and the bsAb can be expected in the presence of PEG. The observation made under linear gradient elution also suggests that stepwise elution can be developed and substantial clearance of half antibody can be achieved by selecting an appropriate wash condition.

In addition to HA, another mixed-mode resin, Capto MMC, can also provide good clearance of half antibody under appropriate conditions. Capto MMC possesses CEX and hydrophobic moieties. Proteins bound to the column can be eluted by linear salt or pH gradient. In general, half antibody binds weaker than bsAb and is found in early elution fractions (authors' unpublished work). Based on the results of linear gradient elution, a wash step can be introduced to selectively remove half antibody in a step elution procedure. Finally, if the half antibody has a pI that is significantly different from that of the bsAb, then separation of these two species can be achieved by using IEX chromatography.

Symmetric bsAb

For bsAbs adopting a symmetric format, bispecificity is usually achieved by fusion of a second antigen-binding unit to either terminus of the LC or HC [5,6]. As symmetric bsAbs achieve their bispecificity through chain extension rather than introducing distinct chains, normally the number of associated by-products is fewer than that found with asymmetric bsAbs. Nevertheless, bsAbs in symmetric format have shown significantly increased tendency to form aggregates [33,34]. In a certain case, aggregate content was found to be as high as 50% [34]. Aggregates are likely formed through intermolecular domain swapping as a result of increased chain length and flexibility [35]. For symmetric bsAbs, aggregate removal becomes a major task of downstream processing. Several types of chromatography that provide good aggregate clearance are individually introduced in the following sections.

Protein A chromatography

In general, Protein A chromatography under typical conditions is less effective at removing aggregates than some alternative chromatographic strategies. Although aggregates are known to bind more strongly than monomer, they are often co-eluted with the latter and adjusting elution pH alone usually is not sufficient for good separation. Recently, we developed a method that can significantly improve Protein A's aggregate removing capability, allowing removal of the majority of aggregates at this capture step [36]. In particular, we showed that adding a PEG/calcium chloride or PEG/sodium chloride

combination to the mobile phase can significantly improve monomer-aggregate resolution. For the case used for method development and demonstration, the optimized procedure allows aggregates to be reduced from greater than 20% to approximately 3%–4% [36]. This approach alleviates the burden of aggregate removal on post-Protein A polishing steps and improves the overall robustness of downstream process.

Hydrophobic interaction chromatography (HIC)

HIC is commonly used for aggregate removal and has been shown to be highly effective [37,38]. HIC is typically run in FT mode for this purpose. Aggregates are generally more hydrophobic than monomer, and an appropriate condition can be chosen under which monomer flows through whereas aggregates bind. HIC resins with various degrees of hydrophobicity are available from the same or different vendors. Depending on the particular HIC resin used, salt concentration in the load needs to be adjusted to achieve the best result. For example, in one study it was learned that for the same load, 0.5 M ammonium sulfate is needed when using Phenyl 650 M, a HIC resin with moderate hydrophobicity, and only 50 mM sodium chloride is required when using POROS Benzyl Ultra, a HIC resin with high hydrophobicity [39].

Capto MMC ImpRes and Capto adhere chromatographies

Capto MMC ImpRes is a resolution-improved version of Capto MMC [40,41]. In comparison with Capto MMC, Capto MMC ImpRes has both a reduced bead size and ligand density, allowing for enhanced selectivity between monomer and aggregates. According to the vendor's application note, high binding capacities are obtained between pH 5.0–7.0 and sodium chloride concentration 0–150 mM, whereas high purity is achieved at pH 5.0 [40]. In a previous study, we showed that good aggregate clearance was achieved when the column was eluted with sodium chloride, but the elution peak tailed significantly [39]. This is not surprising considering hydrophobic interactions are involved in binding. Peak broadening can be remedied without sacrificing resolution by eluting the column with arginine, which disrupts both electrostatic and hydrophobic interactions.

Capto adhere is another mixed-mode resin capable of removing aggregates [42]. Its ligand contains groups that are capable of AEX, hydrophobic interaction and hydrogen bonding. For aggregate removal, Capto adhere is typically performed in FT mode [43]. According to the vendor's application note, aggregate clearance is influenced by pH, conductivity and load [42]. In general, higher pH, higher conductivity and/or lower load results in better aggregate clearance. In case where good aggregate removal is not achieved under FT mode, bind-elute mode is worth a try [39].

HA chromatography

HA chromatography is well-known for its strong aggregate removing capability [32,44]. For example, one study showed that HA reduces aggregate levels from above 60% to less than 0.1% [44]. HA is a mixed-mode resin which is capable of phosphoryl CEX and calcium metal affinity interactions. These two interactions contribute cooperatively but one of them

can dominate the retention for a particular protein. Phosphate is widely employed for elution as it disrupts both types of interaction. Sodium chloride, on the other hand, has a negligible effect on metal affinity interaction. Most antibodies bind mainly through CEX interaction, with aggregates binding stronger than monomers [32]. In general, eluting using a sodium chloride gradient with a fixed phosphate concentration gives better resolution than a phosphate gradient. It had been shown previously that PEG, when added to the mobile phase, enhances protein retention on a HA column and the degree of enhancement is proportional to protein size, suggesting that PEG preferentially enhances aggregate retention and therefore allows more complete separation between monomer and aggregates [31,45].

CEX chromatography

CEX chromatography is widely used as a polishing step in antibody purification. However, it only has limited aggregate-resolving capability under typical conditions. Whereas aggregates often bind more tightly than monomeric protein, resolution between the two species is usually low especially when the aggregates are dimers (the aggregate peak usually elutes as a shoulder on the tail side of the monomer peak) [46–49]. Thus, yield has to be sacrificed in order to achieve adequate aggregate clearance. CEX chromatography is commonly operated under acidic conditions, which is distant from the pIs of most antibodies. Nevertheless, a recent study showed that alkaline pH CEX chromatography achieved improved aggregate removal [50]. We experienced poor separation between monomer and aggregates in a previous study using POROS XS and Capto SP ImpRes at acidic pH under linear salt gradient elution [39]. However, we observed that separation was significantly improved with both resins when 5% PEG 3350 was added to wash and elution buffers [39]. As in the case of HA chromatography, PEG increases protein retention and the magnitude of this effect is proportional to protein size. In the presence of PEG, aggregates bind much tighter and mainly appear in the strip.

Purification roadmap

A roadmap for IgG-like bsAb purification, created by summarizing information provided in preceding sections, is shown in Fig. 8.3. For each type of commonly observed by-product, one or more paths that leads to its removal is provided. For a particular type of by-product, complementary purification paths can be used sequentially to achieve the best clearance. For example, Protein A and MMC are both capable of removing half antibody, and therefore when combined they should be able to provide more complete removal of this by-product. Similarly, better aggregate clearance can be expected when Protein A is combined with MMC or HA chromatography.

Concluding remarks

In general, IgG-like bsAbs are generated through reassembling chains from two different antibodies or attaching an antigen-binding moiety to an existing antibody, resulting in asymmetric and symmetric molecules, respectively. Construction of asymmetric bsAbs involves

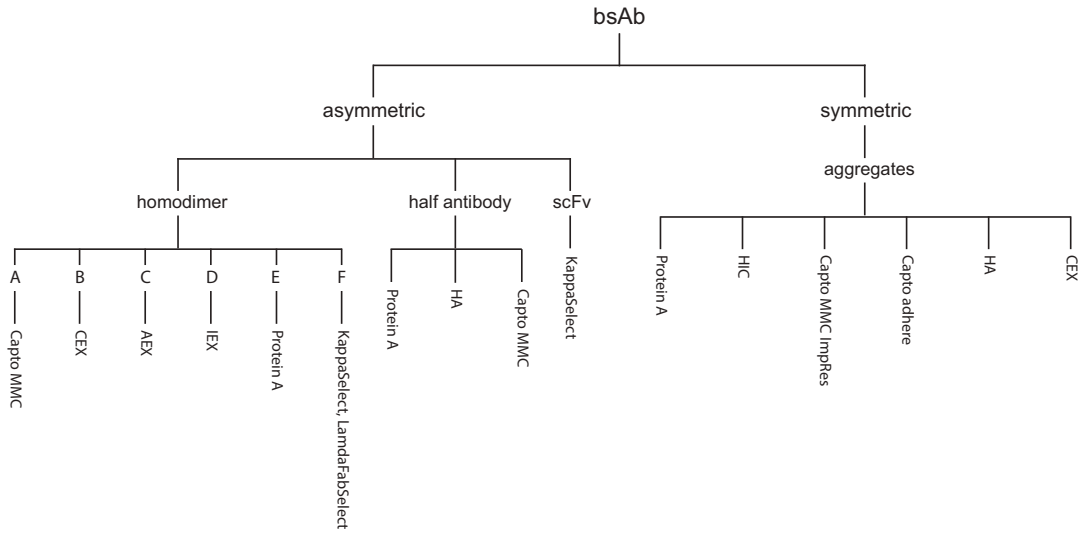

FIG. 8.3 A roadmap for IgG-like bsAb purification. For each type of commonly observed by-product, one or more paths that leads to its removal is provided. A-F refer to the different designs shown in Fig. 8.2.

multiple distinct chains, and consequently expression of this type of bsAb is often accompanied with various by-products due to random chain pairing and unbalanced chain expression. Although several strategies have been developed to promote correct assembly, formation of by-products cannot be completely avoided. Construction of symmetric bsAb involves fewer chains, but for this type of bsAb the extended chain length usually leads to decreased stability and an elevated level of aggregates. For bsAbs in either format, the associated impurities pose considerable challenges to downstream purification. In particular, as the species and relative amount of by-products vary on a case by case manner, it is difficult to develop a platform approach that meets the purification needs of each individual bsAb. By gathering information for bsAb-specific impurity removal available in the literature, we have generated a roadmap which provides general guidance on bsAb purification. Whereas bsAb associated by-products eventually need to be removed by the purification process, solving impurity issues unique to bsAb should not totally rely on the downstream process. It is highly recommended that optimization be made at upstream stages (i.e., molecular design, cell line development and cell culture development) to mitigate the downstream burden.

