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Complete List of Authors:	Liu, Weijing; University of Notre Dame, Department of Chemistry Pang, Yongle; Michigan State University, Chemistry Patel, Nitin; Takara Bio, Inc Jokhadze, Gia; Takara Bio, Inc Tan, Hui Yin; University of Notre Dame, Chemistry Guthals, Adrian; Mapp Biopharmaceutical, Inc. Bruening, Merlin; University of Notre Dame, Chemical and Biomolecular Engineering and Chemistry;

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Enzyme-Containing Spin Membranes for Rapid Digestion and Characterization of Single Proteins†

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Weijing Liu,‡[a] Yongle Pang,‡[b] Hui-Yin Tan,[a] Nitin Patel,[c] Gia Jokhadze,[c] Adrian Guthals,[d] and Merlin L. Bruening[*,a,e]

Proteolytic digestion is an important step in characterizing protein sequences and post-translational modifications (PTMs) using mass spectrometry (MS). This study uses pepsin- or trypsin-containing spin membranes for rapid digestion of single proteins or simple protein mixtures prior to ultrahigh-resolution Orbitrap MS analysis. Centrifugation of 100 µL of pretreated protein solutions through the functionalized membranes requires less than 1 min and conveniently digests proteins into large peptides that aid in confirming specific protein sequence variations and PTMs. Peptic and tryptic peptides from spin digestion of apomyoglobin and four commercial monoclonal antibodies (mAbs) typically cover 100% of the protein sequences in direct infusion MS analysis. Increasing the spin rate leads to a higher fraction of large peptic peptides for apomyoglobin, and MS analysis of peptic and tryptic peptides reveals mAb PTMs such as N-terminal pyroglutamate formation, C-terminal Lysine clipping and glycosylation. Relative to overnight in-solution digestion of mAbs, spin digestion yields higher sequence coverages. Spin-membrane digestion followed by infusion MS readily differentiates a mAb to the Ebola virus from a related antibody that differs by addition of a single amino acid.

Introduction

This paper develops spin-membrane proteolysis for rapid digestion of simple protein mixtures prior to mass spectrometry (MS) and/or tandem mass spectrometry (MS/MS) analysis. Bench-top centrifugation through these spin membranes can occur in any laboratory. Moreover, the resulting peptides, which are often large, readily confirm antibody post-translation modifications (PTMs) and single amino acid variations. Proteolysis is often a crucial step in characterizing protein sequences and post-translational modifications (PTMs) through mass spectrometry (MS) and/or tandem mass spectrometry (MS/MS) analysis.[1] Compared to MS and MS/MS characterization of intact proteins, analyses of proteolytic peptides yield greater sequence information as well as greater resolution in separations with liquid chromatography (LC).[2] However, conventional peptide generation using in-solution digestion employs long incubation times (up to 24 h) because of the low enzyme concentrations required to avoid self-digestion.[3-7] Unfortunately, oxidation or other protein modifications may occur during long digestions.[8, 9] Considering the time for digestion and desalting before or after the digestion, the whole process may take more than one day. Filter-aided sample preparation (FASP) applies an ultrafiltration membrane device as a 'proteomic reactor' for detergent removal, buffer exchange, chemical modification and protein digestion.[10, 11] This device shortens the sample preparation time to 1.5-18 h.[10] However, digestion is still the 'rate-determining step' because proteolysis occurs with low enzyme concentrations in the solution above a membrane.

Several research groups and companies developed immobilized-enzyme reactors for rapid protein digestion.[12, 13] The high enzyme-to-protein ratio in these reactors greatly improves the digestion rate, and immobilization can also increase enzyme stability and decrease autolysis[3-7, 14] Solid supports employed to create immobilized-enzyme reactors include monoliths,[15-19] capillaries,[20, 21] magnetic particles,[22-24] resins,[25-28] microfluidic chips,[29, 30] and membranes.[31-34] We are particularly interested in membrane supports because they are inexpensive, and varying the flow rate through these thin structures affords solution residence times that range from msec to sec.

Initially, enzyme immobilization in membranes relied on hydrophobic interactions in poly(vinylidene difluoride).[34, 35] Xu and coworkers later formed a trypsin-containing membrane through sequential adsorption of poly(styrene sulfonate) (PSS) and trypsin in porous nylon.[31] PSS adsorbs strongly to nylon, presumably through multiple hydrophobic interactions, to create a negatively charged surface. With a pI of ~10.5, trypsin is positively charged in acidic solution and electrostatically adsorbs to negatively charged PSS-modified membranes. This adsorption procedure gives a membrane reactor with a local concentration of ~10 mg of trypsin per mL of membrane pores, which is 450 times higher than the typical trypsin concentration for in-solution digestion. The short radial diffusion distances within the microporous membrane pores further facilitate

[a]*Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556, United States*
[b]*Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, United States*
[c]*Takara Bio USA, Inc., Mountain View, California 94043, United States*
[d]*Mapp Biopharmaceutical, Inc., San Diego, California 92121, United States*
[e]*Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, Indiana 46556, United States*
E-mail: mbruenin@nd.edu; Tel: +1(574)631-3024
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‡These authors contributed to this paper equally.

rapid digestion. Tan et al. used a similar strategy to form pepsin-containing membranes.[32]

Recently, we exploited a pepsin-containing membrane to facilitate monoclonal antibody (mAb) characterization.[33] By varying the antibody residence time (from 3 ms to 3 s) in the membrane, we obtained "bottom-up" (1-2 kDa) to "middle-down" (5-15 kDa) sized peptides, and these peptides covered the entire sequences of two different antibodies. Both the method of protein immobilization and membrane pore diameters may also affect peptide sizes.[36] However, for all the aforementioned membrane-based digestions, protein passage through the membrane employed relatively cumbersome systems that included syringe or peristaltic pumps. To overcome this challenge, we developed a membrane fitting that attaches to a disposable pipette tip.[37] This allows rapid digestion, but loading the membrane into the ferrule fitting, achieving a good seal, and extended production of the device are challenging.

This paper describes protein digestion using spin membranes containing immobilized pepsin or trypsin. During centrifugation, protein solutions pass through the spin membrane in 1 min or less to yield proteolytic peptides for subsequent direct infusion analysis with an Orbitrap ultrahigh resolution mass spectrometer. Although now less common than LC-MS/MS, direct infusion MS analysis with peptide mass fingerprinting can rapidly characterize sequences and post-translational modifications in simple protein mixtures. Infusion analysis of apomyoglobin and four commercial monoclonal antibodies (Herceptin, Avastin, Rituxan and Vectibix) typically yields 100% sequence coverage for each protein and identifies PTMs. To further demonstrate the potential application of such spin devices, we digested a mixture containing two anti-Ebola mAbs that differ by a single lysine insertion. The spectra reveal the sequence difference between the antibodies.

