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High-Affinity Recombinant Antibody Fragments (Fabs) Can Be Applied in Peptide Enrichment Immuno-MRM Assays

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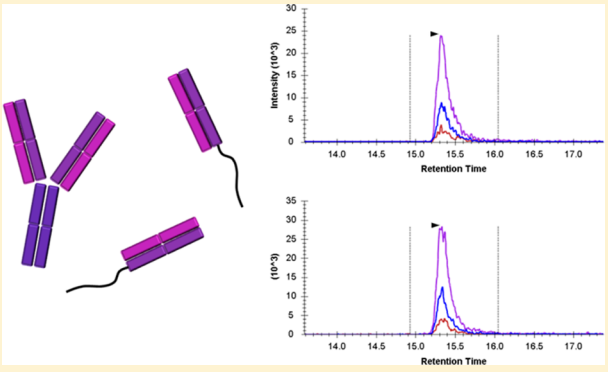
ABSTRACT: High-affinity antibodies binding to linear peptides in solution are a prerequisite for performing immuno-MRM, an emerging technology for protein quantitation with high precision and specificity using peptide immunoaffinity enrichment coupled to stable isotope dilution and targeted mass spectrometry. Recombinant antibodies can be generated from appropriate libraries in high-throughput in an automated laboratory and thus may offer advantages over conventional monoclonal antibodies. However, recombinant antibodies are typically obtained as fragments (Fab or scFv) expressed from *E. coli*, and it is not known whether these antibody formats are compatible with the established protocols and whether the affinities necessary for immunocapture of small linear peptides can be achieved with this technology. Hence, we performed a feasibility study to ask: (a) whether it is feasible to isolate high-affinity Fabs to small linear antigens and (b) whether it is feasible to incorporate antibody fragments into robust, quantitative immuno-MRM assays. We describe successful isolation of high-affinity Fab fragments against short (tryptic) peptides from a human combinatorial Fab library. We analytically characterize three immuno-MRM assays using recombinant Fabs, full-length IgGs constructed from these Fabs, or traditional monoclonals. We show that the antibody fragments show similar performance compared with traditional mouse- or rabbit-derived monoclonal antibodies. The data establish feasibility of isolating and incorporating high-affinity Fabs into peptide immuno-MRM assays.



KEYWORDS: *multiple reaction monitoring, immunoglobulin, monoclonal antibody, scFv, immunoaffinity, targeted proteomics*

INTRODUCTION

Multiple reaction monitoring (MRM) is a targeted mass spectrometry technique that enables specific, precise, and sensitive measurements of target analytes,[1−3] including proteotypic peptides released upon proteolysis of biospecimens. However, for many peptide analytes of interest, an enrichment step must be performed in order for MRM assays to have sufficient sensitivity to quantify endogenous levels of analyte in a complex biological matrix. For most analytes, sufficient sensitivity can be achieved by coupling a peptide immunoaffinity enrichment step with MRM, resulting in a peptide immuno-MRM assay. Immuno-MRM offers excellent specificity, sensitivity, wide dynamic range, and ease of sample handling for measuring endogenous proteins in a variety of sample types.[4−6] It is also reproducible across laboratories[7] and capable of multiplexing many analytes together.[8]

To date, most work with immuno-MRM has centered on the use of affinity-purified polyclonal antibodies[9−11] with an increasing number of studies using monoclonal antibodies (McAbs).[12−17] Although McAbs are more desirable than polyclonals due to their renewable nature, specificity profile, and uniform affinity, the lead time and cost associated with generating hybridoma-based McAbs has hindered the generation of immuno-MRM assays on a large scale. One potential alternative to traditional McAbs are recombinant antibodies isolated from large naive libraries, which offer several advantages. First, using recombinant libraries, antibody isolation is performed in vitro, allowing control of selection parameters,[18−20] including affinity. Second, unlike animal-based systems (e.g., rabbit, mouse), isolation of antibodies is possible for any antigen including toxic, conserved, or self-antigens. Third, the process can be completed in a relatively short time frame, and it is amenable to automation.[21] Fourth, because the sequence of the antibody is known and the genes are available on plasmids, there is a plethora of genetic engineering possibilities, including conversion into different formats like antibody fragments, full immunoglobulins, and fusion proteins as well as enhancement of antibody affinity or specificity via mutagenesis. Finally, recombinant antibody fragments are produced in bacteria, which is easier and faster than using animals or mammalian cell-culture techniques.

Given these potential advantages, we sought to determine the feasibility of isolating and using recombinant antibodies as an

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affinity reagent in peptide immuno-MRM. Generation of antibodies with high affinity ($K_D \leq 10^{-8}$ M) for linear peptides can be very challenging because peptides have a flexible structure in the unbound state, and hence there is a loss of entropy upon binding of an antibody. Employing in vitro selection technologies has proven to be a successful route for selection of such antibodies, albeit in only a few very specific examples. With designed phage display antibody libraries focused toward peptide binding, Cobaugh at al. could isolate antibodies that bind linear peptides with an affinity of up to 18 nM.[22] In another example, rigorous directed evolution was applied to an existing high-affinity antipeptide antibody using multiple rounds of ribosome display to further increase affinity to the low picomolar range.[23] To our knowledge, it has not yet been shown that high-affinity antibodies against short linear peptides can be selected from a naïve antibody library. Furthermore, recombinant antibodies are typically obtained as fragments (Fab or scFv) expressed from *E. coli*, and it is not known whether such fragments are compatible with the established technique of immunocapture of small linear peptides.

Here we describe the generation, expression, and characterization of monovalent Fab antibody fragments for application in immuno-MRM assays using three peptide antigens as examples, chosen because high-affinity monoclonal benchmark antibodies had been generated previously by traditional immunization. For better comparison, we also produced the selected recombinant antibodies as full-length IgG antibodies. We then compared the Fabs and their derivative IgGs to the traditional monoclonal antibodies by evaluating their performance in response curves. The recombinant antibody fragments show similar performance compared with monoclonal antibodies, demonstrating that such recombinant antipeptide antibodies have sufficient affinities for peptide capture and providing the first demonstration of the application of Fabs for immuno-MRM.