For asymmetric bsAbs, an appropriate strategy that enforces correct chain pairing needs to be selected. In addition, the selected strategy can be combined with an isolation-facilitating strategy, which provides an effective means for removal of potential by-products that can still be generated in small amounts with the selected chain pairing-promoting strategy. For example, in one study, highly efficient heterodimerization (>90%) and straightforward separation of bsAb from homodimeric by-products was achieved by combining KiH technology with pI engineering [19]. In another case, Skegro et al. achieved high yield of heterodimer (90%–95%) and robust removal of homodimer traces by combining HC heterodimerization-promoting technology with an asymmetric Protein A-binding strategy [24]. For symmetric bsAbs, optimal engineering of the appended domain and the linker is

critical for minimizing aggregation. For example, in the case of an IgG-scFv the amount of aggregates could be dramatically reduced by stabilizing the scFv moiety with a VH-VL interchain disulfide bond and adjusting the length of the linker that connected the scFv to the remainder of the molecule [34].

In addition to molecular design, balancing the expression of individual chains is critical for good bsAb yield. In certain cases, discordant chain expression can result in increased amounts of by-products and hence a reduced percentage of correctly assembled bsAb [16]. However, on the other hand, one study suggests that slight overexpression of LC is favorable as it promotes bsAb assembly and avoids the generation of $^{3}/_{4}$ antibody (antibody lacking one LC) [51]. Fine tuning is likely required to obtain the best result. In addition, culture conditions can also have an impact on the resulting impurity profile [11]. Thus, in the case of bsAbs, an increased number of vector constructs, plasmid ratios, minipools and culture conditions need to be screened in order to achieve good productivity with low product related impurities.

In conclusion, whereas team effort is critical for the production of any therapeutic antibody, this is especially true for bsAb due to the significantly increased complexity of this type of molecule. The purification roadmap provided in this work can fulfill its maximum usefulness only when upstream work is fully optimized.

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References

[1] Yu S, Li A, Liu Q, Yuan X, Xu H, Jiao D, Pestell RG, Han X, Wu K. Recent advances of bispecific antibodies in solid tumors. J Hematol Oncol 2017;10(1):155.
[2] Dahlén E, Veitonmäki N, Norlén P. Bispecific antibodies in cancer immunotherapy. Ther Adv Vaccines Immunother 2018;6(1):3–17.
[3] Duell J, Lammers PE, Djuretic I, Chunyk AG, Alekar S, Jacobs I, Gill S. Bispecific antibodies in the treatment of hematologic malignancies. Clin Pharmacol Ther 2019;106(4):781–91.
[4] Peyrin-Biroulet L, Demarest S, Nirula A. Bispecific antibodies: the next generation of targeted inflammatory bowel disease therapies. Autoimmun Rev 2019;18(2):123–8.
[5] Spiess C, Zhai Q, Carter PJ. Alternative molecular formats and therapeutic applications for bispecific antibodies. Mol Immunol 2015;67(2):95–106.
[6] Brinkmann U, Kontermann RE. The making of bispecific antibodies. mAbs 2017;9(2):182–212.
[7] Klein C, Sustmann C, Thomas M, Stubenrauch K, Croasdale R, Schanzer J, Brinkmann U, Kettenberger H, Regula JT, Schaefer W. Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies. mAbs 2012;4(6):653–63.
[8] Krah S, Sellmann C, Rhiel L, Schröter C, Dickgiesser S, Beck J, Zielonka S, Toleikis L, Hock B, Kolmar H, Becker S. Engineering bispecific antibodies with defined chain pairing. Nat Biotechnol 2017;39(Pt B):167–73.
[9] Ridgway JB, Presta LG, Carter P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 1996;9(7):617–21.
[10] Schaefer W, Regula JT, Bähner M, Schanzer J, Croasdale R, Dürr H, Gassner C, Georges G, Kettenberger H, Imhof-Jung S, Schwaiger M, Stubenrauch KG, Sustmann C, Thomas M, Scheuer W, Klein C. Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies. Proc Natl Acad Sci USA 2011;108(27):11187–92.

[11] Gomez N, Wieczorek A, Lu F, Bruno R, Diaz L, Agrawal NJ, Daris K. Culture temperature modulates half antibody and aggregate formation in a Chinese hamster ovary cell line expressing a bispecific antibody. Biotechnol Bioeng 2018;115(12):2930–40.
[12] Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, Carter P. An efficient route to human bispecific IgG. Nat Biotechnol 1998;16(7):677–81.
[13] Carter P. Bispecific human IgG by design. J Immunol Methods 2001;248(1–2):7–15.
[14] Zhang HM, Li C, Lei M, Lundin V, Lee HY, Ninonuevo M, Lin K, Han G, Sandoval W, Lei D1, Ren G1, Zhang J, Liu H. Structural and functional characterization of a hole-hole homodimer variant in a "knob-into-hole" bispecific antibody. Anal Chem 2017;89(24):13494–501.
[15] Gunasekaran K, Pentony M, Shen M, Garrett L, Forte C, Woodward A, Ng SB, Born T, Retter M, Manchulenko K, Sweet H, Foltz IN, Wittekind M, Yan W. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem 2010;285(25):19637–46.
[16] De Nardis C, Hendriks LJA, Poirier E, Arvinte T, Gros P, Bakker ABH, de Kruif J. A new approach for generating bispecific antibodies based on a common light chain format and the stable architecture of human immunoglobulin G1. J Biol Chem 2017;292(35):14706–17.
[17] Xu J, Wang Z, Li J. Novel bispecific polypeptide complexes. World intellectual property organizati on: International Patent Application Publication WO, 2019/057122 A1.
[18] Seiyaku C, Kaisha K. Methods of modifying antibodies for purification of bispecific antibodies: United States patent application publication US, 2009/0263392 A1.
[19] Sampei Z, Igawa T, Soeda T, Okuyama-Nishida Y, Moriyama C, Wakabayashi T, Tanaka E, Muto A, Kojima T, Kitazawa T, Yoshihashi K, Harada A, Funaki M, Haraya K, Tachibana T, Suzuki S, Esaki K, Nabuchi Y, Hattori K. Identification and multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VIII cofactor activity. PloS One 2013;8(2). e57479.
[20] Bernett MJ, Moore G, Desjarlais J, Chu S, Rashid R, Muchhal U. Heterodimeric antibodies that bind cd3 and cd38: United States patent application publication US, 2016/0215063 A1.
[21] Moore GL, Bernett MJ, Rashid R, Pong EW, Nguyen DT, Jacinto J, Eivazi A, Nisthal A, Diaz JE, Chu SY, Muchhal US, Desjarlais JR. A robust heterodimeric Fc platform engineered for efficient development of bispecific antibodies of multiple formats. Methods 2019;154:38–50.
[22] Sharkey B, Pudi S, Wallace Moyer I, Zhong L, Prinz B, Baruah H, Lynaugh H, Kumar S, Wittrup KD, Nett JH. Purification of common light chain IgG-like bispecific antibodies using highly linear pH gradients. mAbs 2017;9(2):257–68.
[23] Tustian AD, Endicott C, Adams B, Mattila J, Bak H. Development of purification processes for fully human bispecific antibodies based upon modification of protein A binding avidity. mAbs 2016;8(4):828–38.
[24] Skegro D, Stutz C, Ollier R, Svensson E, Wassmann P, Bourquin F, Monney T, Gn S, Blein S. Immunoglobulin domain interface exchange as a platform technology for the generation of Fc heterodimers and bispecific antibodies. J Biol Chem 2017;292(23):9745–59.
[25] Fischer N, Elson G, Magistrelli G, Dheilly E, Fouque N, Laurendon A, Gueneau F, Ravn U, Depoisier JF, Moine V, Raimondi S, Malinge P, Di Grazia L, Rousseau F, Poitevin Y, Calloud S, Cayatte PA, Alcoz M, Pontini G, Fagète S, Broyer L, Corbier M, Schrag D, Didelot G, Bosson N, Costes N, Cons L, Buatois V, Johnson Z, Ferlin W, Masternak K, Kosco-Vilbois M. Exploiting light chains for the scalable generation and platform purification of native human bispecific IgG. Nat Commun 2015;6:6113.
[26] Moore G, Bernett M, Rashid R, Desjarlais J. Novel heterodimeric proteins. United States patent application publication US 2014/0294835 A1.
[27] GE Healthcare Life Sciences. A platform approach for the purification of antibody fragments (Fabs). 2013. Application note, 29-0655-41, AA.
[28] Rodrigo G, Gruvegård M, Van Alstine JM. Antibody fragments and their purification by protein L affinity chromatography. Antibodies 2015;4:259–77.
[29] Cooke HA, Arndt J, Quan C, Shapiro RI, Wen D, Foley S, Vecchi MM, Preyer M. EFab domain substitution as a solution to the light-chain pairing problem of bispecific antibodies. mAbs 2018;10(8):1248–59.
[30] Bertl S, Duerr H, Schaubmar A. Separation of bispecific antibodies and bispecific antibody production side products using hydroxyapatite chromatography: United States patent application publication US 2016/0376304 A1.