Experimental

Materials

Nylon membranes (LoProdyne LP, nominal pore size 1.2 μm, 110 μm thickness) were purchased from Pall Corporation. Trastuzumab (Herceptin, Genentech), Bevacizumab (Avastin, Genentech), Rituxan (Rituximab, Genentech) and Panitumumab (Vectibix, Amgen) were obtained in their commercial formulations as a gift from Dr. Muhammad Chisti of Michigan State University. Trypsin from bovine pancreas (TPCK-treated, lyophilized powder, ≥10,000 BAEE units/mg protein), pepsin from porcine gastric mucosa (lyophilized powder, 3200-4500 units/mg protein), ammonium bicarbonate (≥99%), iodoacetamide (IAM, ≥99%), dithiothreitol (DTT, ≥99.5%), polystyrene sulfonate (PSS, average molecular weight ~70,000), formic acid (FA, ≥98%) and acetonitrile (ACN, HPLC grade, ≥99.9%) were purchased from Sigma Aldrich. Sequencing grade modified trypsin for in-solution digestion was obtained from Promega. NaCl (ACS grade) and HCl (ACS grade) were purchased from CCI Chemicals. Other chemicals include urea (≥98%, Invitrogen), tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl, >98%, Fluka), trifluoroacetic acid (TFA, EMD), acetic acid (HOAc, ACS, Macron Fine Chemicals), and methyl alcohol (anhydrous, MeOH, Macron Fine Chemicals). Solutions were prepared in deionized water (DI water, Milli-Q, 18.2 MΩ·cm at 25 °C). C4 ZipTips were purchased from EMD Millipore, and Pierce C18 spin columns were used to isolate tryptic peptides after digestion. Amicon ultra 0.5 mL centrifugal filters (MWCO of 10 kDa) were employed to desalt samples before pepsin in-membrane digestion, and an Eppendorf centrifuge (5415D) was used to conduct spin digestion.

Functionalized membrane-containing spin columns

Trypsin- and pepsin-containing membranes were prepared using a slight modification of our literature procedure.[31-33] Membranes were UV/ozone-cleaned for 10 min, and 10 mL of 0.02 M PSS in 0.5 M NaCl (pH=2.3) was circulated through the membrane for 10 min using a peristaltic pump, followed by rinsing with passage of 30 mL of DI water. For trypsin-containing membranes, after adsorption of PSS and rinsing, 5 mL of 1 mg/mL trypsin (TPCK-treated) in 2.7 mM HCl was circulated through the membrane for 1 h. Subsequently, the membrane was rinsed with 30 mL of 1 mM HCl, dried with N_2, and stored in a desiccator. For pepsin-containing membranes, 4 mL of 2 mg/mL pepsin in 5% FA was circulated through the PSS-modified membrane for 1 h. Then, the membrane was rinsed with 30 mL of 5% FA, dried with N_2, and stored in a desiccator. Flow rates during membrane modification were 2 mL/min. The modified membranes were embedded in spin devices at Takara Bio USA, Inc., (Mountain View, CA). These devices expose a membrane surface with a diameter of ~1.8 mm. Dry membranes have a shelf life up to one year when containing trypsin and up to 5 months when containing pepsin.

Apomyoglobin spin digestion with pepsin- and trypsin-containing membranes

Apomyoglobin (10 μg) was dissolved in 100 μL of 10 mM NH_4HCO_3 for trypsin digestion, and in 100 μL of 5% FA for pepsin digestion. The spin column was rinsed with 100 μL of 10 mM NH_4HCO_3 or 100 μL of 5% FA before tryptic or peptic spin digestion, respectively. Both enzymatic digestions were conducted at two spin rates corresponding to 500 g and 10,000 g. The centrifugation time was 1 min, and the digests were dried with a SpeedVac after spin digestion and immediately reconstituted for MS analysis.

mAb spin digestion with pepsin- and trypsin-containing membranes

For pepsin digestion, Herceptin (He), Avastin (Av), Rituxan (Ri) and Vectibix (Ve) were each diluted in deionized water to prepare stock solutions with 1 mg/mL of antibody. Afterward, 2 μL of 0.1 M HOAc and 2 μL of 0.1 M TCEP-HCl were added to stock solution containing 20 μg of antibody prior to incubation at 75 °C for 15 min. Subsequent buffer exchange with 5% FA employed 3 cycles of centrifugation with an Amicon ultra 0.5 mL centrifugal filter (MWCO of 10 kDa). About 25 μL of solution remained after each centrifugation, and 475 μL of 5% FA was added prior to the following centrifugation. Residues were diluted to 200 μL with 5% FA to make 0.1 mg/mL solutions.

For trypsin digestion, 4 μL of 10 mg/mL antibody stock solutions of each of the four mAbs were diluted separately in 14 μL of 2 mM TCEP-HCl solution in 0.1% HOAc containing 8 M urea. The mixtures were incubated at 50 °C for 10 min prior to addition of 14 μL of 20 mM IAM in 2 M NH_4HCO_3 containing 8 M urea, and incubation in the dark for 30 min. Finally, 12 μL of 30 mM DTT in 100 mM NH_4HCO_3 containing 8 M urea was added followed by incubation in the dark for 20 min to quench the IAM. After reduction and alkylation, the residual solutions were diluted with DI water to create 0.1 mg/mL solutions. For nanogram samples, the antibody solution was further diluted to contain 10, 50, 250, 500, or 1000 pg/μL of antibody in 10 mM NH_4HCO_3. Thus, 100 μL solutions contained from 1 to 100 ng of antibody.

