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Review Article

AN OVERVIEW OF BIOLOGICS: SCIENCE BEHIND PROTEIN THERAPEUTICS

Inderjeet Kaur[1], Manoj G Pillai[1,] and Nirpendra Singh[1,2*]

[1]*LabIndia Lifesciences Private Limited, Plot no. 372, Udyog Vihar II, Gurgaon, Haryana, India*
[2]*Central Instrumentation Facility, Biotech Center, University of Delhi South Campus, New Delhi, India*

Abstract: Biosimilars or 'follow-on biologics' are new biopharmaceutical agents that are 'similar' but not identical to a reference biopharmaceutical product. Biosimilars are considered 'comparable' to the reference product, but this does not ensure therapeutic equivalence. Inherent differences between biosimilars may produce dissimilarities in clinical efficacy, safety, and immunogenicity. Therefore accurate measurement and characterization of these differences and absolute quantification of biosimilars is the significant requirement at present. Mass spectrometry coupled with high performance liquid chromatography offers the most sensitive and accurate solution for this. This review discusses the potential strategies for the absolute quantification of biosimilars using mass spectrometry.

Keywords: Biosimilars; follow-on biologicals; follow-on protein products; protein therapeutics; mass spectrometry

Introduction

The area of drug discovery was revolutionized by the advent of biological molecules, especially recombinant proteins, as therapeutic agents to treat various diseases. The first recombinant protein drugs, like Eli Lilly's insulin (developed by Genentech, Inc.), were launched in the 1980's. Since then approximately 165 biopharmaceutical products have gained approval (Walsh, 2007) and several hundred are in clinical trials (PhRMA, 2006). This implies that these "biopharmaceuticals" are very well established now and have opened new therapy options for several ailments including anemia, diabetes, cancer, hepatitis and multiple sclerosis (Avidor *et al.*, 2003). But the patent and regulatory data protection periods for the first and second waves of biopharmaceuticals based on recombinant proteins have started to expire. This has opened the way for other manufacturers to produce copies of the already existing biopharmaceuticals and introduced the field of biomedicine to the era of "biosimilars" or "follow-on" products.

Several terms are used in various countries for ''intended copy" products of the biopharmaceuticals such as biosimilars, follow-on biologicals, follow-on protein products, subsequent-entry biologicals and similar biological medicinal products (European Medicines Agency guideline 2006). Biosimilars can be defined as biological medicinal products which are similar in terms of quality, safety and efficacy to an already licensed, well-established reference medicinal product, marketed by an independent applicant following expiry of patent and regulatory data/market exclusivity periods of the reference product, and authorized for marketing through a procedure based on the proof of similarity to the reference product, using

Corresponding Author: **Nirpendra Singh**
E-mail: nirpendrasingh@gmail.com

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certain preexisting scientific and regulatory knowledge. Biosimilars are not generic versions of innovator products. Conventional generics are considered to be therapeutically equivalent to a reference once pharmaceutical equivalence (i.e. identical active substances) and bioequivalence (i.e. comparable pharmacokinetics) have been established and do not require formal clinical efficacy and safety studies. Hence the term 'biogeneric' would imply that the active substance of a biosimilar could be readily characterized and shown to be identical to the active substance of the reference product. But this is simply not the case with biosimilars as the active substance of a biopharmaceutical is a large recombinant protein molecule produced from genetically modified cell lines and extracted through complex and lengthy purification procedures. Due to the variability inherent in such bioprocesses, identical copies of the original product cannot be manufactured and therefore "biogenerics" can not exist (Crommelin *et al.*, 2005; Schellekens, 2005). Hence the term "biosimilars" has been coined.

The protein science behind biosimilars

Biopharmaceuticals are copies of endogenous human proteins such as erythropoietin (EPO), insulin, growth hormones and cytokines. Proteins are biochemical compounds comprising of one or more polypeptide chains folded into a globular or fibrous manner in a biologically functional way. A polypeptide is a linear polymer of amino acids in which the 20 amino acid residues are joined together by a peptide bond between the amino and the carboxyl terminal of adjacent amino acids in a sequence encoded by the genetic code. This makes proteins very large and highly complex molecules.