EXPERIMENTAL SECTION

Reagents

Bulk human plasma was obtained from Sigma (St. Louis, MO) and stored at −80 °C. Urea, Trizma base, dithiothreitol (DTT), iodoacetamide, formic acid, and (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (CHAPS) were obtained from Sigma. Acetonitrile (LCMS grade), water (LCMS grade), and phosphate-buffered saline (PBS) were obtained from Fisher (Pittsburgh, PA).

The peptide sequences used in this study were proteotypic peptides to three proteins, empirically observed by mass spectrometry: GDSLAYGLR (9 aa from region 145−153 of Uniprot entry P10923, Spp1, mouse Osteopontin), NWAPGEPNNR (10 aa from region 95−105 of Uniprot entry P16581, SELE, human E-Selectin), and VDNEELLPK (9 aa from region 189−197 of Uniprot entry P78536, ADA17, human ADAM17). For each sequence, the following peptides were obtained from JPT Peptide Technologies (Berlin, Germany) as freeze-dried products with a purity of >90%, as analyzed by HPLC and mass spectrometry: the sequence with free amino- and C-termini, the sequence with addition of N-terminal cysteine (e.g., C-GDSLAYGLR), the sequence with C-terminal cysteine (e.g., GDSLAYGLR-C), the sequence with N-terminal biotin conjugated by 4,7,10-trioxa-1,13-tridecanediamine (Ttds) (e.g., bio-Ttds-GDSLAYGLR), and the sequence with C-terminal biotin added by biotinyl lysine (e.g., GDLSAYGLR-Ttds-Lys-bio). All peptides were dissolved in PBS, and N-terminal and C-terminal cysteine peptides were conjugated to carrier proteins bovine serum albumin (BSA) and human transferrin (Trf) using *N*-hydroxysuccinimide/1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (NHS/EDC) chemistry. For mass spectrometry peptide standards, >95% purity synthetic peptides (light peptides) and stable isotope-labeled peptide standards (heavy peptides) were obtained from Sigma (St. Louis, MO) and New England Peptide (Gardner, MA). Labeled peptides contain >99% [^{13}C] and [^{15}N] isotopic purity at the C-terminal arginine for GDSLAYGLR and 99% [^{13}C] isotopic purity at the C-terminal arginine or lysine residue for the other peptides. Peptide standard concentration was determined by amino acid analysis at Dana Farber Cancer Institute (Boston, MA) and New England Peptide.

Generation of Antibodies from the HuCAL PLATINUM Library

The HuCAL PLATINUM library[24] was used for the generation of recombinant antibodies against the peptides from mouse osteopontin, human E-selectin, and human ADAM17. Because we were aiming for antibodies with very high affinity to the free peptides, RapMAT technology[25] as well as inhibition screening with free peptide was included in the antibody generation process.

Phage display selection (panning) consists of antigen immobilization on magnetic beads, incubation with the HuCAL library, removal of unspecific antibodies by wash steps on a Kingfisher instrument (Thermo Scientific), followed by elution of the phage encoding the enriched antibodies. The eluted phage is amplified by infection of *E. coli* and production of new Fab displaying phage for the next panning round. Two rounds of panning were performed on the peptide carrier protein conjugates coupled to Dynal M-450 Epoxy beads (Invitrogen) using the respective Trf peptide conjugate in the first and the BSA peptide conjugate in the second panning round. One panning was on the peptides with N-terminal cysteine, and in another panning the conjugates with the peptides with C-terminal cysteine were used. After the second panning round, the gene region coding for CDR3s of the antibody light chains (LCDR3) from the preselected pool of Fab genes was exchanged with a highly diverse LCDR3 maturation cassette generated by trinucleotides,[25] and *E. coli* MC1061F′ cells were transformed with the ligated DNA. HuCAL libraries containing antibodies with kappa or lambda light chain were kept separate to avoid the formation of mixed frameworks. The obtained maturation libraries contained between (2 and 9) × 10^8 members. With the Fab displaying phage produced from these libraries, another two rounds of selection using increased stringency (extended washing steps and reduced amount of antigen coupled beads) were performed. In one setting, the selection was again performed on the peptide carrier protein conjugates coupled to magnetic beads using decreasing amounts of antigen coupled beads in the panning rounds 3 and 4. In a second selection, biotinylated peptides captured on streptavidin-coated magnetic beads (Invitrogen) were used. The phages from the selection on the peptides coupled via N-terminal cysteine were incubated on the N-terminally biotinylated peptides, and the C-terminally biotinylated peptides were used for the phages from the selections on the peptides coupled via C-terminal cysteine.

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After the two rounds of RapMAT panning, the pool of Fabs genes was subcloned into an expression vector, leading to functional periplasmic expression of monovalent Fab equipped with two peptide tags, the so-called V5 tag (GKPIPNPLLGLDST), and a double extended Strep tag (**SAWSHPQFEK**GGGSGGGSGGGS**SAWSHPQFEK**; Strep tag sequences in bold), which was used later for antibody affinity purification and capture in immuno-MRM experiments by StrepTactin beads. *E. coli* TG1F cells (TG1 depleted for the F pilus) were transformed with the ligated expression vectors, and 368 individual colonies were randomly picked for each panning and grown in microtiter plates. After induction of antibody expression with 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG) overnight at 22 °C, the cultures were chemically lysed, and the crude extracts were tested in enzyme-linked immunosorbent assay (ELISA) for binding to biotinylated peptide captured by neutravidin, which was coated on a microtiter plate. In addition, on a separate plate, competition with free peptide was measured by adding free peptide to the captured biotinylated peptide at 10 μM final concentration before applying the *E. coli* lysate. Detection of bound Fab was with an alkaline phosphatase-labeled antihuman IgG F(ab')$_2$ specific antibody (AbD Serotec, no. STAR126A).