[31] Gagnon P. Improved antibody aggregate removal by hydroxyapatite chromatography in the presence of polyethylene glycol. J Immunol Methods 2008;336(2):222–8.
[32] Gagnon P, Beam K. Antibody aggregate removal by hydroxyapatite chromatography. Curr Pharmaceut Biotechnol 2009;10(4):440–6.
[33] Michaelson JS, Demarest SJ, Miller B, Amatucci A, Snyder WB, Wu X, Huang F, Phan S, Gao S, Doern A, Farrington GK, Lugovskoy A, Joseph I, Bailly V, Wang X, Garber E, Browning J, Glaser SM. Anti-tumor activity of stability-engineered IgG-like bispecific antibodies targeting TRAIL-R2 and LTbetaR. mAbs 2009;1(2):128–41.
[34] Schanzer J, Jekle A, Nezu J, Lochner A, Croasdale R, Dioszegi M, Zhang J, Hoffmann E, Dormeyer W, Stracke J, Schäfer W, Ji C, Heilek G, Cammack N, Brandt M, Umana P, Brinkmann U. Development of tetravalent, bispecific CCR5 antibodies with antiviral activity against CCR5 monoclonal antibody-resistant HIV-1 strains. Antimicrob Agents Chemother 2011;55(5):2369–78.
[35] Luo Y, Raso SW, Gallant J, Steinmeyer C, Mabuchi Y, Lu Z, Entrican C, Rouse JC. Evidence for intermolecular domain exchange in the Fab domains of dimer and oligomers of an IgG1 monoclonal antibody. mAbs 2017;9(6):916–26.
[36] Zhang Y, Wang Y, Li Y. A method for improving protein A chromatography's aggregate removal capability. Protein Expr Purif 2019;158:65–73.
[37] McCue JT, Engel P, Ng A, Macniven R, Thömmes J. Modeling of protein monomer/aggregate purification and separation using hydrophobic interaction chromatography. Bioproc Biosyst Eng 2008;31(3):261–75.
[38] Lu Y, Williamson B, Gillespie R. Recent advancement in application of hydrophobic interaction chromatography for aggregate removal in industrial purification process. Curr Pharmaceut Biotechnol 2009;10(4):427–33.
[39] Zhang X, Chen T, Li Y. A parallel demonstration of different resins' antibody aggregate removing capability by a case study. Protein Expr Purif 2019;153:59–69.
[40] GE Healthcare Life Sciences. Polishing of monoclonal antibodies using Capto™ MMC ImpRes in bind and elute mode. 2013. Application note, 29-0373-49, AA.
[41] GE Healthcare Life Sciences. Capto™ MMC ImpRes. 2015. Data file, 29-0356-74, AB.
[42] GE Healthcare Life Sciences. Selective removal of aggregates with Capto adhere. 2007. Application note, 28-9078-93, AA.
[43] Gao D, Wang LL, Lin DQ, Yao SJ. Evaluating antibody monomer separation from associated aggregates using mixed-mode chromatography. J Chromatogr A 2013;1294:70–5.
[44] Gagnon P, Ng P, Aberrin C, Zhen J, He J, Mekosh H, Cummings L, Richieri R, Zaidi S. A ceramic hydroxyapatite based purification platform: simultaneous removal of leached protein A, aggregates, DNA, and endotoxins. BioProcess Int 2006;4(2):50–60.
[45] Arakawa T, Kita Y, Ejima D, Gagnon P. Solvent modulation of column chromatography. Protein Pept Lett 2008;15(6):544–55.
[46] Yigzaw Y, Hinckley P, Hewig A, Vedantham G. Ion exchange chromatography of proteins and clearance of aggregates. Curr Pharmaceut Biotechnol 2009;10(4):421–6.
[47] Suda EJ, Thomas KE, Pabst TM, Mensah P, Ramasubramanyan N, Gustafson ME, Hunter AK. Comparison of agarose and dextran-grafted agarose strong ion exchangers for the separation of protein aggregates. J Chromatogr A 2009;1216(27):5256–64.
[48] Xu Z, Li J, Zhou JX. Process development for robust removal of aggregates using cation exchange chromatography in monoclonal antibody purification with implementation of quality by design. Prep Biochem Biotechnol 2012;42(2):183–202.
[49] Kluters S, Wittkopp F, Jöhnck M, Frech C. Application of linear pH gradients for the modeling of ion exchange chromatography: separation of monoclonal antibody monomer from aggregates. J Separ Sci 2016;39(4):663–75.
[50] Hall T, Wilson JJ, Brownlee TJ, Swartling JR, Langan SE, Lambooy PK. Alkaline cation-exchange chromatography for the reduction of aggregate and a mis-formed disulfide variant in a bispecific antibody purification process. J Chromatogr B 2015;975:1–8.
[51] Ho SC, Koh EY, van Beers M, Mueller M, Wan C, Teo G, Song Z, Tong YW, Bardor M, Yang Y. Control of IgG LC:HC ratio in stably transfected CHO cells and study of the impact on expression, aggregation, glycosylation and conformational stability. J Biotechnol 2013;165(3–4):157–66.

CHAPTER

9

High-throughput, parallelized and automated protein purification for therapeutic antibody development

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List of abbreviations

ATPS aqueous two phase system
CE-SDS capillary electrophoresis, sodium dodecyl sulfate
CIP clean in place
DBC dynamic binding capacity
DoE Design of Experiments
HCP host cell protein
HIC hydrophobic interaction chromatography
IEX ion-exchange chromatography
LH liquid handler robotics platform
LC-MS liquid chromatography, mass spectrometry
mAb monoclonal antibody
PD process development
PEG polyethylene glycol
SEC size-exclusion chromatography
SPR surface plasmon resonance
UPLC-SEC ultra performance liquid chromatography, size exclusion chromatography

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Introduction

Downstream processing continues to be challenged by the increasing complexity and higher quantities of product resulting from upstream processes, with DSP often described as a bottleneck in the production of therapeutic antibodies. Early stage antibody therapeutic development has different challenges than does the later stages. Initially, there are often many constructs that require production and purification at small to intermediate milligram scale in order to evaluate product attributes and identify manufacturing liabilities via panels of *in vitro* and *in vivo* assays. Various levels of product purity may be required, depending on the kinds of questions to be addressed. Speed, cost and simplicity of operation, where possible, are key factors to be considered in the resulting purification workflows. While parallelization of purification can be readily achieved at microgram and low milligram scale using a variety of liquid handling automation equipment, purification automation and parallelization becomes progressively more challenging as antibody production volumes and product quantity for capture increase. Both commercial equipment and a variety of adapted "in-house" solutions have been described to try to overcome this problem. Here, a survey of chromatographic and non-chromatographic purification approaches are presented in the context of automation, parallelization and their potential for high sample throughput. The survey is by no means exhaustive and the interested reader is referred to previous reviews of this subject area [1–4] for additional perspectives.

Small-scale equipment and strategies

Liquid handling platforms

Scaled-down purification strategies that can handle large numbers of constructs from small volume productions in minimal time are crucial in early-stage discovery projects. The scale-down approach offers the possibility of minimizing volumes for production using transient transfection in mammalian cells, as well as reducing the burden on analytical and characterization studies by optimizing high-throughput analytical methods to analyze many samples in parallel. The ability to parallelize and automate also reduces costs and provides sufficient opportunity to sample the required design space.

A variety of small scale platform and process development related purification methods have been developed using various liquid handling (LH) robotics equipment from a variety of commercial vendors. The most common methods employ the use of resin filled filtration plates that can be utilized in either vacuum filtration, positive pressure or centrifugal mode, magnetic beads to which an affinity resin is coupled to be used with a plate-compatible magnet, small pre-packed columns, OPUS® RoboColumns (Repligen Corp), through which liquids are applied using LH fixed tips, 24 or 96-well filter plates containing membranes immobilized with Protein A, Protein G or ion-exchange functionalities or chromatography resin-filled tips, a common product called PhyTips™ (Phynexus Inc.).

Resin-filled purification tips

Resin filled tips offer the advantage of being readily amenable to a variety of automation platforms. These tips are offered in a wide variety of sizes and different chemistries

depending on the purification application. Aspiration, washing and elution of purified products is achieved simply by controlled liquid movement into and out of the tip. The point has been made that the bidirectional liquid flow in such tips is not as found in conventional packed columns, where liquid flow is usually in one direction, and for this reason they are more similar to a batch purification process [5]. Automation of PhyTips™ in 12 channel format as a platform purification strategy has been described [6]. Using either custom packed 500 μL tips or commercially available PhyTips containing Protein A resin described in Ref [7], successfully developed HT antibody purification platforms capable of purification of up to 2000 antibodies per day from 1 mL cultures.

Magnetic beads

Para-magnetic beads, for example, cross-linked agarose, to which Protein A, Protein G, Protein A/G or Protein L are covalently attached are commercially available from a number of suppliers. Utilizing an appropriate magnetic base integrated with an automated LH platform, magnetic affinity purification resins can offer a fast way to purify small quantities of antibodies from large numbers of small volume samples. Immobilization of the beads by the magnetic base permits LH tips to aspirate liquids for washing and elution of products. An advantage of magnetic beads is the ability to potentially elute purified antibodies in small volumes, resulting in more concentrated samples for buffer exchange or downstream assays. Some considerations with magnetic beads as a potential automated purification option include their cost per unit of purified product, compatibility with standard CIP procedures for recycling of resin as well as the quantity of purified product required. This scale of purification can be particularly attractive for assays that require small quantities of purified protein, including LabChip microfluidic CE-SDS, SPR, UPLC-SEC and LC-MS.