The complexity of protein molecules

Biopharmaceuticals made up of recombinant proteins are very complex drugs as compared to their conventional counterparts i.e. chemically synthesized small molecules. A typical biopharmaceutical differs from a conventional drug in many aspects such as size, structure and stability. The molecular weight of a small-molecule conventional drug is typically between 100 -1000 Da. In contrast, generally proteins have molecular weights ranging between 10,000 to more than 200,000 Da. Therefore proteins are typically 100-1000 fold larger than the conventional drugs.

The structure of conventional drugs is quite less complex than a protein molecule as the latter has to be precisely folded three dimensionally to adopt correct secondary, tertiary and quaternary structures to be biologically active. In small molecules, it is often known that every atom of the molecule will play a role in defining the clinical profile of the compound whereas the structure– function relationship is usually unknown, or at best partially known, for proteins. Thus, the impact of differences in the molecular structure in most cases cannot be predicted. Moreover, proteins are inherently unstable molecules, and may structurally be damaged by heat, prolonged storage, denaturants, organic solvents, oxygen, pH changes, and by other factors, leading to reduction or complete loss of biological activity.

The prime concern about biopharmaceuticals is the fact that mammalian proteins undergo a variety of post translational modifications such as glycosylation, acetylation, sulphation and phosphorylation. Some of the proteins even undergo proteolysis to achieve a functionally active isoform. This introduces heterogeneity in protein preparation. Furthermore, the modifications can be incorporated in a protein molecule even during manufacturing process such as oxidation, deamidation, partial denaturation and aggregation (Jenkins *et al.*, 2008). Additional heterogeneity may arise if the protein is intentionally modified, for example by multi-site pegylation (Caliceti *et al.*, 2003). Even amino acid substitutions were observed at a significant level of 2% in simple, unglycosylated human growth hormone expressed in bacteria when analyzed mass spectrometrically (Hepner *et al.*, 2005). In complex, glycosylated proteins, the heterogeneity is much greater. A typical immunoglobulin G molecule preparation may comprise of as large as 108 different species considering only a limited number of all possible glycosylation variants (Kozlowski *et al.*, 2006), as represented in Figure 1.

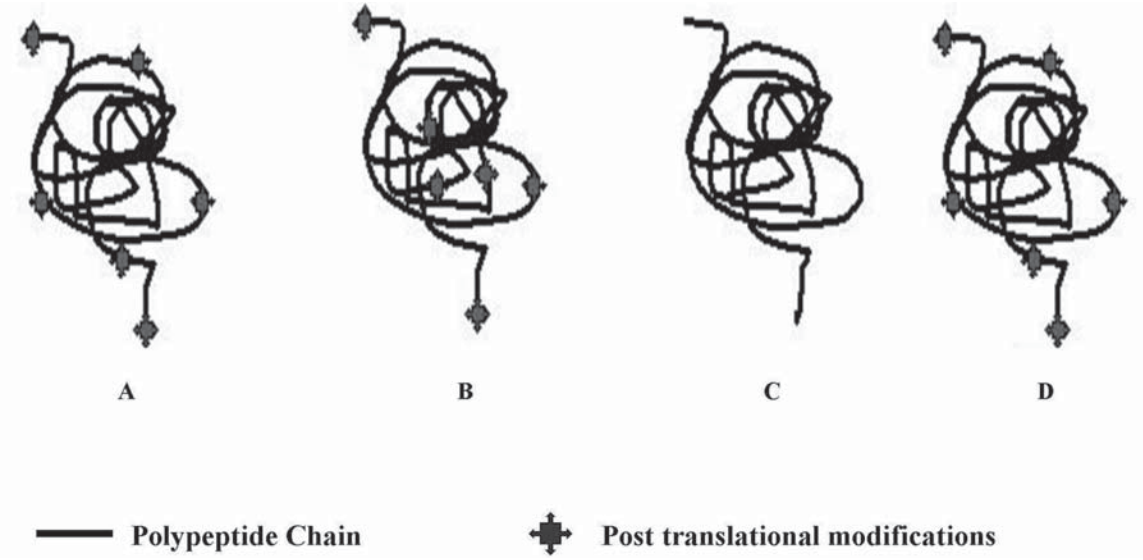

Figure 1: **Sketch diagram of polypeptide chain showing the difference between native polypeptide and synthetic chain of amino acids in different systems. A. Patented active biomolecule, B. Biomolecule expressed in some other organism so it has post translational modification at different positions, C. Polypeptide chain without any post translational modifications, D. Biosimilars molecules having the same sequence and same number of post translational modifications at same position of sequences**