The sequences of the antibody VH and VL complementarity-determining regions (CDRs) were determined from a selection of the clones that gave a strong signal on the biotinylated peptide in the ELISA (at least five-fold above the background signal) and which also showed a strong signal reduction in the presence of free peptide. Clones containing antibodies with unique sequence were chosen for subsequent expression and purification via the Strep tag.[26] Antibody concentrations were determined by measuring the absorption at 280 nm.

Conversion of Fab into Human IgG1

To allow direct comparison of peptide affinity enrichment protocols using protein G beads developed for traditional full-length IgGs, we converted the Fab antibodies to the full-length human IgG1 isotype. Variable domain VH and VL gene fragments from selected antibodies were subcloned into the pMORPH2_h_Ig vector series for human IgG1 expression.[27,28] These vectors carry the human constant region and the human lambda or kappa constant region, respectively. Eukaryotic HKB11 cells[29] were transiently transfected with the human IgG1 and the human light-chain expression constructs. Cell culture supernatants were subjected to protein A affinity chromatography. The purified antibody was rebuffered to PBS and finally sterile-filtered.

Affinity Determination using Solution Equilibrium Titration

Electrochemiluminescence (ECL)-based solution equilibrium titration (SET) measurements were performed essentially as previously described.[30] In brief, a constant amount of monovalent Fab was incubated with different concentrations of the peptides GDSLAYGLR, NWAPGEPNNR, and VDNEELLPK, respectively, until equilibrium was reached. The concentration of free antibody in the equilibrated solution was determined by applying the solution onto a 384-well multiarray plate (Meso Scale Discovery) coated with the respective peptide coupled to BSA or Trf, followed by incubation with a Sulfo-Tag (Meso Scale Discovery)-labeled goat antihuman F(ab')$_2$ specific antibody (AbD Serotec). ECL signals were detected using a SECTOR Imager 6000 (Meso Scale Discovery). Evaluation and K_D calculation were done using XL-fit software (version 5.2.0.0, IDBS) applying a customized 1:1 equilibrium fit model.[30]

Generation of Rabbit or Mouse Monoclonal Antibody

Rabbit or mouse monoclonal antibodies against the same peptides, as previously described for mouse osteopontin, human E-Selectin, and human ADAM17, were produced by Epitomics (Burlington, CA), as previously described.[13] In brief, animals were immunized with peptide antigen coupled to KLH (keyhole lympet hemocyanin). Splenocytes from animals with the highest titers were harvested and the hybridomas were screened to identify positive clones. Antibody produced by the best performing hybridoma was purified by Protein-A affinity chromatography.

Plasma Digestion

Human plasma was used as a background matrix for peptide immunoaffinity enrichment experiments. The plasma was denatured by the addition of 9 M urea in 300 mM Tris, pH 8.0 (final conc. 6 M) and 500 mM dithiotreitol (final conc. 20 mM) and incubated at 37 °C for 30 min on a shaker (700 rpm). Following denaturation, 500 mM iodoacetamide was added to the mixture (final conc. 50 mM) to alkylate the sulfhydryls, and plasma was incubated in dark under ambient condition for 30 min. Before the addition of trypsin, 100 mM Tris buffer, pH 8.0 was added to reduce urea concentration to ~0.6 M; then, sequencing-grade trypsin (Fisher Scientific) was added to the mixture at ratio of 1:50 (enzyme: protein). The plasma was incubated at 37 °C for 16 h. The trypsin activity was quenched by the addition of concentrated formic acid (final conc. 1%). The digested plasma was desalted on an Oasis HLB cartridge (Waters). The cartridge was conditioned with 3 × 1 mL of 0.1% formic acid in 80% acetonitrile and equilibrated with 4 × 1 mL of 0.1% formic acid. The plasma digest was applied on the cartridge and washed with 4 × 1 mL of 0.1% formic acid. Peptides were eluted with 3 × 400 μL of 0.1% formic in 80% acetonitrile. The plasma digest was dried by SpeedVac and resuspended in PBS to the original volume.

Immunoaffinity Enrichment of Peptides

The pH of digested plasma was adjusted with the addition of 1 M Tris to pH 8.0 before the addition of antibodies. For immunoaffinity enrichment experiments, 10 μL of original digested plasma, 1 μg of antibodies, a variable amount of heavy peptides (0−200 fmol), a constant amount of light peptides (10 fmol), and 5 μL of magnetic beads were mixed together into a final volume of 200 μL in PBS, 0.03% CHAPS. Magnetic beads were adjusted according to the affinity reagent. StrepTactin beads (Qiagen, no. 36311) were used for fragment antibodies containing the Strep tag. Protein G beads (Dynabeads Protein G, Invitrogen) were used for full-length IgG and monoclonal antibody reagents. The mixture was incubated overnight at 4 °C. Beads washing and peptide elution steps were performed on a Kingfisher Magnetic Particle Processor (Thermo). The beads were washed 2 × 200 μL in PBS, 0.03% CHAPS, and 1 × 200 μL in reduced strength (1/10) PBS, 0.03% CHAPS. Peptides were eluted using 5% acetic acid, 3% acetonitrile for 5 min. For recovery efficiency experiments, heavy peptides were captured by the antibodies, and light peptides were added to the elution buffer following the affinity enrichment to calculate the recovery. For all immunoaffinity enrichment experiments, the eluted peptides were stored at −80 °C until analysis by mass spectrometry.