Within 1.5 h of antibody pretreatments, 100-200 μL of each nonalkylated antibody solution was added to a pepsin spin column, and 100-200 μL of each alkylated antibody solution was added to a trypsin spin column. The solutions were centrifuged through the

membrane for 1 min at 500 g. For nanogram input samples, an additional 100 µL of 10 mM NH_4HCO_3 elution buffer was subsequently spun through the membranes to increase peptide recovery. Pepsin spin digestion samples were collected for direct infusion MS analysis, whereas trypsin spin digestion samples were first desalted using Pierce C18 spin cartridges (following the manufacturer's protocol) before infusion analysis. The C18 spin column was activated with 50% MeOH and equilibrated in 0.5% TFA in 5% ACN. Then, the sample was loaded onto the column, followed by washing with 0.5% TFA in 5% ACN. Finally, the peptides were eluted from the spin column with 70% ACN. The nanogram input samples were desalted using C18-µZipTips using the same buffers employed with the C18 spin column. In-membrane antibody spin digestions were also monitored by SDS-PAGE. The reproducibility of the spin-membrane digestion was tested by running triplicate pepsin digestions of Av with a separate membrane for each digestion. Sequential peptic and tryptic digestions with the same membrane employed rinses with 5% formic acid and 10 mM NH_4HCO_3, respectively, prior to subsequent digestions. In a few cases, we allowed antibody solutions to sit above the membrane for 30 min without spinning through the membrane. These solutions show a few proteolytic peptides, but most of the protein is intact. In most cases, the user would add the protein to the spin cartridge and perform centrifugation within a minute, so any digestion above the membrane is minimal.

mAb in-solution digestion with pepsin and trypsin

We conducted in-solution peptic and tryptic digestion of four antibodies to compare to in-membrane spin digestion. For peptic in-solution digestion, 5 µL of 0.2 µg/µL pepsin solution was added to 200 µL of the 0.1 mg/mL nonalkylated, reduced, buffer-exchanged antibody solution prior to incubation at 37 °C for 16 h. The reaction was quenched with 200 µL of ACN. Samples were then dried with a SpeedVac before MS analysis. For tryptic in-solution digestion, 5 µL of 0.2 µg/µL sequencing grade or TPCK-treated trypsin solution was added to 200 µL of the 0.1 mg/mL alkylated antibody solution prior to incubation at 37 °C for 16 h. The reaction was quenched by adding 5 µL of acetic acid. Samples were then desalted with a C18 spin column and dried with a SpeedVac before reconstitution and infusion MS analysis. To study the in-solution digestion efficiency in terms of the digestion duration, we performed in-solution digestion of He and Av for 5, 30, or 60 min and of apomyoglobin for 5, 15, 30, or 60 min with incubation at 37 °C.

Mass spectrometry and data analysis

In-membrane spin digests and in-solution digests were dried with a SpeedVac and reconstituted in 1% acetic acid, 49% H_2O, and 50% methanol within 1 day. Then, 40 µL of each sample was loaded into a Whatman multichem 96-well plate (Sigma–Aldrich) and sealed with Teflon Ultrathin Sealing Tape (Analytical Sales and Services, Prompton Plains, NJ). An Advion Triversa Nanomate nanoelectrospray ionization (nESI) source (Advion, Ithaca, NY) was used to introduce the sample into a high-resolution accurate mass Thermo Fisher Scientific LTQ Orbitrap Velos mass spectrometer (San Jose, CA) that was equipped with a dual pressure ion trap, HCD cell, and ETD. The spray voltage and gas pressure were set to 1.4 kV and 1.0 psi, respectively. The ion-source interface had an inlet temperature of 200 °C with an S-Lens value of 57%. High-resolution mass spectra were acquired in positive ionization mode across the m/z range of 400–1800, using the FT analyzer operating at a mass resolving power of 100,000. Spectra were the average of 100 scans. Signals with >1% of the base peak intensities and S/N>3 were analyzed. Peptide identification was performed manually using ProteinProspector to generate a list of peptide masses (v 5.14.1, University of California, San Francisco, CA) with carbamidomethyl as a constant modification for tryptic digests. Using the generated list, we searched for glycosylation manually based on the known asparagine glycosylation sites and glycan masses. We also identified pyroglutamate and lysing clipping manually from the expected mass changes. The mass tolerance for peptide identification was set to 10 ppm.

For LC-MS/MS, Nano-Ultra High Performance Liquid Chromatography MS/MS was performed essentially as described previously.[38] Two-µL injections corresponding to 500 ng of spin-digested tryptic protein (reconstituted in 0.1% FA) were loaded onto a 100 mm x 75 µm C18-BEH column (Waters Billerica, MA), and separated over a 90 min gradient from 5-35%B on a nano-Acquity system (Waters) flowing at 500 nL/min. Solution A was 0.1% FA in H_2O, and solution B was 0.1% FA in ACN. MS/MS was performed on an LTQ-Velos Orbitrap-FTMS instrument (Thermo, San Jose, CA) running a top-20 data-dependent method, where a single MS at a resolution of 60,000 was acquired, and the top-20 precursors were selected for fragmentation.

Raw LC-MS/MS files were processed by MaxQuant version 1.5.6.0. MS/MS spectra were searched against the Cricetulus griseus (Chinese hamster) proteome (23,884 proteins). The database also included common contaminants and the antibody sequences. MaxQuant analysis parameters included a precursor mass tolerance of 20 ppm for the initial search, a precursor mass tolerance of 6 ppm for the main search, and an FTMS MS/MS match tolerance of 20 ppm. We set trypsin as the specific enzyme. Variable modifications included oxidation (M), deamidation (NQ), and Gln->pyro-Glu, while the fixed modification was carbamidomethyl on cysteine. The minimal peptide length was set to 6 amino acids, the maximum peptide mass was 8000 Da, and the maximum number of missed cleavages was 5.

Results and discussion

Workflow for digestion in membrane-containing spin columns

Fig. 1 shows the workflow that we use to conduct protein digestion in spin membranes. After protein pretreatment, the solution simply passes through the membrane reactor during centrifugation. Digestion of 100 µL of protein solution requires a centrifugation time less than 30 sec. The high concentration of enzyme in the membrane pores affords rapid digestion of protein, and we can control the digestion by varying the spin rate. Moreover, the tiny dead volume (0.275 µL) of the spin membrane should minimize peptide loss during digestion.

Fig. 1 Workflow for protein spin digestion and analysis.

Apomyoglobin spin digestion with pepsin- and trypsin-containing

membranes

We chose apomyoglobin (17 kDa, 153 AA), a common standard for peptide mapping, to initially test the spin digestion. Apomyoglobin has a compact hydrophobic core at neutral pH and undergoes slow in-solution proteolysis at pH 8.[39] Reduction and alkylation are not necessary for digesting this protein because it has no disulfide bonds. Using both trypsin and pepsin spin membranes and different spin rates prior to infusion MS analysis, we always observed 100% apomyoglobin sequence coverage (percentage of the protein sequence covered by the identified proteolytic peptides) after spin digestion in a single pass through the membrane.