An example of how magnetic protein A based purification has been described for small-scale purification of monoclonal antibodies secreted by yeast cell lines [8]. In these studies, mAbs from 96-well plate cultures were purified using 20 μL per well quantities of BioMag Protein A beads (Qiagen) using a Biomek NX LH platform. The resulting purified samples were subsequently analyzed by HT E-PAGE pre-cast electrophoresis system (Invitrogen). Combined with gel imaging analysis, such experiments can be used to rank-order clones for protein productivity.

New generations of immuno-magnetic beads are being developed from materials other than cross-linked agarose with new properties, allowing for faster antibody purification. Examples include either Protein A or Protein A/G covalently immobilized to magnetic SiO_2 microspheres [9] or Protein A coated and glutaraldhehyde-crosslinked Nickel particles [10]. Improved magnetic bead technologies along with reductions in costs will expand the scope of use for these affinity resins in automated antibody purification.

Plate-based membrane chemistries

A wide variety of membrane-based separations in purification have been described although mainly for larger-scale antibody purification, usually as an intermediate polishing step (reviewed in Ref. [11]). A major advantage of membranes compared to bead-based resins

is that mass-transfer is convection instead of diffusion limited, resulting in much faster binding kinetics at higher flow rates. Another advantage of the membrane-based format is the ability to elute products with a smaller volume of elution buffer, resulting in more concentrated samples prior to desalting or other post-purification treatments. Buffer consumption is also reduced due to the small membrane area. A potential limitation of such an approach is the dynamic binding capacity may be limited as the membrane surface area in a 96-well plate format will be small.

While the options for antibody affinity purification using membranes in plate format are limited, commercially available Protein A and Protein G immobilized in a 24 or 96-well membrane plate format recently have appeared, one product being Capturem™ plates (Takara Bio Inc.). A number of vendors supply 96-well or other plate formats with ion exchange or other membrane chemistries that can alternatively be used for high-throughput antibody purification. Examples include Mustang E and Mustang Q ion exchange chemistries in 96-well plate format (Pall Corp) as well as S- (cation exchange), Q- and Stick PA (anion exchange) and Phenyl (HIC) chemistries in Sartobind 96-well plate format (Sartorius).

Batch purification in plates

Batch purification methods can also be scaled down into different plate formats for execution using liquid handler systems. Clarification options include both centrifugation and vacuum driven filtration. The clarification could be performed off line or integrated as part of the automated purification workflow. Filtration plates can either be obtained commercially, such as Multi-Trap purification plates (GE Healthcare) or made in-house using chromatography resins and appropriate filtration plates. As with clarification, both vacuum-based filtration and centrifugation are options for binding, washing and elution of products. Positive pressure manifolds, used for example in solid-phase extraction, can offer more control of liquid flow rates compared to either centrifugation or vacuum based approaches, and are available for a number of LH systems from different suppliers.

An example of the utilization of resin filled filter plates for HT process mAb purification development has been described in Ref. [5]. In these studies, vacuum filtration was employed in the purification and protein quantitation achieved using 96-well UV plates to order binding capacities for different resins. A similar automated batch purification strategy was employed in Ref. [7] with mAb Select SuRe except that centrifugation was utilized for plate washing and product elution. In this study, the batch binding was performed in separate plates prior to transfer of antibody-loaded resin to filter plates for further processing using the LH.

Robo columns

Opus® Robo-columns (Repligen Corp) are small packed bed columns available in a range of bed volumes from 50 to 600 μL. Unlike resins in plate format, they represent a scale down model for a packed chromatography column. Columns are available either pre-packed or packed with user-defined resins. It is possible to perform step gradients and collect fractions from such columns if appropriate plate-shuttle hardware is available for the LH. These

columns have been demonstrated to run with TECAN EVO LH systems (Tecan Trading AG; [5]) as well as the JANUS liquid handler (Perkin Elmer; [12]). One study [5] utilized 600 μL bed volume Robo Columns POROS HS media for DBC studies and 200 μL bed volumes for product elution studies. In another study [12] work, 100 μL bed volume Robo Columns containing mAb Select SuRe for purification were coupled with a CentriPlate P96 buffer exchange column array (emp Biotech) or HT preparative scale HPLC-SEC. One advantage of HPLC SEC columns in this kind of application is the comparatively higher flow rates that are possible compared to soft gels (cross-linked agarose), resulting in faster cycle times.

Buffer exchange and protein concentration

Workflows for the small scale purification of intact mAbs as well as antibody fragments usually involve at least one step where the sample matrix needs to be modified (buffer exchange) or a sample is required at a higher concentration. It is sometimes difficult to find good options to achieve parallelized buffer exchange and concentration of protein samples, leading to a bottleneck in the workflow that results in additional manual effort, time and cost. Resin filled tips (gel filtration Phytips), OPUS Robo-Columns filled with gel filtration media and 96-well plates such as MultiTrap Sephadex G25 plates, (GE Healthcare) or Zeba spin desalting plates (Thermo Fisher Scientific) in 96-well format are all commercially available options. Selection of the correct product depends on sample application volume, the available LH equipment and how to optimally integrate buffer exchange into the overall purification workflow. While individual centrifugal or gravity based desalting columns are readily available from a number of suppliers, they normally require more manual effort than HT-compatible devices. Some solutions to this have been proposed, for example, integrating gravity desalting columns such as PD-10 or any number of other formats into racks such that an LH can apply samples and the resulting samples collected in blocks [13].

There are fewer options for parallelized concentration of samples prior to purification steps such as SEC. Ultrafiltration plates in 96 well format for small volume sample concentration with different molecular weight have been described, for example, Acroprep Advance filter plates (Pall Corp) or Multiscreen Ultracel-10 96-well plates (Millipore).

Mid-scale purification strategies

Batch methods

Many of the more recently developed parallelized, higher throughput protein purification approaches that utilize commercial equipment and chromatography consumables can be prohibitively expensive for academic and other non-industrial lab environments. There can also be some burden with regards to the availability of trained personnel to develop and execute some of these purification workflows. A relatively simple, if not elegant approach consists of batch based purification, a method which can be readily parallelized with minimal investment in materials. Disadvantages of this approach are that it is labor intensive and can result in variable purification performance, as the level of control in the method is more limited than for pump-based liquid handling.

In batch purification using affinity resins, the product capture step can be either coupled directly to the rest of the work flow or uncoupled. When coupled, clarified supernatant or lysate is applied directly to columns containing defined quantities of purification resin(s), allowed to pass through the resin and samples collected in tubes or blocks. Control of residence time is relatively difficult under gravity flow conditions, unless a multiport pump can be utilized, although it can be better controlled using a vacuum manifold so long as all samples in the manifold flow at a similar rate. A multi column plate adapter, MCPA, has been described and can be utilized for such an application under gravity or vacuum conditions [14].

In an uncoupled batch purification process, resin is first incubated with clarified harvest supernatant for some pre-determined period of time prior to moving the resin/supernatant mixture into columns of appropriate size, which can then be processed by gravity flow to collect the flow-thru. Column washes and product elution are all achieved by adding sequential buffers to the columns, either manually or with a pump. The uncoupled approach is more attractive if larger supernatant volumes are to be used, requiring less operator time, as well as alleviating the issues around residence time, since the product capture step has already occurred. Using appropriate CIP conditions, products having the required endotoxin levels can normally be successfully purified.

Automation options

Mid-scale purification, where purified product requirements are in the tens to hundreds of milligrams, represents one of the most challenging areas for parallelization and automation of protein purification. While custom built, specialized high-throughput and highly automated protein purification equipment, such as that found in the GNF protein expression and purification platform [15] does exist, it is expensive, time-consuming and requires a team with the right kinds of expertize and resources to bring it to life. Purification at this scale also often places more demands on the purification platform in order to deliver products with defined endotoxin levels for cell based and*in vivo* studies, as well with minimal protein aggregates. Ideally, such purification strategies are properly integrated with both upstream clarification technologies as well as analytical assays and sample and IT management in order to create a holistic antibody production platform.

Protein Maker

The Protein Maker (Protein BioSolutions) is a liquid handling platform using syringe pumps, originally developed and sold by Emerald BioSystems for high-throughput, mid-scale protein purification [16]. The current version of the instrument consists of 24 syringe pumps, each having an independent flow path consisting of sample inlet tubing, a nine port switching valve, a mixing syringe at position 5 of the valve (for forming step gradients, if desired) and outlet tubing leading to the column manifold. Buffers and cleaning solutions are delivered from bottles via the syringe pumps and tubing for each flow path to the multiport valve. Chromatography columns of 1 or 5 mL bed volume are most commonly employed, although other columns can be connected singly or in series, depending on the available space and required flow rate. There are a total of 20 deck positions, including one for waste, in which SBS-formatted plates or blocks can be located. Plates having 24, 48 or 96 wells can potentially be utilized for sample collection, depending on how column outlets are organized on the gantry.