A typical protocol for manufacturing recombinant proteins is a complex and lengthy process involving several steps starting from cloning of DNA followed by expression, fermentation, purification and finally the formulation of the biopharmaceutical. The whole process has to be performed under strictly controlled, validated conditions in closed systems to assure consistency and avoid any contamination. Even though, the batch-to-batch variations are unavoidable even with well-controlled, consistent protein manufacturing procedures. Therefore it is really difficult for a second manufacturer to replicate the process identically to have a true copy of the innovator biopharmaceutical. This truly signifies the need to precisely perform the impurity profiling as well as quantification of the biosimilars.

The extremely specific and very sensitive immunoassays involving monoclonal or polyclonal antibodies may be conveniently used to detect and quantify non-product-related impurities in the final preparation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most common used analytical technique in the assessment of final product purity (Miles and Saul, 2005). The only drawback relating to this analytical technique is that contaminants of the same molecular mass as the product will go undetected. However this can be overcome using two-dimensional electrophoretic analysis (2D SDS-PAGE). The most common method implies separation of proteins by isoelectric focusing (IEF) in the first dimension, separating proteins on the basis of charge characteristics, followed by separation via SDS-PAGE on the basis of protein size in the second dimension. This technique is also utilized to determine homogeneity of biopharmaceuticals (Sosic *et al.*, 2008; Lopez-Soto-Yarritu *et al.*, 2002), as for example the heterogeneity between 12 commercial EPOá samples has been analyzed by isoelectric focusing (IEF) (Schellekens, 2005). Analysis such as peptide mapping, N-terminal sequencing or amino acid analysis, yield information relating to a polypeptide's primary structure. Higher-order structures (secondary and tertiary structure of polypeptides) may be studied in great detail by X-ray crystallography or NMR spectroscopy; however, routine application of such techniques to biopharmaceutical manufacture is impractical from a technical or economical point of view. However, limited analysis of protein secondary and tertiary structure can be more easily undertaken using spectroscopic methods (e.g. far-UV circular dichroism). Proton-NMR has also

been applied to studying higher orders of protein structure (Deechongkit *et al.*, 2006; Khalilzadeh *et al.*, 2008; Tugarinov *et al.*, 2004; Vanz *et al.*, 2004).

Mass spectrometry to the rescue

Techniques such as high-pressure liquid chromatography and capillary electrophoresis along with advanced mass spectrometry offer a solution to the problems associated with the quantification as well as measuring the purity of biosimilars (Asenjo and Andrews, 2009; Jiang *et al.*, 2009; Jin, 2008). A study by Yuen et al utilized mass spectrometry to compare eight different laboratory preparations of EPO and demonstrated that each sample contained a distinct mixture of isoforms with at least 23 observed glycan structures (Yuen *et al.*, 2003). Another study by Yan et al presented a new strategy to characterize the variants in a human recombinant IgG1 mAb by using RPLC–time-of-flight (TOF)-MS (Yan *et al.*, 2007). In this study the authors have efficiently separated the subdomains (light and heavy chains, Fab and Fc) of mAb containing several specific modifications such as pyroglutamic acid, deamidation, isomerization and oxidation on RPLC and revealed the modification profiles of these variants by TOF-MS. The analysis of intact and deglycosylated mAb showed that the majority of the mAb was a cyclized form with the amino terminus converted to pyroglutamate in the heavy chain, and no C-terminal K residue (Yan *et al.*, 2007).