Fig. 2 shows deconvoluted ESI-Orbitrap mass spectra of peptic apomyoglobin digests obtained using spin digestion at 500 and 10,000 g. Four peptides, amino acids 1-29, 30-106, 107-137, and 138-153 cover the whole sequence after digestion at 500 g, whereas three peptides, amino acids 1-29, 30-106, and 107-153, cover the sequence after digestion at 10,000 g. When the spin rate increases to 10,000 g, the signals of large peptides such as amino acids 30-106 and 107-153 increase dramatically. At the same time, signals for smaller peptides, including amino acids 30-69 and 70-106 decrease. Increasing the spin rate leads to a higher fraction of large peptides, presumably because lower residence times in the membrane decrease the proteolysis time to generate more missed cleavages. By varying the centrifugation rate, we can obtain overlapping peptides using a single enzyme.

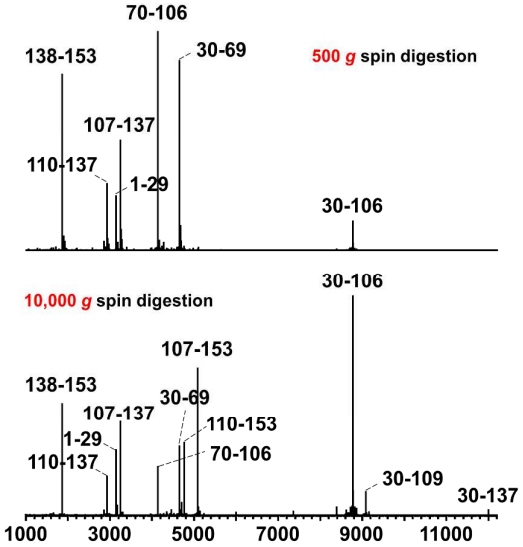

Fig. 2 Deconvoluted ESI-Orbitrap mass spectra of apomyoglobin peptic digests obtained through 500 g (top) and 10,000 g (bottom) spin digestion. Deconvoluted mass spectra were generated with Xtract software and combine signals from different charge states and isotopes to show signals only from the monoisotopic singly charged species. Numbers represent the amino acid sequences of the identified peptides.

The aspartic protease pepsin exhibits less specificity than trypsin.[40] However, extensive studies of pepsin digestion show that this protease prefers to cleave peptide bonds after phenylalanine (F) and leucine (L).[41] Our results match the cleavage site preferences for pepsin. Peptic peptides 30-69, 70-106, 107-153, and 138-153 result from cleavage of the 29L-30I, 69L-70T, 106F-107I, and 137L-138F bonds. Tables S-1 and S-2 of the electronic supplementary information (SI) list the peptic peptides identified from digestion at 500 and 10,000 g, respectively.

Trypsin is the most common enzyme used for protein digestion.[42] Compared with pepsin, it has higher specificity, cleaving proteins and peptides at the C-terminus of lysine (K) and arginine (R), except when followed by proline (P). Moreover, at low pH tryptic peptides carry at least two positive charges, which benefits downstream collision-induced dissociation tandem mass spectrometry (CID-MS/MS) analysis. Fig. S-1 shows the ESI-Orbitrap mass spectrum of a tryptic spin digest of apomyoglobin. A spin at 500 g gives no intact apomyoglobin in one pass through the membrane. We identified 26 tryptic apomyoglobin peptides in MS spectra, and as few as seven tryptic peptides cover 100% of the sequence: amino acids 1-31, 32-47, 48-63, 64-77, 78-96, 97-133, and 134-153. Table S-3 of the SI gives a full list of the identified peptides, most of which contain 1 or more missed cleavage sites. The missed cleavages may prove problematic for quantitation, but they avoid low sequence coverages that result from the formation of undetectable small peptides. Large peptides also simplify the interpretation of mass spectra for simple mixtures. Despite the missed cleavage sites, digestion patterns are reproducible (see below).

Different from peptic spin digestion, we don't see the emergence of large peptides at higher rates of centrifugation in tryptic spin digestion. Instead signals of undigested, intact protein appear. This may stem from the compact structure of apomyoglobin at pH 7-8 as well as the high activity of trypsin. After an initial cleavage, structures of the resulting peptides likely open rapidly to allow further digestion, although the initial cleavage is slow. Thus, increasing the spin rate yields intact protein rather than limited proteolysis. In contrast, for the case of denatured antibody proteolysis (see below), in-membrane tryptic digestion yields peptides with many missed cleavages.

mAb spin digestion with pepsin-containing membranes

Antibodies have unique Y-shaped structures that include inter- and intra-chain disulfide bonds.[43] Thus, we used mAbs to examine digestion of proteins with disulfide crosslinks. Moreover, enzymatic digestion is crucial for antibody characterization and quality control.[44] We previously employed a cumbersome homemade setup with a syringe pump for controlled in-membrane peptic digestion of antibodies.[33] Here we examine spin digestion using both trypsin- and pepsin-containing membranes and four different therapeutic antibodies.

Proper antibody pretreatment, which is vital to effective, reproducible digestion, is different for pepsin and trypsin. For peptic digestion, we used TCEP as the reducing agent. Unlike DTT, TCEP can reduce disulfide bonds under acidic conditions where pepsin has maximum activity. Moreover, antibodies partially denature at pH 2-3, and this should increase access to cleavage sites. Acidic conditions also decrease the rate of some unwanted modifications such as deamidation[45] and prevent reformation of disulfide bonds after reduction, thus avoiding the need to add chaotropic and alkylation agents. Different from our previous workflow, just prior to the spin digestion we added a buffer-exchange step using 10 kDa molecular weight cutoff membranes. Because of the high concentration of salt in the commercial antibody formulation,[46] desalting is important for downstream MS or MS/MS analysis. In digestion, we employed 30 s for spinning 100 µL of desalted antibody solutions through membranes at 500 g, but

the time required for the solution to pass through the membrane is actually less than 30 s.