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Nanoliquid Chromatography–Mass Spectrometry

An Eksigent 2DLC system (Eksigent Technologies, Dublin, CA) equipped with an autosampler was used for liquid chromatography. Solvents were water, 0.1% formic acid (mobile phase A), and 90% acetonitrile, 0.1% formic acid (mobile phase B). The sample was loaded onto a trap column (0.3 × 5 mm, LC Packings PepMap Acclaim C18) for 1.5 min at 10 μL/min with 3% mobile phase B. For peptide elution, the trap was connected in line with a 0.075 × 100 mm IntegraFrit column (New Objective, Wobum, MA) packed with 3 μm Reprosil C18-AQ particles (Dr. Maisch, Germany). The LC gradient was delivered at 300 nL/min with a linear gradient of mobile phase B from 3 to 40% B over 10 min. The trap column was backwashed with 3% mobile phase B buffer at 3 μL/min. The nano-LC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (6500 QTRAP, MDS SCIEX, Foster City, CA) equipped with a CaptiveSpray source (Michrom Bioresources, Auburn, CA). All measurements were made using MRM targeting the peptides of interest. The typical instrument settings included spray voltage of 1.4 kV and an ion source temperature of 110 °C. The optimum transitions and parameters for MRM methods were determined using Skyline software.[31] Transitions for peptides GDSLAYGLR (476.25 > 779.44 (y7), 692.41 (y6), 579.32 (y5), 508.29 (y4)), NWAPGEPNNR (577.77 > 854.41 (y8), 783.37 (y7), 500.26 (y4)), and VDNEELLPK (528.78 > 957.49 (y8), 842.46 (y7), 599.38 (y5)) were monitored along with corresponding transitions for their heavy stable isotope analogs using 10 ms dwell times with a 5 ms interscan delay time. The most abundant transition was used for all quantitative calculations. The presence of multiple transitions at the same retention time was used to confirm the specificity of the peak.

RESULTS AND DISCUSSION

The coupling of peptide immunoaffinity enrichment with quantitative MRM mass spectrometry in an immuno-MRM assay has the potential to significantly impact basic biological and clinical studies by providing highly multiplexable, sensitive, and specific assays. We tested the feasibility of isolating high affinity antibody fragments (Fabs) from a naive phage display library and incorporating them into peptide immuno-MRM assays. We developed antibody fragment-based peptide enrichment immuno-MRM assays for three peptides shown in Table 1. The performance of the high-affinity Fabs was compared with existing assays based on antipeptide rabbit or mouse monoclonal antibodies (McAbs), as described later.

Generation of High-Affinity Antipeptide Fragment and Full-Length Antibodies

The Fab phage display library HuCAL PLATINUM was used for the generation of antibodies binding to the peptides shown in Table 1. The peptide sequences were chosen based on being proteotypic for the protein of interest and on the availability of existing affinity reagents for use in immuno-MRM. The peptide antigens were attached to magnetic beads either via coupling to carrier proteins or via attachment of biotinylated peptides to streptavidin beads. For fast generation of high-affinity antibodies (Figure 1), a pool maturation by LCDR3 exchange after the second panning round was performed (so-called RapMAT;[25]). After each panning, 368 clones were tested in an ELISA screening for binding to biotinylated peptide, and, in parallel, a competition screening using inhibition by free peptide was performed. Many Fabs were obtained for each target and each

Table 1. Target Analytes and Antigens Used for Panning and Screening for the Generation of the Final Candidate Antibodies[a]

protein description	peptide sequence	antibody description	panning first round	panning second round	RapMAT first round	RapMAT second round	primary screening antigen	inhibition screening peptide	K_{CD} (nM)
osteopontin	GDSLAYGLR	Fab AbD18303	mSPP1-C-Trf	mSPP1-C-BSA	bio-mSPP1-C	bio-mSPP1-C	bio-mSPP1-C	mSPP1	0.6 ± 0.5
		Fab AbD18304	mSPP1-C-Trf	mSPP1-C-BSA	bio-mSPP1-C	bio-mSPP1-C	bio-mSPP1-C	mSPP2	0.9 ± 0.5
E-Selectin	NWAPGEPNNR	Fab AbD18279	hSELE-N-Trf	hSELE-N-BSA	bio-hSELE-N	bio-hSELE-N	bio-hSELE-N	hSELE	0.4 ± 0.2
		Fab AbD18288	hSELE-N-Trf	hSELE-N-BSA	bio-hSELE-N	bio-hSELE-N	bio-hSELE-N	hSELE	1.2 ± 0.5
ADAM17	VDNEELLPK	Fab AbD18260	hADAM17-C-Trf	hADAM17-C-BSA	hADAM17-C-BSA	hADAM17-C-Trf	bio-hADAM17-C	hADAM17	58 ± 12
metalloprotease		Fab AbD18307	hADAM17-N-Trf	hADAM17-N-BSA	bio-hADAM17-N	bio-hADAM17-N	bio-hADAM17-N	hADAM17	33 ± 2

[a]Antibody affinities were measured by SET. K_D values are the mean of two (AbD18288), three (AbD18279, AbD18304, AbD18260, and AbD18307), or four measurements (AbD18303). Peptide abbreviations correspond to (mSPP1-N: C-GDSLAYGLR; mSPP1-C: GDSLAYGLR-C; bio-mSPP1-N: bio-Ttds-GDSLAYGLR; bio-mSPP1-C: GDSLAYGLR-Ttds[1]-Lys-bio-mSPP1: GDSLAYGLR; hSELE-N: C-NWAPGEPNNR; hSELE-C: NWAPGEPNNR-C; bio-hSELE-N: bio-Ttds-NWAPGEPNNR; bio-hSELE-C: NWAPGEPNNR-Ttds-Lys-bio; hSELE: NWAPGEPNNR; hADAM17-N: C-VDNEELLPK; hADAM17-C: VDNEELLPK-C; bio-hADAM17-N: bio-Ttds-VDNEELLPK; bio-hADAM17-C: VDNEELLPK-Ttds-Lys-bio; hADAM17: VDNEELLPK). Peptides were conjugated to bovine serum albumin (BSA) or human transferrin (Trf).