Racks located at either the ends or top of the instrument secure samples of various volumes as well as buffer, cleaning and sanitization solutions. Typically, clarified supernatant volumes between 10 or 20 mL and 1 L are applied per flow path. If required, larger volume or higher productivity clarified harvests can be split over multiple columns and eluted products subsequently pooled together. An accessory available for the Protein Maker is a 24-channel, A280 nm absorbance manifold assembly, allowing chromatograms to be recorded and stored for each of the individual flow paths. It is also possible to cool the deck using a circulating cooling bath containing an appropriate solvent system.

In addition to single step column purification, the instrument can also perform automated two-step protein purification of up to 12 samples in parallel by aspirating samples from a plate located on the deck. In this configuration, two sets of 12 columns, for example, 12 Protein A columns and 12 desalting columns, would be connected to gantry A and gantry B, respectively. Products eluted from Protein A into an elution plate would be aspirated and passed over desalting columns located in gantry B, with the final eluted samples collected into a separate receiving plate.

A large variety of therapeutic antibody research and development studies can be executed using the parallelized purification capability of the Protein Maker, with a few of these recently summarized in Ref. [13]. In addition to platform purification of antibodies or antibody fragments at various scales, using a variety of affinity chromatography columns, process development applications, including resin screening studies, Protein A column washing studies to reduce HCPs, or clarified harvest hold stability studies to define optimal hold time and temperature, can be performed. The Protein Maker can also be readily integrated with other platform purification equipment depending on the overall workflow requirements, for example, utilization in combination with a Tecan EVO liquid handler for high-throughput gravity-based buffer exchange of purified samples [13] or utilization of semi-automated size exclusion chromatography using FPLC's. In-process analytical assays including SEC and CE-SDS to analyze product attributes can also be performed.

Modified FPLC and HPLC systems

The general lack in availability, with few exceptions, of commercial higher-throughput, automated protein purification solutions for mid-scale (10−1000 mg) purification has resulted in a number of ingenious adaptations to FPLC and HPLC equipment. In many cases, this involves integration of FPLC's, pumps and switching valves with software and appropriate autosampler equipment. Addition of a standard sample pump, injection valve and sample inlet valve with air sensor can permit automated single-step purification of up to 14 productions of any volume, depending on the instrument and configuration selected. The challenges then become temperature control of supernatant feeds and potentially fractionation capacity for the eluted products.

Commercially available autosamplers, including those from GE/Spark-Holland (ALIAS Bio Cool & Bio-Prep Cool) and Teledyne CETAC (ASX-560) can be readily connected to GE ÄKTA or Bio-Rad NGC systems, as examples, to help improve purification sample throughput. Volumetric dispensers under high pressure, such as the HPD (Spark Holland) offer the possibility of applying larger clarified production volumes to chromatography

columns at flow rates of up to 10 mL/min. Using the HPD system, up to 24 larger volume samples (six samples per unit, utilizing four units) could be automatically loaded onto columns sequentially and purified. As with autosamplers, these and related devices can be integrated with FPLC systems using the appropriate control software and Input/output connections.

For smaller numbers of samples (five or fewer), commercial GE (ÄKTA Pure; [17]) or Bio-Rad (NGC Discover; [18]) instruments can perform automated two or three column purification steps utilizing an additional loop valve and tubing to temporarily store intermediate samples in the purification workflow. A recent strategy utilizing modified ÄKTA PURE or NGC systems to perform an automated sequential Protein A, buffer exchange and SEC workflow on up to seven samples at 500 mg scale has been described [19]. An alternative approach, using legacy equipment such as the ÄKTA Explorer, is to utilize the outlet valve and sample inlet valve position in combination with the sample pump to sequentially reapply eluted samples from one purification step to subsequent columns [20]. Generally, the most common platform purification workflows would include affinity purification using Protein A chromatography followed by buffer exchange or SEC. The introduction of other chromatographic methods, such as HIC, IEX or mixed-mode chromatography would first require development of the appropriate binding, washing and elution conditions suitable for a given product.

The overall approaches possible with newer, off-the-shelf commercial instruments represent improvements to that utilized in the legacy ÄKTAxpress system (GE), where each individual instrument could purify up to 4 samples per day, depending on the type of purification used. Increased throughput is achieved through combining ÄKTAxpress units, with up to 12 modules controlled by one computer. A number of groups have reported successful automated, multi-step purification at multi-milligram scale using these instruments, often for structural studies [21–24,59].

A relatively early effort was made by a group at Amgen to build an integrated HT purification platform utilizing a modified ÄKTA Purifier, and a commercially available CETAC ASX-520 autosampler [25]. This system was named the ÄKTA-AS. A significant challenge at the time was establishing communication between the autosampler and the FPLC system. Modifications to the ÄKTA Purifier were also required, including the addition of valves to direct flow and to be able to move liquid from a waste valve port onto a second column in two-step purification mode. Using this system, samples are served by the autosampler to a 1 mL Protein A affinity column followed by buffer exchange using a 5 mL HiTrap desalting column. Fractionation is achieved in 96-well blocks to facilitate HT analytical experiments. Depending on the sample volumes, the system has a throughput of up to 240 samples (with a maximum volume of 14 mL) or 84 samples (with a maximum volume of 50 mL). Variable sample loading is controlled through integration of an air sensor within the flow path, which is used to stop a pump.

A further modified ÄKTA Purifier or Explorer system has been described, this time the purpose being automated two-step purification of antibodies where the second chromatographic step requires conditioning of the elution from the first step [26]. This second purification step could be one of a variety of chemistries, although in this specific study, cation exchange in bind and elute mode was selected to remove antibody aggregates post Protein A. The elution conditioning for the second purification step is achieved by in-line dilution,

utilizing a static mixer. In this setup, either 5 or 10 mL bed volume Protein A and IEX columns can be integrated to perform two-step purification of up to five samples without manual intervention.

Utilizing Agilent HPLC components an automated, preparative purification system capable of executing consecutive Protein A and SEC chromatography on up to 48 samples at 35 mL production scale has been developed [27]. Using a 10-port valve and two quaternary pumps permitted two SEC columns to run in parallel, with a third quaternary pump dedicated to Protein A purification. As opposed to the normally utilized cross-linked agarose sizing columns, a silica based TSKgel G3000SW column was instead utilized, decreasing the run time approximately 4-fold. Samples were loaded using a CETAC ASX-520 autosampler.

Normally in batch processes, clarification of the harvest is an off-line operation prior to product capture by chromatography. To overcome this challenge, an automated harvesting and two-step purification system using commercially-available components has been described [28]. This system makes use of a separate pump and disposable filtration units to achieve harvest clarification of up to eight, one liter productions. Two ÄKTA Pure 25 systems are connected to the clarification train such that each FPLC instrument can perform both affinity purification, in-line pH adjustment of the eluted product as well as SEC in a continuous manner. The system has been demonstrated to yield products having low endotoxin levels. Using this equipment, the entire process can applied to 8 productions in less than 24 h.

It is also possible to utilize less traditional equipment in order to create an HT purification instrument. An example of this is the development of a positive pressure device for affinity purification by adapting a Gilson ASPEX solid phase extraction system [29]. As configured, this instrument could process up to 60 samples in 18.5 h. While this particular study made use of His-tagged proteins, adaptation to antibody affinity purification would be straightforward. Using a modified Gilson solid phase extraction system with switching valves a 4 channel automated antibody purification instrument, capable of purifying up to 24 samples has been developed [30]. The setup is can also perform two step purification, consisting of Protein A purification and buffer exchange using PD-10 desalting columns in gravity flow mode.

A number of vendors sell Protein A, Protein G, Protein A/G or Protein L spin columns, a convenient format for lab-scale, centrifugation-based antibody purification. Depending on the resin volume of the particular spin column, it is possible to purify several milligrams of product per column. In principle, one could imagine automation of spin columns by building an appropriate column holder, collection reservoir and integration with an automatable centrifuge. There seem to be few examples of commercial solutions to automation of spin columns, one being the QIAcube system (Qiagen), although this appears to be limited to IMAC purification methods [31].

Magnetic resin based separations

Paramagnetic beads to which Protein A or other affinity ligands are covalently attached offer an attractive alternative to traditional resins for small and mid-scale antibody affinity capture and have even been evaluated even at pilot scale [32,33]. A paramagnetic Protein A resin, LOABeads Alkali A, have tolerance to sodium hydroxide for cleaning and are

colored black for easy visibility (Lab on a Bead AB). A particularly attractive feature of these resins is the ability to effectively uncouple the clarification and purification steps, by introducing magnetic beads directly into mammalian culture supernatants to bind secreted products prior to clarification. The cost, however, of these resins can represent a significant investment, especially if used in a single use workflow. Using strong rare earth magnets, it is possible to bind the beads and separate these from cells, permitting rapid isolation of the antibody. Alkali resistant (0.1−0.5 M NaOH) magnetic Protein A (AmMag™) beads have been developed by GenScript, as well as a semi-automated purification system, AmMag®SA. This instrument is able to process up to 12 samples at 5−50 mL production volume concurrently.

A different instrument, the KingFisher system, available in different formats, has also been developed (ThermoFisher Scientific). These instruments can process between 6 and 96 samples to a maximum sample volume of 50 mL, depending on the instrument model and quantity or volume of product to be purified. This instrument contains rods that consecutively transfer and release magnetic affinity resin beads, moving them through binding, washing and elution buffers to effect purification. Protocols that demonstrate applications with either Protein A or Protein G paramagnetic beads have been described.