After in-membrane digestion, direct-infusion MS analysis of peptic peptides from Herceptin (He), Avastin (Av), Rituxan (Ri) and Vectibix (Ve) gives 100% sequence coverage for all of the antibodies to afford rapid antibody characterization. The SI (Figs. S-2 to S-5) provides sequence maps for the four antibodies and Tables S-4 to S-7 give the peptide sequences. The average length of the 365 identified peptic peptides from He, Av, Ri and Ve spin digests is 36 amino acids, indicating that spin proteolysis at 500 g generates middle-down sized peptides that enable rapid confirmation of protein PTMs. We identified glycosylation on the heavy chains of all four antibodies. As an example, Fig. 3(A) shows isotopic envelopes from H265-309 of He containing two glycoforms. The deconvoluted masses of the two envelopes, 6705.1693 and 6867.2208, differ by 162 Da because of an additional galactose unit on the larger peptide. N-terminal pyroglutamate formation appeared on the Ri light chain (Lc), Ri heavy chain (Hc) and Ve Hc. We also saw C-terminal Lysine clipping on Av, Ri and Ve. Fig. 3(B) demonstrates C-terminal Lysine clipping for Ve.

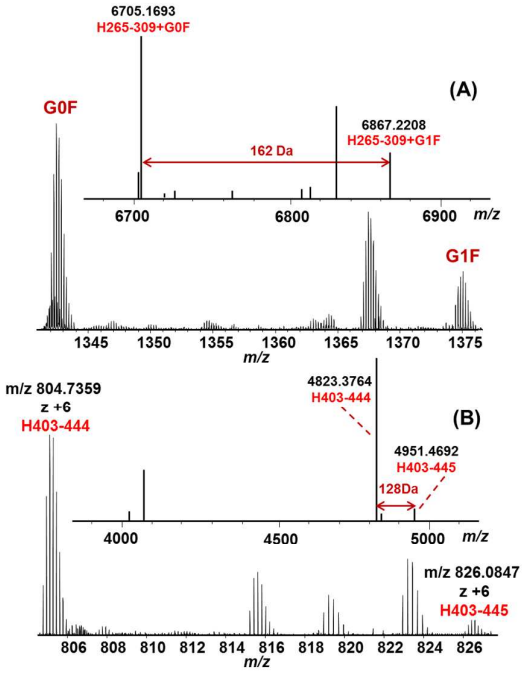

Fig. 3 Identification of PTMs. (A) Part of the mass spectrum of Herceptin showing isotopic envelopes from H265-309 containing 2 N300 glycoforms, G0F and G1F. (B) Part of the mass spectrum of Vectibix showing isotopic envelopes from H403-444 and H403-445. These peptides differ by a C-terminal lysine. The insets show parts of the manually deconvoluted spectra in which normalized intensities are the sums of the intensities for all detected charge states of the given mass range, and the spectra show the signals only at the +1 charge state for the monoisotopic mass.

In the SI, Figs. S-6 through S-9 present the original mass spectra of the four mAbs, and Tables S-4 through S-7 list the identified peptic peptides. Some signals with the same m/z value are present in the spectra of all four antibodies. For example, signals corresponding to M+H of 8858.2650 result from light chain 136-214 (L136-214) of He, L136-214 of Av, L135-213 of Ri, and L136-214 of Ve. Another peptide with M+H of 4823.3821 stems from heavy chain 408-449 (H408-449) of He, H411-452 of Av, H409-450 of Ri, and H403-444 of Ve. More examples appear in the peptide lists in Tables S-4 through S-7, as one would expect because the antibodies He, Av, Ri and Ve share the same sequences in a large part of the Lc and Hc constant regions. The presence of the same peptides in the spectra of four antibodies shows that rapid spin digestion is a powerful method for comparing proteins with similar sequences. Signals that are present in one mass spectrum but not another give hints for the parts that are different in two proteins. Moreover, the similar peptides demonstrate the consistency of the digestion sites.

To further test the reproducibility of spin proteolysis, we digested Av with three different spin membranes. For the twenty highest signals in the mass spectra, standard deviations of the signal intensities (relative to the base peak) from triplicate digestion are <6% (SI Fig. S-10 presents the mass spectra). The spectra do not show signals of intact protein, and gel electrophoresis further confirms no intact protein after digestion of all four antibodies (Fig. 4). Moreover, pepsin spin membranes effectively cleave proteins in repetitive digestion with the same membrane. Each digest from 3 cycles of pepsin proteolysis with the same spin device covers 100% of the Av sequence without a significant signal intensity drop after reuse (SI, Fig. S-11). Gel electrophoresis further supports complete digestion from all 3 cycles (SI, Fig. S-12). Despite the reusability, most researchers would probably employ the membranes only once to avoid any potential contamination.

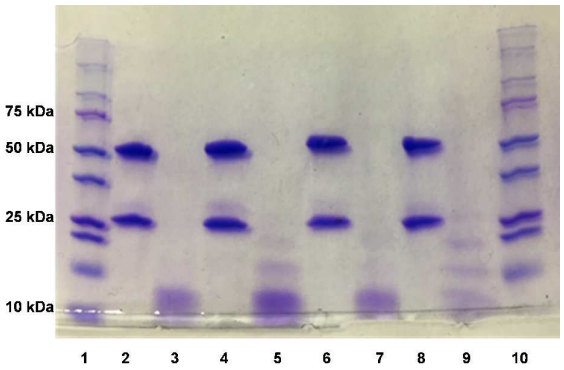

Fig. 4 Gel electrophoresis (SDS-PAGE) analysis of antibodies before and after digestion in a peptic spin column. Lanes 1 and 10: protein standards; Lanes 2, 4, 6 and 8: 5 µg of Herceptin, Avastin, Rituxan and Vectibix, respectively; Lanes 3, 5, 7 and 9: 5 µg of Herceptin, Avastin, Rituxan and Vectibix peptic spin digests (spun at 500 g), respectively.

One concern with in-membrane digestion is that peptide adsorption in the membrane may reduce the digestion yield. We previously showed that passing a complete in-solution peptic apomyoglobin digest through a pepsin-containing membrane at the end of a pipette tip results in minimal signal loss, consistent with low adsorption.[37] To examine the extent of antibody adsorption during spin-membrane digestion, we calculated the digestion yield based on tryptophan fluorescence using a literature procedure.[11, 47] For Av and Ri, which contain 13 and 12 tryptophan residues, respectively, the digestion yields were 80% from pepsin spin digestion (Table S-8), which is comparable to yields using FASP and higher than with simple in-solution digestion. The high digestion efficiency and reproducibility will enable qualitative comparison of

different batches of antibodies despite the large number of missed cleavages. We should note that trypsin leaching from a membrane could increase the calculated digestion yield by ~10% (see the discussion below on trypsin leaching).