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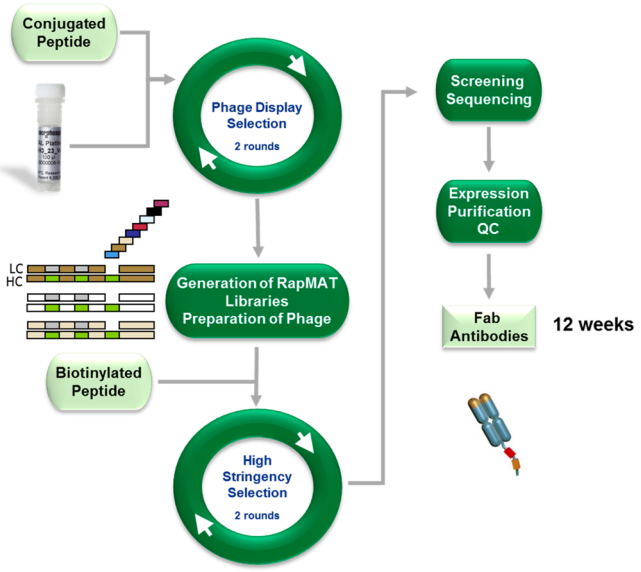

Figure 1. Scheme of the antibody generation process with the HuCAL phage display antibody library and RapMAT technology.

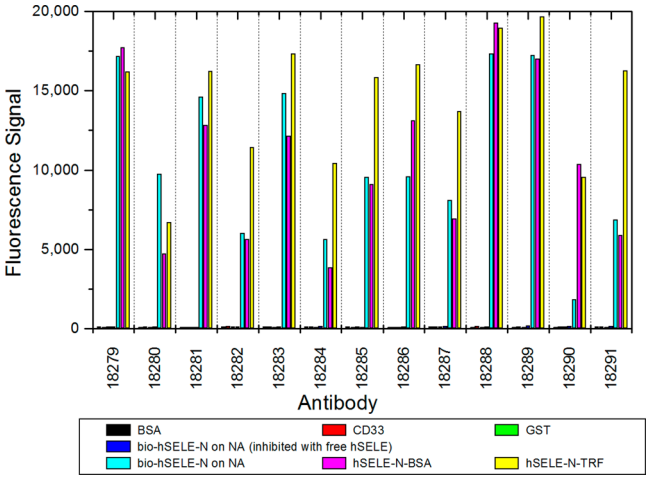

Figure 2. Purified antibodies AbD18279–AbD18291, selected on hSELE-N peptide, were tested in ELISA for specific binding and for competition with free peptide. Unrelated protein antigens as well as peptide-carrier proteins were coated on the plate, or biotinylated antigen peptide was captured on coated neutravidin (NA). For bio-hSELE-N, a competition ELISA was performed using free hSELE peptide at a final concentration of 10 μM as competitor. Detection of binding was performed with antihuman Fab secondary antibody coupled to alkaline phosphatase. All tested antibodies were specific to the peptide and fully inhibited by the free peptide.

panning, which were specific for the peptide and could be inhibited by free peptide in solution. (See Table 2 for a summary of the selections using the biotinylated peptides in the RapMAT panning rounds.) For the selections using the peptide-carrier protein conjugates in the RapMAT panning rounds, about 20 times fewer antibodies were positive in ELISA screening on the biotinylated peptide and could be inhibited by free peptide (data not shown). A subset of unique Fabs was expressed on a 250 mL scale in *E. coli*, purified, and tested again in ELISA on the antigens used in panning, including inhibition with free peptide, and on unrelated control proteins. Figure 2 shows example data for the antibodies selected on the peptide NWAPGEPNNR (coupled to carrier proteins and biotinylated) from E-Selectin. The intrinsic monovalent affinity of a selection of the Fabs showing specific binding and competition by free peptide was determined by SET. Antibodies with subnanomolar affinities could be generated against two of the three antigens (Table 1).

We tested two panning strategies during pool maturation, the use of biotinylated peptides, as well as decreasing amounts of peptide-linker-carrier conjugates in the RapMAT panning rounds. Clearly more antibodies capable of binding to the free peptide in solution were selected using the biotinylated peptides captured by streptavidin. A reason for this could be the fact that biotinylated peptides lack the N- or C-terminal cysteine that was added for coupling purposes, driving the selection toward the core peptide sequences. Hence, panning on the peptide-Trf and BSA conjugates in the first two panning rounds followed by RapMAT using biotinylated peptide appears to be an acceptable general strategy for selection of high-affinity antipeptide antibodies. In the pool maturations using the biotinylated peptides, we found on average 30% positive clones in ELISA, and, on average, 24% of those ELISA positive clones were binding to free peptide (Table 2). It has been shown previously that large in vitro antibody repertoires contain a highly diverse set of different antibodies to a given target.[32] In a previous attempt to generate monoclonal antibodies against the ADAM17 peptide by immunization of six mice, only two clones were finally isolated that bound to the free peptide, and the majority of clones were binding to the chemical linker between the peptide and the carrier protein.[13] In the in vitro approach described here, such binders are excluded already during the panning process because the linker-carrier conjugates are alternated between the panning rounds and a biotinylated antigen version was used during maturation.

Table 2. Results of Antibody Generation for the Selections Using the Biotinylated Peptides in the Two RapMAT Panning Rounds[a]

gene symbol	protein description	peptide sequence	clones screened in ELISA	ELISA positive	binding to free peptide	clones sequenced	unique antibody sequences	affinity range (nM)
Spp1	osteopontin	GDSLAYGLR	1472	408 (28%)	81 (6%)	40	9	0.6–380
SELE	E-Selectin	NWAPGEPNNR	1472	325 (22%)	192 (13%)	42	20	0.4–243
ADA17	ADAM17 metalloprotease	VDNEELLPK	1472	577 (39%)	40 (3%)	33	17	33–91

[a]Clones with a signal at least five times above the background in ELISA on the respective biotinylated peptide captured by coated streptavidin were considered positive. The clones which in addition showed an at least five times lower signal on the biotinylated peptide in the presence of 10μM peptide with free amino- and C-termini were considered positive for binding to free peptide. For Spp1 and ADA17, also a few clones with less than five-fold signal reduction in the competition screening were sequenced. The affinity to the free peptide was determined for 5 (Spp1), 17 (SELE), and 5 antibodies (ADA17).