In another comprehensive study, Xie and coworkers demonstrated the utility of peptide mapping/sequencing and glycan analysis using LC-MS in assessing comparability of a biosimilar with an innovator mAb (Xie *et al., 2010*) while Ren *et al.* utilized the RPLC-MS strategy for the characterization of chemical modifications in mAb in a site-specific manner (Ren *et al.*, 2008). Peptide mapping, determining the composition of carbohydrate moieties and location of glycosylation sites, chemical modification of amino acid residues and the location of disulphide linkages have been reported by Jiang *et al* for comparison of innovator and biosimilar TNK-tissue plasminogen activator (Jiang *et al.*, 2010). Chemical modification of the TNK-tissue plasminogen activator occurred in a position where it could affect clot lysis activity and reduce efficacy. In another study, the same group used peptide mapping with LC-MS of three sources of human growth hormone, and identified different levels of oxidation of methionine residues, deamidation of asparagines and cleavage at two sites between an innovator, a biosimilar and a counterfeit product (Jiang *et al.*, 2009). These differences were attributed to variations in the manufacturing, formulation and storage conditions.

Apart from impurity profiling and characterization of biosimilars, mass spectrometry is also a powerful tool for the quantification of these biologicals as Xie *et al simultaneously* identified and quantified intact protein mass, primary sequence, PTMs, and the micro-differences between the two mAbs (Xie *et al., 2010*). Ultra sensitive and high throughput mass spectrometry provides a platform for more accurate and absolute quantification of different biosimilars.

Mass spectrometry-based approaches for absolute quantification of proteins

Absolute quantification of biopharmaceuticals is an absolute need at the validation stage of drug development. Validation criteria are easy to fulfill when addressing small molecules in plasma or serum owing to the fact that they are easily extracted from the bulk of high molecular weight proteins simply by selective precipitation. But the same does not apply to the biopharmaceuticals that comprise of large and complex protein molecules. The quantification of higher molecular weight proteins is very challenging due to the complexity and large dynamic range of proteins present in the biological samples. This challenge becomes much bigger if working with plasma, which comprise of approximately 10^6 different types of proteins out of which 1200 have been identified (Anderson and Anderson, 2002). The quantitative distribution for all these different proteins is not uniform in the plasma as they exhibit a wide dynamic range of protein abundance ranging from 50,000,000,000 pg/ml for albumin to 5 pg/ml for Interleukin-6 (Anderson and Anderson, 2002). Mass spectrometry coupled with high/ultra performance liquid

chromatography offers solutions to the problems associated with the quantification of protein molecules. In MS-based absolute quantification of proteins, a known amount of isotope-labeled authentic standard is mixed with the analyte, and the mixture is introduced into mass spectrometer. The absolute amount of the analyte is calculated from the ratio of ion intensity between the analyte and its standard. Accordingly, known amounts of stable isotope-labeled synthetic peptides, proteins, or peptide concatemers have been used as a standard for absolute or stoichiometric quantification of proteins. Therefore, the most suitable standard should be selected, depending on the purpose of the experiment, or on whether it intends to quantify a small number of targets including their post-translational modifications, obtain highly accurate data for a single unique protein, or measure absolute or stoichiometric abundance of many proteins. Based on this, following approaches which are commonly used for protein quantitation may be utilized for the quantification of biosimilars by mass spectrometry.

Quantification with Stable Isotope-Labeled Peptide Standard

The use of isotope-labeled synthetic standards has a long history in quantitative mass spectrometry. Originally described in the early 1980s (Desiderio and Kai, 1983), it is now becoming more broadly applied as a method commonly known as AQUA (absolute quantification of proteins) (Gerber *et al.*, 2003). For this targeted quantitative proteomics, a method called multiple reaction monitoring (MRM) (Kirkpatrick *et al.*, 2005) is employed in which the (triple quadrupole) mass spectrometer monitors both the intact peptide mass and one or more specific fragment ions of that peptide over the course of an LC-MS experiment. The combination of retention time, peptide mass, and fragment mass practically eliminates ambiguities in peptide assignments and extends the quantification range to 4–5 orders of magnitude (Wolf-Yadlin *et al.*, 2007).

Kusmierz *et al.* have used multiple reaction monitoring (MRM) mode for absolute quantification of enkaphalin in human tissue utilizing the stable isotope-labeled peptide standard approach (Kusmierz *et al.*, 1990). In the original report that uses a stable isotope-labeled peptide as an internal standard for MS-based absolute quantification (Desiderio and Kai, 1983), the amount of endogenous enkephalin in thalamus extract was measured with an ^{18}O-incorporated standard peptide.