mAb spin digestion with trypsin-containing membranes

We previously digested an antibody using a trypsin membrane connected to a pipet tip, even without protein alkylation.[37] However, reforming and/or scrambling of the disulfide bonds might occur under basic conditions. In developing a general protein pretreatment, we decided to conduct protein alkylation. Desalting is also necessary because of the large amount of denaturation and alkylation agents. We initially tried to desalt the reduced antibody before trypsin spin digestion. However, when desalting at the protein level using a C4 ZipTip, MS spectra did not reveal identifiable peptides after spin digestion, presumably because of a large sample loss during desalting. Zhao et al. found that reduced antibodies tend to precipitate during elution with 50% ACN and 0.1% FA.[48]

To avoid the instability of reduced, desalted antibodies, the desalting step should occur after tryptic spin digestion. We were concerned that the salt and chaotropic agents in the digestion mixture, especially 0.8 M urea, might overcome electrostatic interactions between PSS and trypsin in the digestion membrane. However, this protocol yielded detectable tryptic peptides that cover 100% of the He, Av and Ve sequences, and 94% of the Ri sequence (infusion MS). Prior studies indicate that in-solution tryptic digestion generates peptides with average lengths of ~14 amino acids.[49] In contrast, spin digestion gives tryptic peptides with up to 10 missed cleavages. Based on 311 tryptic peptides identified from He, Av, Ri and Ve, the average antibody tryptic peptide length is 37 amino acids after spin digestion. A limited proteolysis time apparently leads to missed cleavage sites. As mentioned, this may lead to challenges in quantitative methods, but the larger peptides facilitate rapid characterization of antibody complementarity determining regions (CDRs) with simple infusion MS. For example, a large tryptic peptide, L1-108 of Ve, covers all the light-chain CDRs, which makes characterization of three CDRs possible with a single peptide. As with peptic digestion, spin-membrane tryptic digestion enables identification of PTMs such as glycosylation, N-terminal pyroglutamate formation and C-terminal Lysine clipping. Fig. 5 contains the sequence map of Ve and shows the location of PTMs. In the SI, Figs. S-13 through S-16 present the original mass spectra of the four mAbs. Tables S-9 through S-12 (SI) present the full list of identified tryptic peptides, and Figs. S-17 to S-19 give sequence maps for the other antibodies.

Unlike the pepsin-containing membranes, the trypsin membranes show a decline in the extent of digestion during second and third uses. Gel electrophoresis indicates that the tryptic spin column continues to digest Av over the three cycles, but the second and third cycles contain some intact heavy chain and light chain (SI, Fig. S-20). MS spectra confirm that all three cycles of digestion give detectable peptides, but the signal intensities decrease after the first and second digestions (SI, Fig. S-21). The decline in digestion after the first use may result from some trypsin leaching or inactivation (see below). Spin membranes with covalent immobilized native trypsin or dimethylated trypsin may provide a potential solution to achieve better reusability.

In-solution digestion with pepsin and trypsin

For all four mAbs, we compared the performance of in-solution and spin-membrane peptic and tryptic digestion. Not surprisingly, overnight in-solution digestion gives smaller proteolytic peptides than spin digestion due to fewer missed cleavages. In-solution pepsin digestion yielded an average peptide size of 16.3 amino acids from 340 identified peptides, and trypsin in-solution digestion gave an average peptide size of 22.9 amino acids from 89 assigned peptides. In-solution digestion also gave lower sequence coverages (infusion MS) than spin digestion. For pepsin in-solution digestion, the

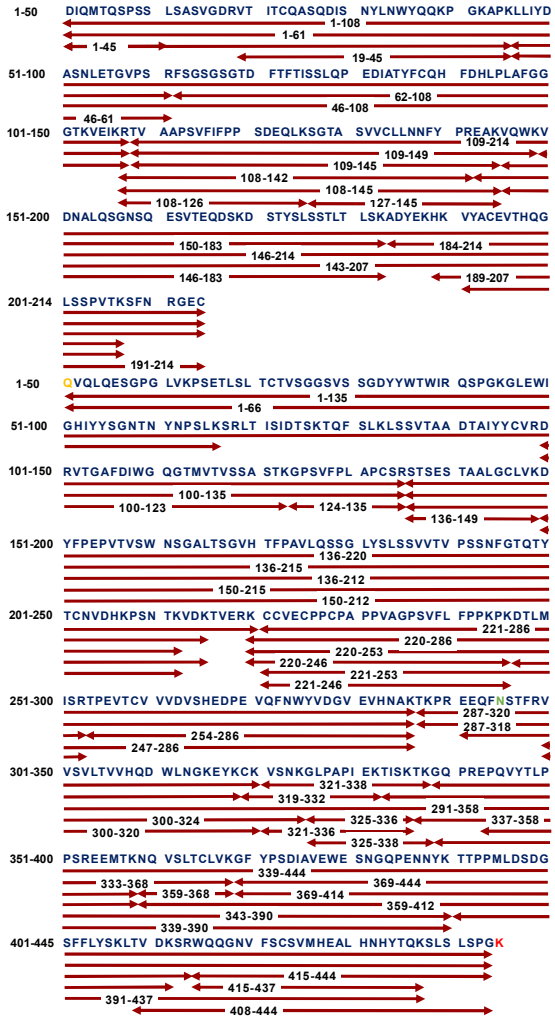

Fig. 5 Sequence map of the peptides identified from infusion ESI-Orbitrap analysis of an in-membrane tryptic digest of Vectibix. The orange "Q" indicates N-terminal pyroglutamate formation, the light green "N" denotes the glycosylation site, and the red "K" represents the C-terminal clipping.

sequence coverages of He, Av, Ri and Ve were 81.9%, 81.7%, 86.9%, and 87.1%, respectively. For trypsin in-solution digestion, the sequence coverages of He, Av, Ri and Ve were 67.1%, 68.6%, 73.5%, and 76.3%, respectively. This is significantly lower than the typical 100% sequence coverage achieved with spin-membrane digestion, where larger peptides lead to the high coverage. Fig. 6 compares the number of missed cleavage sites in the proteolytic peptides from all four mAbs after tryptic spin and overnight in-solution digestion. The broader distribution and increased number of identified peptides from spin digestion facilitate the sequencing of mAbs and determination of PTM sites.[50] Tables S-9 to S-12 and S-17

to S-20 of the SI list the identified tryptic peptides.