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Table 3. Performance Characteristics of Reagents Used in Immuno-MRM Assays[a]

gene symbol	protein description	peptide sequence	light precursor m/z	heavy precursor m/z	fragment ion	antibody description	bead system	recovery (%) in immuno-MRM	limit of detection in immuno-MRM (ng/mL)
Spp1	osteopontin	GDSLAYGLR	476.25	481.25	y5	Fab AbD18303	StrepTactin	87	0.3
						Fab AbD18304	StrepTactin	86	
						IgG AbD18303	Protein G Dynabeads	97	0.3
						IgG AbD18304	Protein G Dynabeads	98	
						Rabbit Mab	Protein G Dynabeads	84	1.0
SELE	E-Selectin	NWAPGEPNNR	577.77	580.78	y7	Fab AbD18279	StrepTactin	95	0.5
						Fab AbD18288	StrepTactin	86	
						IgG AbD18279	Protein G Dynabeads	91	0.5
						IgG AbD18288	Protein G Dynabeads	82	
						rabbit Mab	Protein G Dynabeads	110	0.5
ADA17	ADAM17	VDNEELLPK	528.78	531.79	y7	Fab AbD18260	StrepTactin	23	
						Fab AbD18307	StrepTactin	37	2.9
						IgG AbD18260	Protein G Dynabeads	5	
						IgG AbD18307	Protein G Dynabeads	2	1860
						Mouse Mab	Protein G Dynabeads	49	2.9

[a]For each peptide, the transition for the listed fragment ion was used for quantitation. Recovery is the average of three replicates. The antibody with greatest recovery was used in response curves. Limit of detection is the lowest point detected on a response curve with signal greater than three times the standard deviation of the noise. Protein concentration (ng/mL) is calculated assuming complete trypsin digestion of 10 μL plasma.

For each antigen, the two Fabs with the highest affinity for the free peptide were converted to the hIgG1 format and produced in mammalian cell culture. The selection and affinity data for these six antibodies are shown in Table 1. Both the purified Fab and hIgG1 products of these six clones were assessed as reagents in immuno-MRM assays.

Comparison of Performance Characteristics of Fabs and Mabs in Immuno-MRM

We initially compared the performance of the available capture reagents by estimating peptide recovery using each reagent (Table 3). Recovery was determined by measuring the amount of spiked light peptide (relative to heavy peptide) prior to and following the enrichment process. Specifically, two samples were prepared. In the first, a known amount of light synthetic peptide was measured relative to the stable isotope standard with no enrichment. To the second sample, the light peptide was added to diluted plasma digest and captured by the antipeptide antibody. Following the enrichment, the stable isotope standard was added and the ratio (light/heavy) measured by mass spectrometry. The relative ratio of light/heavy peptide in the two samples (before and following the enrichment process) was used to estimate the recovery efficiency.

Recoveries of the three peptides for each affinity reagent tested are shown in Table 3. Overall, the performance of the Fabs compares very well to the monoclonal antibodies. For two out of three peptides (GDSLAYGLR from Osteopontin and NWAPGEPNNR from E-Selectin), the Fabs and the hIgG1 antibodies have recoveries greater than 85%, similar to the rabbit monoclonal antibodies. For the peptide VDNEELLPK (ADAM17), the best recovery was obtained using the mouse monoclonal antibody. The Fab was successful in capturing the peptide, but little recovery was obtained using the converted hIgG1 antibody. In each case, the antibody fragment showing the highest recovery corresponded to the antibody clone with the highest measured affinity.

To further assess the performance characteristics of the reagents in the immuno-MRM assay format, we assessed the linear range, limit of detection (LOD), and precision for each combination of peptide/antibody in a response curve in a complex plasma matrix. To eliminate any interference in signal from endogenous analyte, we varied the amount of heavy stable isotope-labeled synthetic peptide while keeping the light synthetic peptide at the same concentration in each sample (10 fmol). Response curves were constructed using the equivalent of 10 μL of digested neat plasma as background matrix, and the enrichment step was performed in triplicate for each concentration point. For each peptide sequence, the antibody fragment and the respective IgG showing the highest recovery (Table 3) were selected for use in the curve.

Figure 3 shows the response curves for each peptide. The curves resulting from enrichment of a given analyte using each affinity reagent (Fab, hIgG1, and Mab) are plotted together. Overall, the dynamic range of response was at least three to four orders of magnitude. The curves overlay each other for the affinity reagents tested, with the exception of the full length IgG antibody for VDNEELLPK (ADAM17). LODs were deter-