Membrane-based and monolith devices

As with 96-well membrane plate formats, small membrane capsule devices are commercially available for Protein A affinity purification. Both 1 and 3.5 mL GORE™ protein capture devices for antibody purification are available (W.L. Gore & Associates Inc.). These devices are capable of purification of tens of milligrams of products and are amenable to repeated CIP using 0.1 M NaOH as a cleaning agent. With the lure lock connectors, several units could be utilized with an appropriate column valve and integrated autosampler on an FPLC platform, or alternatively, replace packed beds on an instrument such as a the Protein Maker. A distinct advantage over small packed beds would be the higher flow rates, resulting in comparable quantities of purified products in less time.

Another device is the Sartobind Protein A membrane absorber (Sartorius stedim biotech GmbH) available in a 2 mL format. This device can also be connected via lure lock connectors to FPLC, peristaltic pump or related equipment using flow rates of 5−10 mL/min. As with other Protein A formats binding capacity varies depending on IgG isotype and species. The protein A ligand utilized in these devices is partially stabile under 0.05 M NaOH CIP conditions.

As with 96-well format plates, a number of small (1−5 mL) capsule devices are available in anion exchange, cation exchange and HIC membrane chemistries from various suppliers including Pall and Sartorius, which can be utilized in a similar manner as with small chromatography columns. Depending on the application, these devices can be utilized in either bind-and-elute or flow-thru mode.

Monolith columns having large flow channels share the high mass transfer properties under high flow rates of membrane based devices but with the potential of larger binding capacity. Protein A, Protein G and Protein L devices are available in 1 mL format and larger sizes as well as in a variety of other chemistries (BIA Separations). These devices also circumvent the need for column packing and offer a lower shear environment, due to the large channels, for large molecules. Like other devices, these monoliths can be connected to a variety of

FPLC or related instruments. The application of similar devices for the isolation of immunoglobulins from serum as part of a high-throughput purification workflow has been described [34].

Applications to HT process development

Establishing purification unit operations as part of developing an appropriate downstream purification process remains a significant challenge, facing multiple pressures relating to minimizing time and process costs, achieving required final drug substance/drug product attributes, operate within practical equipment feasibility limits and remain both scalable and technically robust within a cGMP environment. The demands are further exacerbated with complex therapeutic antibody modalities, which may possess inherent design-related liabilities and limitations that have not yet been fully explored nor are well understood until downstream process development is well underway. Inevitably, the time-line for downstream process development shrinks, compacted by continued upstream process development changes on one side and the demands for large quantities of purified product for analytical assay development, product characterization, formulation and pre-clinical*in vivo* studies on the other. High-throughput purification tools combined with statistical methods including Design of Experiment can, in some instances, increase process development efficiency and reduce overall time and effort. Several reviews that cover various aspects of HT downstream process development of therapeutic molecules including mAbs have been published previously [1,35,36,60].

Commercial suppliers related to protein purification products provide a number of HT purification consumables that can be adapted into antibody product process development studies. One of these, OPUS® Robo-columns (Repligen Corp), integrated with an appropriate LH system capable of collecting fractions, have been utilized in a number of studies, including determination of adsorption isotherms [37], column washing and elution studies [38], dynamic binding capacity studies [12] and yield and purity in both bind and elute as well as flow through mode [39]. More detailed descriptions of Robo-column operation on the TECAN EVO [40] or JANUS LH platforms [12,41] are available for the interested reader.

While the miniature column formats do offer benefits, including purification parallelization, reduced requirements for feedstocks and an increased number of automated purification experiments per unit time, there are also limitations. Some of these are related to the nature of the columns themselves. The small bed height as well as diameter mean a reduced number of theoretical plates in the packed bed compared to a longer column, impacting separation resolution, as well as increased wall effects [5]. These would be important considerations in polishing steps. An additional limitation, apt for any small-scale purification method, is the availability of an appropriate set of analytical methods with the corresponding performance characteristics to provide meaningful data for the samples generated [38,41]. Integration of an LH platform capable of multiple modes of HT purification in various formats (columns, tips, plates) with analytical methods including protein quantitation by absorbance and microfluidic electrophoresis (LabChip) has been described as one possible solution to this challenge [41].

An alternative to commercial pre-packed miniature columns is the packing of slightly larger columns (e.g. 2 mL bed volume) of more representative bed height, whose performance may be more predictive to that of lab-scale and larger columns while also allowing for larger product loads [42]. This approach may offer an acceptable compromise between the limitations of miniature packed beds and the increased sample requirements and longer run times of larger lab-scale columns. While these studies were performed using an ÄKTA Explorer 100 system for resin and chromatography condition screening, greater performance could be obtained today utilizing a similar approach with more modern FPLC instrumentation integrated with an auto-sampler and fractionation capability in 96-well plates.

In addition to use of small chromatography columns, batch binding studies in 96-well plate format are often performed as part of antibody purification process development. Commercially available filter plates pre-filled with resin, such as Predictor plates (GE Life Science), Resin seeker plates (TOSOH Bioscience) or Foresight™ pre-packed plates (Bio-Rad Laboratories) are amenable to automation using LH instruments and can be used for chromatographic media scouting, batch uptake studies to determine binding capacity, determine adsorption isotherms under various conditions, understand resin performance with respect to CIP cycles as well as screen resin washing and elution conditions. Filter plates can also be filled in-house with bulk resins from a defined slurry, although there are a number of important considerations when preparing such plates, including the type of filter plate used, agitation conditions for the resin and a quantitative method to dispense resins [43]. Other kinds of studies, such as HCP washing experiments with various buffers [44], and estimation of dynamic binding capacity from uptake studies [45] can also be performed in a similar filter plate format.

Non-chromatographic HT purification approaches

While affinity chromatography, particularly the use of Protein A, has dominated antibody purification for some time, there has been increasing interest in the potential of non-chromatographic approaches, both technically and from a cost of goods perspective. While not yet necessarily adopted, non-chromatographic purification unit operations, especially as part of primary clarification prior to product capture, are applied as required for purification of some products. These steps are often combined with chromatographic steps in designing the overall downstream purification process. Examples of these approaches include large scale crystallization, aqueous two phase extraction, precipitation and polymer-mediated flocculation. Some of these approaches are amenable to high-throughput screening in combination with the appropriate assays to read out on purification performance.

Batch crystallization

Crystallization of antibodies and antibody fragments has been explored as a purification step at both smaller and larger scale as well as a formulation method to increase product stability and even as a subcutaneous delivery format (reviewed in Ref. [46]). While small-scale

crystallization screening experiments can be performed manually, these are in many cases readily amendable to high-throughput robotics, either using traditional liquid handlers or specialized protein crystallization automation equipment. Microbatch under oil screening experiments [47–49] and scaled up (5 mL–1 L scale) stirred tank crystallizers [48,49] can both allow for parallelization of batch crystallization studies.

Precipitation

In addition to using salts such as ammonium sulfate, reversible precipitation of antibodies and related products as a potential purification strategy has been described using ethanol [50], various anionic polyelectrolytes [61], cationic polyelectrolytes [51], polyamines [52] or different molecular weight polyethylene glycols [53–55]. High-throughput experimental methods can be adopted to screen precipitation conditions in plates, including the generation of solubility curves, as well as determine re-solubilization conditions for the precipitated product. Using a TECAN EVO platform a series of polyethylene glycol molecular weights and two pH values were screened with a series of mAbs in filter plates with the protein recovery determined based on A280 nm measurements has ben performed [55]. A related study used a series of linear and branched polyamines at various concentrations to effect precipitation of a CHO produced antibody [52]. In this work, various conditioning of the antibody feed was used in order to evaluate removal of host cell proteins and recovery of product. In these experiments 96-well plates were used for precipitation studies with corresponding filter plates for removing precipitated protein. Utilizing the clarified culture supernatant containing an IgG4 mAb a high-throughput PEG-based precipitation screening system was developed [53]. Using this system, the clarified harvest conditions, including product concentration and pH, as well as optimal PEG molecular weight and concentration leading to the best IgG4 purity and recovery could be readily determined.

Finally, flocculation of CHO produced clarified harvests using polyethylenimine (PEI) or other polymers, such as benzylated poly(allylamine) or SmartPolymer (SmP) can be utilized to reduce host impurities at the clarification step and be combined with filtration to improve subsequent downstream purification processes [56]. As polymer concentration and feed conditions need to be optimized in order to optimize residual clearance and product recovery, high-throughput screening techniques can be brought to bear on the early stages of flocculation clarification development.

Aqueous two-phase systems

Aqueous two-phase processing (ATPS) for protein purification consists of utilizing mixtures of polymer-salt (e.g. phosphate and polyethylene glycol) or polymer-polymer (e.g. dextran and polyethylene glycol) such that the monoclonal antibody product of interest will partition preferentially into one of the aqueous phases, resulting in some degree of purification (reviewed in Ref. [57]). Newer generations of compounds designed for this application, such as self-buffering ionic liquids, hold promise for wider applicability of this method for antibody purification in the future [58].

Using an automated liquid handling system, a high-throughput screening method was developed for ATPS analysis of PEG-salt mixtures as applied to two monoclonal antibodies [62]. Miniaturization of ATPS screening conditions results in reduced requirements for material and the potential for the application of a DoE-based approach to screening as well as appropriate sample replicates for statistical analysis of the data.

Summary and conclusions

An effort has been made here to provide a brief synopsis of the current state of parallelized, automated and high-throughput approaches to antibody purification. Balancing the needs for platform and process development purification applications, as well as appreciating the competition between sample throughput and purified product quantity, does present challenges (Fig. 9.1). While there are no universal solutions, there is often more than one acceptable technical solution at a given purification scale, depending on the specific needs of the user and the equipment available (Table 9.1).