A study by Barr et al showed the absolute quantification of apolipoprotein A-I in which three H^2- and C^{13} labeled peptides from the same protein were used as standards to quantify apolipoprotein A-I in human serum using SRM (selected reaction monitoring) or MRM mode (Barr *et al.*, 1996). Expression level of G protein-coupled receptor rhodopsin in rod outer segment membrane was quantified using a H^2-labeled synthetic peptide (Barnidge *et al.*, 2003). Similarly, absolute amount of C-reactive protein, a well-known diagnostic marker for rheumatoid arthritis, and GST-α, a marker for acute hepatocyte damage, were measured in human serum and liver tissue respectively (Kuhn *et al.*, 2004; Zhang *et al.*, 2004).

However, for quantification of low abundance proteins, enrichment of the target protein is necessary. For instance, abundance of Sir2 and Sir4 were determined as ~10^3 copies per cell following enrichment (Gerber *et al.*, 2003) [38]. In this study, yeast extract was separated by SDS-PAGE and a gel slice containing the target protein was excised, mixed with ^{13}C and ^{15}N-labeled standard peptides, and subjected to trypsin digestion followed by MS. More recently, stable isotope-labeled synthetic peptides were applied to simultaneous quantification of multiple proteins. For instance, the stoichiometry among the 10 components of human spliceosomal U1 small ribonucleoprotein complex was determined using chemical labeling of sample derived and synthetic standard peptides with isotope-coded reagents after trypsin digestion (Hochleitner et al., 2005). Similarly, concentrations of 8 endogenous proteins in human serum were quantified by spiking isotope-labeled standard peptides (Pan *et al.*, 2005). In another study, isotope-labeled peptides were applied to absolute quantification of 32 key proteins in the postsynaptic density of rat, including calmodulin-dependent protein kinase, synaptic GTPase-activating protein,

glutamate receptors, and scaffold proteins (Cheng *et al.*, 2006); the absolute and stoichiometric abundance obtained from this study provided valuable information on the abundance of receptor subtypes and protein interactions.

Peptide standard can be used to quantify not only protein abundance but also post-translational modifications. Isotope labeled unphosphorylated and phosphorylated peptides can be used to quantify phosphorylation stoichiometry. For instance, stoichiometry of phosphorylation at Ser-1126 of separase was measured in different stages of the cell cycle either in a peptide ion scanning mode (Stemmann et al., 2001) or in SRM mode (Gerber *et al.*, 2003). Modifications other than phosphorylation can be quantified. For instance, abundance of polyubiquitin chain branched at the Lys-48 was measured in mammalian cells treated with or without the proteasome inhibitor MG132 (Kirkpatrick *et al.*, 2005). In a study aiming at absolute quantification of ubiquitin conjugated sites, a peptide bearing diglycine-conjugated Lys residue was synthesized and used as an isotope-labeled standard. Topology of polyubiquitin chain of an *in vitro* ubiquitinated protein, or which of the seven Lys residues is used for branching, was also analyzed using ubiquitin-standard peptides (Kirkpatrick *et al.*, 2006).

Taken together, isotope-labeled synthetic peptide is definitely a powerful tool for absolute quantification not only for cultured cells, but also for tissue or blood samples from animals and human. However, it is too expensive to synthesize multiple stable isotope-labeled peptides for quantification of multiple proteins.

Quantification with Stable Isotope-Labeled Intact Protein

Trypsin digestion of a protein yields a large number of proteolytic peptides which differ in terms of cleavage efficiency since the efficiency of trypsin digestion differs from one site to another (Kuhn *et al.*, 2004). Therefore efficiency of protease digestion becomes critical for accuracy when proteolytic peptides are used for absolute quantification as the incomplete digestion of the analyte leads to underestimation of its concentration. While some studies have optimized and monitored cleavage efficiency for specific peptides (Barr *et al.*, 1996; Barnidge *et al.*, 2003), it is difficult to assess a large number of peptides in terms of cleavage efficiency. Therefore stable isotope labeled protein itself is an ideal standard for absolute quantification of a particular protein, because it can be spiked at the earliest stage of sample preparation to minimize experimental errors and shares exactly the same efficiency of protease digestion with the target proteins in the sample. For instance, concentrations of insulin in sera of normal individuals and diabetic patients were quantified with an isotope-labeled recombinant standard protein expressed in and purified from *E. coli* (Kippen *et al.*, 1997). Similarly, ^{15}N-labeled recombinant standard proteins were used for absolute quantification of 6 proteins localized in postsynaptic density (Peng *et al.*, 2004), and expression level of alcohol dehydrogenase isozyme ADH1C1 was quantified in human liver tissue using a ^{13}C- and ^{15}N-labeled recombinant intact protein (Janecki *et al.*, 2007).