Like spin digestion, in-solution tryptic digestions for shorter times can potentially yield large peptides that increase sequence coverage. With the same TPCK trypsin used to fabricate membranes, however, even 1-h in-solution digestions still give significant amounts of intact He and Av (electrophoretic gels, see SI, Fig. S-22) and relatively low sequence coverages (~70%). In contrast, short-time in-solution digestion with sequencing grade trypsin enhances the sequence

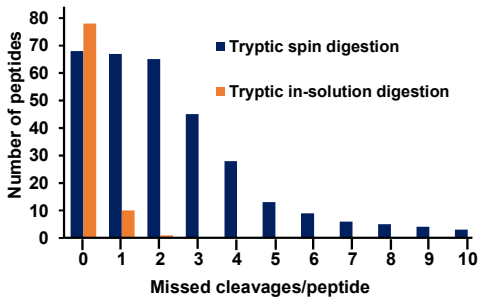

Fig. 6 Distribution of the number of peptides containing missed cleavages after either tryptic spin digestion or overnight digestion in solution.

coverage significantly. We obtained no intact antibody (based on gel electrophoresis) and 100% sequence coverage in infusion MS analysis of He and Av after in-solution tryptic and peptic digestions as short as 5 min. Even with only 5 min of incubation, however, in-solution peptic digestion of He and Av generates relatively small peptides, which complicates peptide identification from MS data. Perhaps some digestion occurs after the acetonitrile quenching and during drying of the digest.

With apomyoglobin tryptic in-solution digestion, proteolytic peptides cover the whole sequence regardless of the in-solution incubation time, but even with sequencing grade trypsin, digests generated with 5 and 15 min of in-solution digestion reveal intact proteins in both electrophoretic gels and MS spectra (SI, Figs. S-23 and S-24). Apomyoglobin is a globular protein, and we did not denature it prior to proteolysis, so its tryptic digestion is significantly slower than that of reduced, denatured mAbs. In contrast, tryptic spin digestions yields no intact apomyoglobin because of the high immobilized-protease concentration. Overall, compared to in-solution digestion, the spin membrane overcomes the shortcoming of TPCK-treated trypsin, gives more complete tryptic digestion of apomyoglobin, and separates much of the enzyme from the digest. This separation may allow the use of spin membranes for consecutive digestion with multiple proteases without methods for protease removal. With pepsin, in-membrane digestion also gives larger peptides than in-solution digestion.

LC-MS/MS analyses.

Direct infusion nanoESI is a powerful method for peptide mapping because of its short sample analysis time (<3 min for data collection). Also, injection of all peptides into the mass spectrometer avoids the peptide losses that are inevitable in LC.[51] However, peptides have different ionization efficiencies,[52] and ion suppression may occur during infusion MS analysis.[53] Moreover, with protein mixtures spin digestion may generate hundreds or thousands of peptides, making effective direct infusion analysis impossible.

LC-MS/MS readily analyzes complex mixtures of digested proteins, and its well-developed bioinformatics software makes data analysis possible. As an initial test of whether spin-membrane digestion enables analysis of antibody sequences using LC-MS/MS, we examined the antibody tryptic spin digests. (Peptic digests are more difficult to analyze in LC-MS/MS due to the limited cleavage specificity.[40]) Because mAbs are typically expressed in Chinese hamster ovary cell lines,[54] we identified proteolytic peptides through comparison to the hamster reference proteome from Uniprot, with the addition of the mAb sequences to the database. Using MaxQuant data analysis with this protein data base, we compared the sequence coverage and number of unique peptides identified after tryptic digestion either in a spin membrane or in solution.

As Table 1 shows, for all four antibodies tryptic spin digestion gives higher or essentially equal sequence coverages and more unique peptides than in-solution digestion. The missing sequences in the light chain after in-solution digestion likely result from undetectable small peptides with 3 or 4 amino acids. Heavy-chain sequence coverages are lower in LC-MS/MS analysis than in direct infusion analysis primarily because some of the large peptides are missing and we didn't consider the glycosylation on the heavy chain in the MaxQuant search. Glycosylated peptides also show low ionization efficiencies.[55] Enzymatic removal of the glycans prior to digestion will likely give heavy-chain sequence coverages near 100%. In comparing in-solution and spin digestion, the additional unique peptides from spin digestion may enhance protein identification in database searching with protein mixtures.

Table 1. Antibody Sequence Coverages and Numbers of Unique Peptides Obtained From LC-MS/MS Analyses of Tryptic Spin and In-solution Digests* (Lc = light chain and Hc = heavy chain).

Tryptic Spin Digestion					
		Herceptin	Avastin	Rituxan	Vectibix
Sequence Coverage	Lc	100%	100%	100%	100%
	Hc	81.3%	83.6%	77.8%	100%
Unique Peptides	Lc	42	41	32	37
	Hc	65	72	46	56
Tryptic In-solution Digestion					
		Herceptin	Avastin	Rituxan	Vectibix
Sequence Coverage	Lc	87.4%	93.5%	93.4%	94.9%
	Hc	83.7%	73%	74.7%	76.4%
Unique Peptides	Lc	16	17	18	16
	Hc	32	33	32	31

*Peptides were identified using MaxQuant Software with comparison to a Chinese hamster proteome modified with antibody sequences.

Up to 5 peptides that arise from trypsin autolysis also appeared in nanoLC-MS/MS analyses, and the combined intensity of tryptic peptide ions was ~10% of the combined intensity of the antibody peptides. The ratio of total antibody passing through the membrane (10 µg) to trypsin in the pores (~1.3 µg) is ~7. This ratio along with the signals from the tryptic peptides suggest that a significant fraction of trypsin may leach (perhaps due to the high concentration of urea) or self-digest in the membrane. Quenching the reaction with acetic acid immediately after spinning the protein solution through the membrane still led to no remaining intact antibody (observed with gel electrophoresis), so most of the digestion likely occurs in the membrane and not in the membrane effluent. Moreover, the number and composition of identified

peptides were similar between digestions that were immediately quenched and those that that were dried down over 30 min, again indicating that most of the digestion occurs in the membrane. Preliminary data show that the use of dimethylated trypsin (to minimize autolysis) decreases the intensity of trypsin autolysis peptide ions down to 1% of the intensity of antibody peptide ions. Thus, there are strategies for reducing signals from tryptic peptides if needed.