While many off-the-shelf options exist when small quantities of purified protein are required, fewer commercial instruments capable of parallelized purification are available for mid-scale platform purification. More commercial equipment options for mid-scale, parallelized protein purification are required in order to advance the technological capabilities in this area. This deficiency has been tackled by downstream and automation scientists in biotech and biopharma to come up with hybrid solutions using commercial FPLC equipment combined with various components to solve this problem. Integration of FPLCs with auto samplers and other pumping equipment will provide more options for automated, sequential purification at sub-gram purification scale. We can expect to see greater adoption of membrane and monolith chromatography in platform mode, given the advantage of greater mass transfer at higher flow rates and the new products released by vendors. On the process development side, unit operations involving non-chromatographic based separations offer the potential of lower cost, scalable downstream processes that can be integrated with chromatography in a holistic manner. As always, protein purification does not exist in isolation,

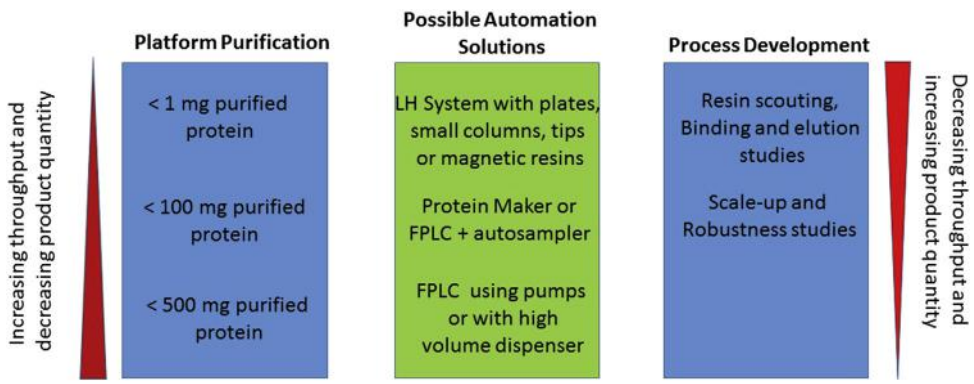

FIG. 9.1 Potential automation solutions for protein purification in both platform and process development modes.

TABLE 9.1 Automation and parallelized antibody purification examples at various scales.

Equipment	Scale	Max Samples/day	Application	Comments
LH + 96 well resin filled plates	μg to low mg	Several hundred	Platform & PD	Vacuum, centrifugation or positive pressure
LH + Robo columns	μg to low mg	96+	Mainly for PD	Selected LH systems. Sample throughput depends on column bed volume and sample volume(s) applied
LH + magnetic resins	μg to low mg	Several hundred	Mainly platform	Difficult to scale due to cost
LH + resin filled tips	μg to low mg	Several hundred	Platform & PD	
Protein Maker	<100 mg	12 to 24 or 48 depending on clarified harvest volume and method used	Platform & PD	Larger supernatant volumes can be divided over additional 5 mL columns. Max number of samples is 12 for two-step purification methods
Batch using columns	<100 mg	~10 per person	Platform	Manual unless peristaltic pump is used. Minimal control of liquid flow rate without a pump
ÄKTAxpress	<100 mg	~4 (2-step method)	Platform	Instrument discontinued by GE, legacy platform
FPLC with sample pump	Low mg to < 1 g	Depends on clarified harvest volume & productivity	Platform & PD	Variable number of samples possible depending on number of sample inlets. In-line air sensor desirable
FPLC with autosampler	<100 mg	Depends on clarified harvest volume, productivity and auto sampler	Platform & PD	Alternative to sample pump for small volume samples, depending on specific auto sampler
Customized FPLC	<500 mg	Depends on clarified harvest volume, productivity and equipment specifics	Mainly platform	Case-by-case setups depending on specific needs and availability of equipment and components

and benefits the most when automation and parallelization are integrated on both the upstream and analytical sides in combination with robust data management and sample tracking capabilities.

References

[1] Chhatre S, Titchener-Hooker NJ. Review: microscale methods for high-throughput chromatography development in the pharmaceutical industry. J Chem Technol Biotechnol 2009;84:927–40.
[2] Konczal J, Gray CH. Streamlining workflow and automation to accelerate laboratory scale protein production. Protein Expr Purif 2017;133:160–9.
[3] Massé F, Parat M, Matte A, Thauvette L, Helie G, Durocher Y, Vercauteren F. Parallelized protein purification: opportunities and challenges in early-stage biotherapeutics research and development. Am Pharmaceut Rev 2017;20(7):16–24.

[4] Rathore AS, Kumar D, Kateja N. Recent developments in chromatographic purification of biopharmaceuticals. Biotechnol Lett 2018;40:895–905.
[5] Welsh JP, Petroff MG, Rowicki P, Bao H, Linden T, Roush DJ, Pollard JM. A practical strategy for using miniature chromatography columns in a standardized high-throughput workflow for purification development of monoclonal antibodies. Biotechnol Prog 2014;30:626–35.
[6] Hopkins R, Esposito D, Gillette W. Widening the bottleneck: increasing success in protein expression and purification. J Struct Biol 2010;172:14–20.
[7] Luan P, Lee S, Aena TA, Paluch M, Kansopon J, Viajar S, Begum Z, Chaing N, Nakamura G, Hass PE, Wong AW, Lazar GA, Gill A. Automated high throughput microscale antibody purification workflows for accelerating antibody discovery. mAbs 2018;10(4):624–35.
[8] Barnard GC, Kull AR, Sharkey NS, Shaikh SS, Rittenhour AM, Burnina I, Jiang Y, Li F, Lynaugh H, Mitchell T, Nett JH, Nylen A, Potgieter TI, Prinz B, Rios SE, Zha D, Sethuraman N, Stadheim TA, Bobrowicz P. High-throughput screening and selection of yeast cell lines expressing monoclonal antibodies. J Ind Microbiol Biotechnol 2010;37:961–71.
[9] Slimi K, Usta DD, Koçer I, Çelik E, Tuncel A. Protein A and protein A/G coupled magnetic SiO_2 microspheres for affinity purification of immunoglobulin G. Int J Biol Macromol 2018;111:178–85.
[10] Gao J, Li Z, Russell T, Li Z. Antibody affinity purification using metallic nickel particles. J Chromatogr B 2012;895–896:89–93.
[11] Orr V, Zhong L, Moo-Young M, Chou CP. Recent advances in bioprocessing applications of membrane chromatography. Biotech Adv 2013;31:450–65.
[12] Schmidt PM, Abdo M, Butcher RE, Yap M-Y, Scotney PD, Ramunno ML, Martin-Roussety G, Owczarek C, Hardy MP, Chen C-G, Fabri LJ. A robust robotic high-throughput antibody purification platform. J Chromatogr A 2016;1455:9–19.
[13] Hélie G, Parat M, Massé F, Gerdts CJ, Loisel TL, Matte A. Application of the Protein Maker as a platform purification system for therapeutic antibody research and development. Comp Struct Biotechnol 2016;14:238–44.
[14] Dominguez MJ, Lantz BJ, Rhode RJ, Sharp ZL, Finney KC, Martinez VJ, Stollar EJ. A multi-column plate adaptor provides an economical and versatile high-throughput protein purification system. Protein Expr Purif 2018;152:84–91.
[15] Rue SM, Anderson PW, Gaylord MR, Miller JJ, Glaser SM, Lesley SA. A high-throughput system for transient and stable protein production in mammalian cells. Methods Mol Biol 2019;2025:93–142.
[16] Smith ER, Begley DW, Anderson V, Raymond AC, Haffner TE, Robinson JI, Edwards TE, Duncan N, Gerdts CJ, Mixon MB, Nollert P, Staker BL, Stewart LJ. The Protein Maker: a system for high-throughput parallel purification. Acta Crystallogr 2011;F67:1015–21.
[17] Ferguson DE, Mahan ER, Ma W, Bitzas G, Zhong X, Zollner R, D'Antona AM. Parallel loading and complete automation of a 3-step mAb purification process for multiple samples using a customized preparative chromatography instrument with networked pumps. J Chromatogr A 2018;1542:50–60.
[18] Becker W, Scherer A, Faust C, Bauer DK, Scholtes S, Rao E, Hofmann J, Schauder R, Langer T. A fully automated three-step protein purification procedure for up to five samples using the NGC chromatography system. Protein Expr Purif 2019;153:1–6.
[19] Winters D, Tran M, Yoo D, Walker KW. Development of BioRad NGC and GE ÄKTA Pure systems for highly automated three column protein purification employing tandem affinity, buffer exchange and size exclusion chromatography. Protein Expr Purif 2020;165:105497.
[20] Camper DV, Viola RE. Fully automated protein purification. Anal Biochem 2009;393:176–81.
[21] Ludwig C, Wear MA, Walkinshaw MD. Streamlined, automated protocols for the production of milligram quantities of untagged human cyclophilin-A (hCypA) and untagged human profilerating cell nuclear antigen (hPCNA) using AKTAxpress™. Protein Expr Purif 2010;71:54–61.
[22] Mlynek G, Lehner A, Neuhold J, Leeb S, Kostan J, Charnagalov A, Stolt-Bergner P, Dijinović-Carugo K, Pinotsis N. The Center for optimized structural studies (COSS) platform for automation in cloning, expression and purification of single proteins and protein-protein comeplxes. Amino Acids 2014;46:1565–82.
[23] Nowicki MW, Blackburn EA, McNae IW, Wear MA. A streamlined automated protocol for the production of milligram quantities of untagged recombinant lactate dehydrogenase A using AKTAxpress™. PLoS One 2015. https://doi.org/10.1371/journal.pone.0146164.