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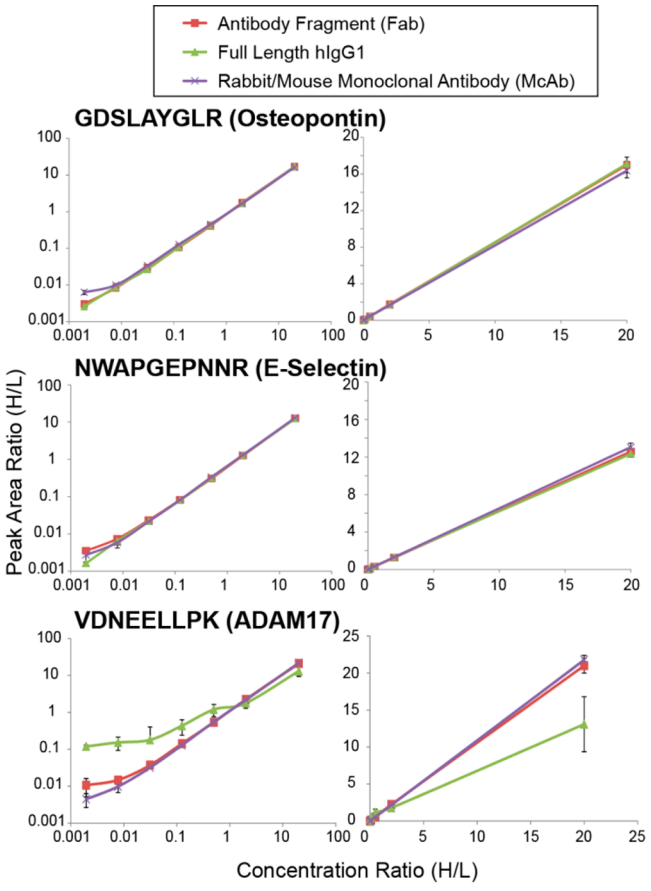

Figure 3. Response curves for each peptide/affinity reagent. The concentration of heavy peptide was varied and measured relative to the light peptide signal. One antibody fragment and full-length IgG was used for each peptide, GDSLAYGLR (AbD 18303), NWAPGEPNNR (AbD 18279), and VDNEELLPK (AbD 18307). For each peptide, the curve is plotted on a log 10 scale and a linear scale. Curves obtained from using antibody fragments (red), IgG (green), and rabbit or mouse monoclonal antibodies (purple) are overlaid. Error bars are the standard deviation of three replicate measurements.

mined by taking the concentration on the response curve nearest a signal intensity corresponding to three times the standard deviation of the noise. The LODs (Table 3) are comparable among the reagents tested. For the Osteopontin peptide, GDSLAYGLR, the Fab and full length IgG1 showed slightly improved sensitivity compared with a rabbit monoclonal antibody. Further investigation of the absolute peak areas (Figure 4) shows higher signals for the GDSLAYGLR peptide using the Fabs and IgG. Given that recovery efficiencies for these antibodies were similar, the increase in peak area is likely due to a decrease in ion suppression from the background, that is, a decrease in nonspecific binding. LODs for the peptide VDNEELLPK (from ADAM17) were comparable between the Fab and McAb, but the converted IgG antibody was not successful in detecting peptide below the highest concentration level. The precision (expressed as percent coefficient of variation, %CV) for the replicate captures is presented in Table 4. The average CVs are comparable for each affinity reagent, showing similar performance.

The performance of the Fabs in the immuno-MRM application was excellent. Results in the recovery experiment and the full response curve were comparable or superior to existing assays employing traditionally developed monoclonal

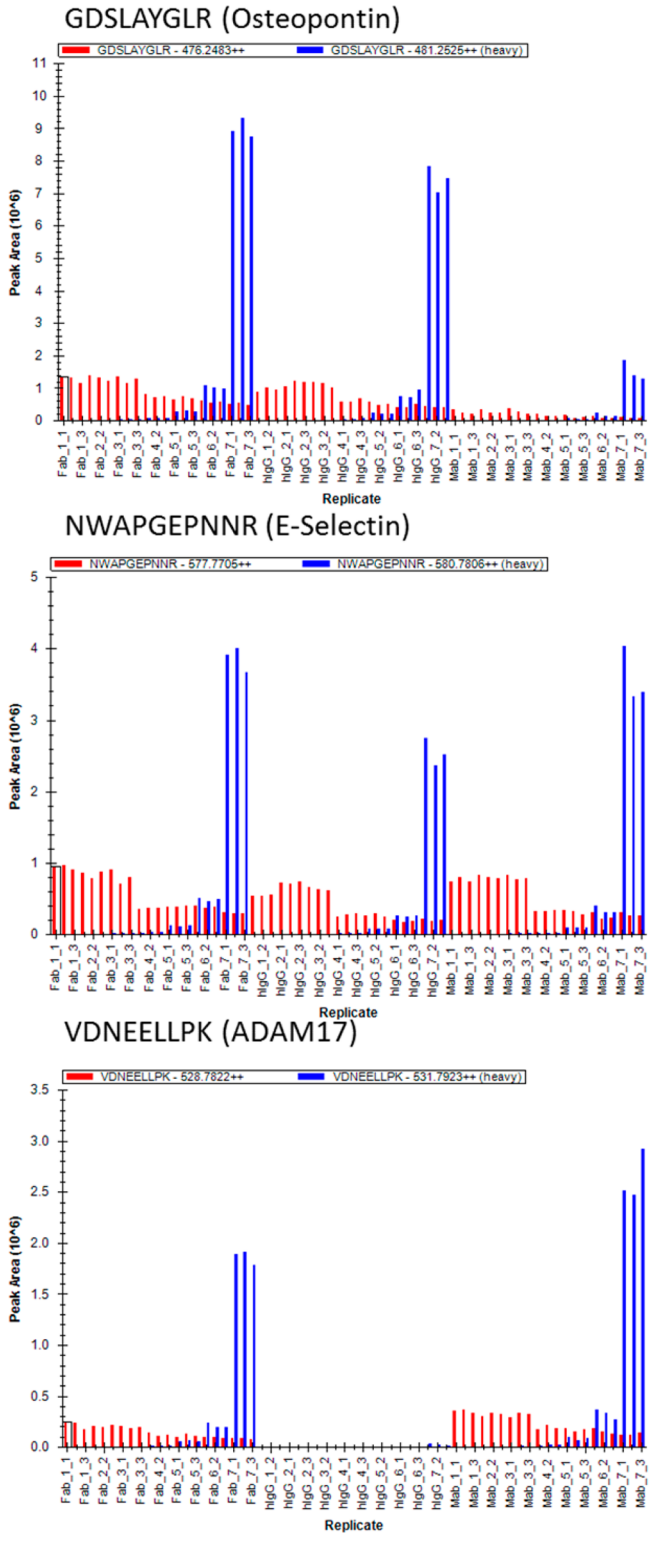

Figure 4. Peak areas for each replicate of the response curves. Light signal (red) and heavy signal (blue), comprising the sum of three transitions, are shown for each replicate point of the response curve. Each antibody type is denoted on the *x* axis (Fab, IgG, Mab). For the osteopontin peptide (GDSLAYGLR), the Fab and IgG show higher overall peak areas. Peak area is similar for the NWAPGEPNNR peptide for each reagent type. For the peptide VDNEELLK, the Fab and Mab perform similarly, but the IgG form did not effectively capture the peptide.