Quantification with Stable Isotope-Labeled Peptide- Concatenated Standard

The strategies using synthetic peptides or intact proteins as stable isotope-labeled standards are a powerful tool for absolute quantification of protein molecules. However a large scale analysis requires preparation and handling of many standard peptides/ proteins, thereby raising many concerns. The cost and purity of synthesizing many stable isotope-labeled synthetic peptides is the prime concern. It is also very difficult to express and purify many recombinant proteins as stable isotope-labeled standards. Apart from cost and purity, the equimolarity of the individual standards is another concern during the quantification process. These obstacles were overcome by the advent of a strategy that uses a peptide concatemer as a standard, namely QconCAT (Beynon *et al.*, 2005) and PCS for peptide-concatenated standard (Kito *et al.*, 2007). In this, tryptic peptides used for quantification are concatenated into a single artificial protein. This protein was metabolically labeled with stable isotope in *E. coli*, purified, and mixed with a protein sample to obtain absolute or

stoichiometric quantities of multiple proteins. The QconCAT approach provided absolute quantitative data of more than 10 proteins in chick skeletal muscle of different developmental stages (Beynon *et al.*, 2005; Rivers *et al.*, 2007). Absolute amounts of 13 proteins in human plasma of 20–10,000 fmol/µl concentrations were successfully measured in an MRM mode using this approach (Anderson and Hunter, 2006).

However, it should be noted that, as discussed above, one of the critical key for accurate quantification is to equalize cleavage efficiency between the analyte and its standard. In contrast to QconCAT, PCS contains each standard peptide with its natural flanking sequences on both sides to faithfully recapitulate the efficiency of tryptic cleavage of parental proteins or analytes. The involvement of flanking sequences was demonstrated to improve the accuracy of quantification, and led to accurate quantification of stoichiometry among 5 subunits in eIF2B stable complex of yeast within 5% measurement error (Kito *et al.*, 2007). Similarly, others successfully quantified absolute and stoichiometric abundance of each subunit of transducin, a heterotrimeric G-protein, using a PCS incorporating the flanking sequences of each tryptic peptide (Nanavati *et al.*, 2008). These studies highlight the importance of incorporation of flanking sequences into the standard to ensure highly accurate absolute or stoichiometric quantification.

Thus, in total biosimilars are new drug entities emerging from the genetically modified organisms that need a thorough activity guided characterization and quantification for clinical trials. Most of the strategies that are used for the protein analysis by mass spectrometry, find application in the biologics analysis too. The characterization part comprises of intact mass analysis, its confirmation similarity with the innovator drug, peptide sequencing and analysis of post translational modifications like phosphorylation, acetylation, nitration, deamidation, glycosylation and PEGylation etc. Most suitable analytical platform will be a mass spectrometer which can provide a high resolution MS as well as MS/MS data that can help in achieving the complete sequence coverage of the protein. The mass accuracy of the mass spectrometer also plays an important role as some of the modifications to be identified, such as deamidation, has only 1Da difference in the parent mass. In a protein with molecular mass of 10000Da identification of such kind of modification is very difficult and thus the mass accuracy of the instrument becomes crucial which need to be addressed at the chromatographic level. Even though there are several reports for the quantitation of proteins using mass spectrometry as mentioned earlier, the absolute quantification of biologics is still in a developing stage; whereas for the characterization workflows, high-resolution mass spectrometry is aptly used.

Conclusion

The absolute quantification of biosimilar drugs is greater than ever obligation in today's world. Mass spectrometry definitely is the most powerful device for the quantification of biosimilar drugs because it offers an array of strategies for the absolute quantification and impurity profiling of these biopharmaceuticals. However each strategy has its own applications and limitations.

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