[24] Kim Y, Babnigg G, Jedrzejczak R, Eschenfeldt WH, Li H, Maltseva N, Hatzos-Skintges C, Gu M, Makowska-Grzyska M, Wu R, An H, Chhor G, Joachimiak AJ. High-throughput protein purification and quality assessment for crystallization. Methods 2011;55:12–28.
[25] Yoo D, Provchy J, Park C, Schulz C, Walker K. Automated high-throughput protein purification using an AKTA purifier and a CETAC autosampler. J Chromatogr A 2014;1344:23–30.
[26] Winters D, Chu C, Walker K. Automated two-step chromatography using an ÄKTA equipped with in-line dilution capability. J Chromatogr A 2015;1424:51–8.
[27] Schmitz S, Schönfeld DL, Freitag B, Götzberger-Schad C, Fischer M, Linden L. Keeping pace with the increasing demand for high quality drug candidates in pharmaceutical research: development of a new two-step preparative tandem high performance chromatographic system for the purification of antibodies. J Chromatogr B 2019;1104:18–28.
[28] Holenstein F, Ericksson C, Erlandsson I, Norman N, Simon J, Danielsson Å, Milicov A, Schindler P, Schlaeppi J-M. Automated harvesting and 2-step purification of unclarified mammalian cell-culture broths containing antibodies. J Chromatogr A 2015;1**418**:103–9.
[29] Steen J, Uhlen M, Hober S, Ottosson J. High-throughput protein purification using an automated set-up for high-yield affinity chromatography. Protein Expr Purif 2006;46:173–8.
[30] Zhang C, Long AM, Swalm B, Charest K, Wang Y, Hu J, Schulz C, Goetzinger W, Hall BE. Development of an automated mid-scale parallel protein purification system for antibody purification and affinity chromatography. Protein Expr Purif 2016;128:29–35.
[31] McGraw J, Tatipelli VK, Feyijinmi O, Traore MC, Eangoor P, Lane S, Stollar EJ. A semi-automated method for purification of milligram quantities of proteins on the QIAcube. Protein Expr Purif 2014;96:48–53.
[32] Brechmann NA, Ericksson P-O, Ericksson K, Oscarsson S, Buijs J, Shokri A, Hjalm G, Chotteau V. Pilot-scale process for magnetic bead purification of antibodies directly from non-clarified CHO cell culture. Biotechnol Prog 2019;35(3).
[33] Ebeler M, Lind O, Norman N, Palmgren R, Franzreb M. One-step integrated clarification and purification of a monoclonal antibody using Protein A mag Sepharose beads and a cGMP-compliant magnetic separator. N Biotech 2018;42:48–55.
[34] Martinović T, Andjelkovic U, Klobučar M, Černlgoj U, Vldlč J, Lučić M, Pavellć K, Joslć D. Affinity chromatography on monolithic supports for simultaneous and high-throughput isolation of immunoglobulins from human serum. Electrophoresis 2017;38:2909–13.
[35] Bhambure R, Kumar K, Rathore AS. High-throughput process development for biopharmaceutical drug substances. Trends Biotechnol 2011;29:127–35.
[36] Chollangi S, Jaffe NE, Cai H, Bell A, Patel K, Fischl M, Russell RJ, Cheng K-C, Wang M-J. Accelerating purification process development of an early phase Mab with high-throughput automation. BioProcess Int (April) 2014:32–41.
[37] Pirrung SM, da Cruz DP, Hanke AT, Berends C, Van Beckhoven RFWC, Eppink MHM, Ottens M. Chromatographic parameter determination for complex feedstocks. Biotechnol Prog 2018;34:1006–18.
[38] Treier K, Hansen S, Richter C, Diederich P, Hubbard J. High-throughput methods for miniaturization and automation of monoclonal antibody purification processes. Biotechnol Prog 2012;28:723–32.
[39] Petroff MG, Bao H, Welsh JP, van Beuninen-de Vaan M, Pollard JM, Roush DJ, Kandula S, Machielsen P, Tugon N, Linden TO. High-throughput chromatography strategies for potential use in the formal process characterization of a monoclonal antibody. Biotech Bioeng 2016;113:1273–83.
[40] Konstantinidis S, Goh H-Y, Bufájer JMM, de Galbert P, Parau M, Velayudhan A. Flexible and accessible automated operation of miniature chromatography columns on a liquid handler station. Biotechnol J 2017. https://doi.org/10.1002/biot.201700390. e1700390.
[41] LeSaout X, Costioli M, Jordan L, Lambert J, Beighley R, Provencher L, Gerwe B, McGuire K, Verlinden N, Barry A. Automated small scale protein purification and analysis for accelerated development of protein therapeutics. Eng Life Sci 2016;16:179–84.
[42] Teeters M, Bezila D, Alred P, Velayudhan A. Development and application of an automated, low volume chromatography system for resin and condition screening. Biotechnol J 2008;3:1212–23.
[43] Li X, de Roo G, Burgers K, Ottens M, Eppink M. Self-packed filter plates: a good alternative for pre-packed filter plates for developing processes for therapeutic proteins. Biotechnol J 2012;7:1269–76.

[44] Sisodiya VN, Lequieu J, Rodriguez M, McDonald P, Lazzareschi KP. Studying host cell protein interactions with monoclonal antibodies using high throughput protein A chromatography. Biotechnol J 2012;7:1233–41.
[45] Bergander T, Nilsson-Välimaa K, Öberg K, Lacki KM. High-throughput process development: determination of dynamic binding capacity using microtiter filter plates filled with chromatography resin. Biotechnol Prog 2008;24:632–9.
[46] Hekmat D. Large-scale crystallization of proteins for purification and formulation. Bioprocess Biosyst Eng 2015;38:1209–31.
[47] Zang Y, Kammerer B, Eisenkolb M, Lohr K, Kiefer H. Towards protein crystallization as a process step in downstream processing of therapeutic antibodies: screening and optimization at microbatch scale. PLoS One 2011;6(9). e25282.
[48] Hebel D, Huber S, Stanislawski B, Hekmat D. Stirred batch crystallization of a therapeutic antibody fragment. J Biotechnol 2013;166:206–11.
[49] Smejkal B, Agrawal NJ, Helk B, Schulz H, Giffard M, Mechelke M, Ortner F, Heckmeier P, Trout BL, Heckmat D. Fast and scalable purification of a therapeutic full-length antibody based on process crystallization. Biotechnol Bioeng 2013;110:2452–61.
[50] Tscheliessnig A, Satzer P, Hammerschmidt N, Schulz H, Helk B, Jungbauer A. Ethanol precipitation for purification of recombinant antibodies. J Biotechnol 2014;188:17–28.
[51] Peram T, McDonald P, Carter-Franklin J, Fahrner R. Monoclonal antibody purification using cationic polyelectrolytes: an alternative to column chromatography. Biotechnol Prog 2010;26:1322–31.
[52] Ma J, Hoang H, Myint T, Peram T, Fahrner R, Chou JH. Using precipitation by polyamines as an alternative to chromatographic separation in antibody purification processes. J Chromatogr B 2010;878:798–806.
[53] Knevelman C, Davies J, Allen L, Titchener-Hooker NJ. High-throughput screening techniques for rapid PEG-based precipitation of IgG4 mAb from clarified cell culture supernatant. Biotechnol Progress 2009;26:697–705.
[54] Giese G, Myrold A, Gorrell J, Persson J. Purification of antibodies by precipitating impurities using polyethylene glycol to enable a two chromatography step process. J Chromatogr B 2013;938:14–21.
[55] Oelmeier SA, Ladd-Effio C, Hubbuch J. Alternative separation steps for monoclonal antibody purification: combination of centrifugal partitioning chromatography and precipitation. J Chromatogr A 2013;1319:118–26.
[56] Schmitt MG, Rajendra Y, Hougland MD, Boyles JS, Barnard GC. Polymer-mediated flocculation of transient CHO cultures as a simple, high-throughput method to facilitate antibody discovery. Biotechnol Prog 2017;33(5):1393–400.
[57] Azevedo AM, Rosa PAJ, Ferreira IP, Aries-Barros MR. Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. Trends Bioechnol 2009;27:240–7.
[58] Taha M, Almeida MR, Silva FA, Domingues P, Ventura S, Coutinho JAP, Freire MG. Novel biocompatible and self-buffering ionic liquids for biopharmaceutical applications. Chem Eur J 2015;21:4781–8.
[59] Bhikhabhai R, Sjöberg A, Hedkvist L, Galin M, Liljedahl P, Frigård T, Pettersson N, Nilsson M, Sigrell-Simon JA, Markeland-Johansson C. Production of milligram quantities of affinity tagged-proteins using automated multistep chromatographic purification. J Chromatogr A 2005;1080:83–92.
[60] Lacki KM. High-throughput process development of chromatography steps: advantages and limitations of different formats used. Biotechnol J 2012;7:1192–202.
[61] McDonald P, Victa C, Carter-Franklin JN, Fahrner R. Selective antibody precipitation using polyelectrolytes: a novel approach to the purification of monoclonal antibodies. Biotechnol Bioeng 2009;102:1141–51.
[62] Oelmeier SA, Dismer F, Hubbusch J. Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. Biotechnol Bioeng 2011;108:69–81.

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