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Table 4. Precision of Measurements at Each Concentration Point in the Response Curve[a]

analyte	curve concentration (ng/mL)	%CV Fab	%CV IgG	%CV Mab
osteopontin	0.3	4.3	11.7	
(GDSLAYGLR)	1	6.5	6.2	8.9
	4	8.2	13.0	8.5
	16	2.4	4.0	11.9
	65	1.6	2.7	6.5
	649	5.0	1.7	4.8
	average	*4.7*	*6.5*	*8.1*
E-selectin	0.5	10.9	21.1	27.8
(NWAPGEPNNR)	2	3.9	11.1	4.1
	8	11.8	9.6	8.2
	33	6.1	2.1	5.3
	133	0.5	2.2	4.9
	1333	3.2	2.8	3.3
	average	*6.1*	*8.1*	*8.9*
ADAM17	3	14.9		0.9
(VDNEELLPK)	12	10.0		12.3
	47	1.9		7.7
	186	5.8		2.6
	1860	4.7	28.5	2.8
	average	*7.5*	*28.5*	*5.3*

[a]% CV is based on three replicate captures of peptide in 10 μL of plasma digest. Points above the limit of detection are reported. Protein concentration (ng/mL) is calculated assuming complete trypsin digestion.

antibodies. We were also successful in employing StrepTactin beads to bind the Fabs through a double-extended Strep tag, enabling the use of the Fabs directly in the assay. This saves additional preparation steps of converting the Fab to full-length immunoglobulin. Where applications might require the full length antibody, we found two out of the three antibodies in our study to produce working IgG with the same or better performance compared with the existing monoclonal antibodies. In the case of VDNEELLPK (ADAM17), the Fab showed good performance in the immuno-MRM assay, but the IgG form of the antibody did not. The performance of the IgG version of this antibody in the MSD-SET assay was also clearly worse than the Fab format, while the appearance of the purified IgG on a gel was normal (not shown). This discrepancy in performance between the Fab and IgG version is not yet understood.

In summary, we demonstrate the feasibility of isolating and incorporating high-affinity Fabs into peptide immuno-MRM assays. The Fab fragments obtained after selection were functional for peptide immuno-MRM by directly attaching them to magnetic beads via their Strep-tag.

More extensive studies are needed to statistically evaluate the overall success rate in selecting such affinity reagents as a routine approach to assay reagent generation. This includes using more diverse targets spanning a range of properties including length, hydrophobicity, charge, and containing post-translational modifications. While all three antibody fragments showed similar performance to monoclonals in this proof-of-principle study, it is possible that some peptides will not yield high-affinity recombinant antibodies, as observed with traditional immunizations.

The potential advantages in time savings of such an approach are attractive. Using the approach described here, recombinant antibody generation including production and quality control takes about 12 weeks, in contrast with the 6 to 9 months needed for traditional, animal-derived monoclonal antibody generation. Because selection of HuCAL antibodies is done entirely in vitro and has been automated,[21] many projects can be handled in parallel without the corresponding increase in timelines.

HuCAL PLATINUM is a synthetic, highly diverse (45 billion members), and modular library that contains high-affinity (dissociation constant <10 nM) antibodies to the majority of protein antigens tested.[24] However, generating high-affinity antibodies to short linear peptides is considered to be much more demanding. Therefore, we incorporated affinity maturation into the overall antibody generation process. Because of the modularity built into HuCAL, CDRs can be modified on the level of antibody gene pools, allowing affinity maturation during the panning process[25] without significantly expanding the overall process timelines. However, it is not yet clear whether in-process affinity maturation is actually needed, because for one of the peptides (ADAM17) we isolated only medium-affinity antibodies, but the best Fab (K_D = 33 nM) nevertheless performed successfully in immuno-capture experiments. Further experiments will be necessary to determine whether antibodies directly selected from the library without affinity maturation would qualify for immuno-MRM assays, which would further shorten the timelines for antibody generation to 8 weeks.

It is not readily apparent if there are significant cost savings over traditional approaches to monoclonals due to the possibility of multiplexing and the unanswered question of how often affinity maturation will be required. Furthermore, the cost for recombinant antibody generation in an automated way as described here will largely depend on multiplexing, that is, on the number of projects performed in parallel. It is also not known if other recombinant libraries would produce similar antibodies. Numerous antibody libraries similar to the one used here have been generated by others, including academic institutes, and these alternative libraries may also prove capable of producing antibodies with sufficient affinities. Now that feasibility has been established, it would be of great interest to pan additional recombinant libraries for high-affinity binders to linear, tryptic peptide antigens for incorporation into peptide immuno-MRM assays.

■ CONCLUSIONS

This work demonstrates the feasibility of isolating antibody fragments from a naïve antibody library to support robust immuno-MRM-based quantification. Further work on larger sets of analytes will help in determining the overall success rates and feasibility of making recombinant reagents for the enrichment of a diversity of targets, such as modified peptides.

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Notes

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■ ABBREVIATIONS

MRM, multiple reaction monitoring; IgG, immunoglobulin; Fab, antibody fragment; McAb, monoclonal antibody

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