Digestion of low amounts of protein

Spin membranes might prove particularly useful for digesting small amounts of protein. To investigate this possibility we performed spin digestions with 0.1-mL solutions containing from 1 to 100 ng of Herceptin (6.7 to 670 fmoles). NanoLC-MS/MS analysis identified Herceptin peptides even with the lowest antibody concentrations. However both the sequence coverage and the number of identified peptides decrease with the amount of antibody (Table S-21). For the peptide ions detected at all antibody concentrations, nanoLC-MS/MS signals decrease linearly with the amount of digested antibody, suggesting that label-free quantitation is possible (Fig. S-25). Overall, spin digestion is a powerful method for fast protein digestion, and proteolytic peptides from spin digestion are suitable for downstream direct infusion or LC-MS/MS analysis. The technique may prove useful for digestion of small amounts of immunoprecipitates after buffer exchange, but we have not explored this possibility.

Identifying single mutations in antibody sequences

Characterization of sequence variations is crucial in antibody engineering.[56] To demonstrate the utility of in-membrane digestion for identifying antibody mutations, we performed spin digestion of a mixture of a native Ebola antibody (Z) and its mutated form (ZK). The only difference in the sequences of these two antibodies is that ZK has an extra lysine K107 on the light chain. The sequences of Lc amino acids 103-115 and 103-116 for the native and mutated antibodies, respectively, are KLELRTVAAPSVF and KLELKRTVAAPSVF. Residue K107 is next to an arginine on the mutated sequence, so trypsin digestion will give two theoretical small peptides LELK or LELKR if there are no missed cleavages or 1 missed cleavage in this region. Considering the basic residues in these two peptides, the m/z value will be <200 m/z after protonation, and this mass spectral region contains a large amount of noise that will interfere with the analysis. However, with rapid peptic digestion, infusion MS analysis readily identifies a number of relatively large peptides that demonstrate the differences between the two antibodies. In particular, as Fig. 7 shows, the +5 peptide with m/z of 968.489 is L87-131 of Z, whereas the +5 peptide with m/z of 994.1185 comes from L87-132 of ZK. The deconvoluted mass difference between these two peptides is 128.1475, the mass of a lysine residue. Interestingly, in this region many of the cleavage sites are different for the two antibodies, implying that the extra lysine changes the digestion pattern. Table 2 presents some of the peptides that reflect the sequence differences of Z and ZK. Although the digestion patterns in this region of the two antibodies are different, the data clearly show the presence of the two antibodies and indicate that the sequences differ by a single lysine residue.

A tryptic spin digestion also identified the mutation site, but with fewer distinguishing peptides. We identified peptides L108-141 from Z and L 108-142 from ZK with deconvoluted masses of 3724.9126 and 3881.0063 respectively. The mass difference of these two peptides is 156.0937 which corresponds to an arginine residue. These results indicate that both pepsin and trypsin spin membranes can digest mixtures of Z and ZK to differentiate one from the other, even though they have similar sequences. Peptic in-solution digestion gives a different peptide pattern with fewer distinguishing peptides. The number of distinguishing peptides decrease with increasing incubation time from 17 to 14 to 11 with in-solution peptic digestion for 5 min, 30 min and 1 h, respectively. The number of distinguishing peptides from spin peptic digestion is 23 because the generation of longer peptides enhances the possibility that these peptides cover the mutation site. Successful digestion of antibody mixtures with spin membranes could either confirm the antibody sequence for quality control or validate the desired mutation in antibody engineering. Confirmation of mutations is also possible in separate solutions of each antibody.[57]

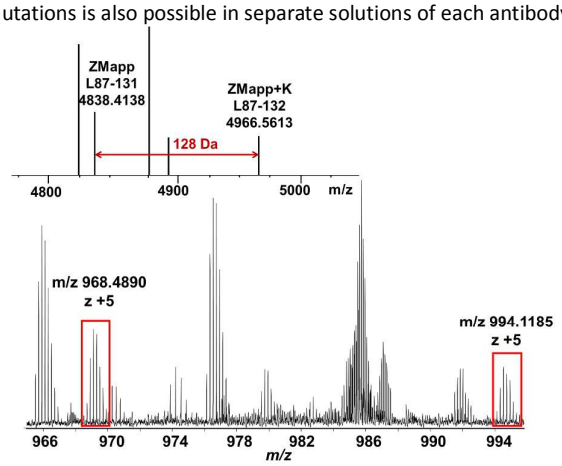

Fig. 7 Mass spectrum containing signals of two peptides representing the sequence variation between Z and ZK. The inset shows part of the deconvoluted spectrum where the normalized intensities are signal intensities for all detected charge states of the given mass range. The deconvoluted spectrum show the signals only at the +1 charge state for the monoisotopic mass.

Table 2. Selected MS signals that correspond to differences in Z and ZK in an analysis of a peptic spin digest of a mixture of these antibodies

m/z	Charge state	Amino acid	Peptide from Z	Peptide from ZK
984.8411	+9	L71-149	✓	×
1329.9656	+3	L78-113	✓	×
815.2025	+5	L84-120	✓	×
1061.2125	+3	L87-115	✓	×
968.4890	+5	L87-131	✓	×
782.7314	+6	L88-131	✓	×
973.8546	+3	L107-134	✓	×
1020.1722	+9	L66-148	×	✓
1537.7625	+6	L78-160	×	✓
1384.7032	+6	L84-158	×	✓
1089.7652	+5	L86-135	×	✓
828.1858	+4	L87-116	×	✓
994.1185	+5	L87-132	×	✓
1235.3133	+3	L88-121	×	✓
1318.7984	+7	L89-172	×	✓

955.9127	+5	L91-135	×	✓

Conclusions

This work used pepsin/trypsin spin membranes as microreactors for reproducible proteolysis prior to MS analysis. The high concentration of enzyme in the membrane pores allows spin digestion of 100 µL of antibody solution within 1 min. Peptic spin digestion avoids protein alkylation because the acidic conditions prevent reforming of disulfide bonds, whereas tryptic spin digestion benefits from alkylation. Moreover, with peptic spin digestion we can control the proteolytic peptide size by varying the spin rate. Direct infusion MS of spin digests is fast and typically provides 100% peptide coverage along with identification of PTMs for Herceptin, Avastin, Rituxan and Vectibix. Spin membranes also enable identification of the mutation site in antibody sequences. In summary, the spin-digestion platform is rapid, simple, and user-friendly, and it may afford control over peptide sizes for various types of subsequent MS analyses. Parallel digestions in spinnable 96-well plates containing membranes should also be possible.

Conflicts of interest

Takara Bio USA now offers commercial spin-digestion membranes.

Acknowledgements

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TOC text

Centrifugation of antibodies through enzyme-containing spin membranes yields large proteolytic peptides that enable confirmation of protein sequences and post-translational modifications.

TOC graphic