Published in final edited form as:
Anal Chem. 2010 December 15; 82(24): 10045–10051. doi:10.1021/ac101857j.

Facile Trypsin Immobilization in Polymeric Membranes for Rapid, Efficient Protein Digestion

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Abstract

Sequential adsorption of poly(styrene sulfonate) and trypsin in nylon membranes provides a simple, inexpensive method to create stable, microporous reactors for fast protein digestion. The high local trypsin concentration and short radial diffusion distances in membrane pores facilitate proteolysis in residence times of a few seconds, and the minimal pressure drop across the thin membranes allows their use in syringe filters. Membrane digestion and subsequent MS analysis of bovine serum albumin provide 84% sequence coverage, which is higher than the 71% coverage obtained with in-solution digestion for 16 h or the <50% sequence coverages of other methods that employ immobilized trypsin. Moreover, trypsin-modified membranes digest protein in the presence of 0.05 wt% sodium dodecyl sulfate (SDS), whereas in-solution digestion under similar conditions yields no peptide signals in mass spectra even after removal of SDS. These membrane reactors, which can be easily prepared in any laboratory, have a shelf life of several months and continuously digest protein for at least 33 h without significant loss of activity.

Proteolytic digestion followed by mass spectrometry (MS) analysis of the resulting fragments is the most common method to identify proteins and investigate post-translational modifications.1 Typically, digestion occurs upon mixing proteases such as trypsin, lys-C, or chymotrypsin with substrate proteins in buffer solutions, where the ratio of protease to substrate protein is low (e.g. 1:20~1:50 for trypsin) to avoid autoproteolysis of the protease. However, the low concentration of proteases frequently requires long incubation times for complete digestion (often more than 16 h).2 Additionally, solution digestion is not very effective in the presence of surfactants, e.g. SDS, which are frequently required for protein solubilization.

This work demonstrates that trypsin immobilized in nylon membranes can digest protein in a few seconds even in the presence of 0.05 wt% SDS. Moreover, compared to other proteolysis methods, including previous studies with immobilized trypsin, digestion of bovine serum albumin (BSA) with these modified membranes leads to 1.4- to 4-fold times as many peptide signals in matrix-assisted laser desorption/ionization (MALDI)-mass spectra. The membrane modification method, electrostatic adsorption, employs inexpensive supports and occurs in about 1.5 h using simple reagents that are readily available.

A number of reports show that immobilization of trypsin on a support can greatly decrease digestion time compared to in-solution digestion.3 The enhanced efficiency stems primarily

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Supporting Information Available
Most material in the SI is mentioned in the text, including experimental procedures, membrane digestion efficiency as a function of concentration, and MALDI-mass spectra. The SI also contains tables of identified peptide sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

from the high enzyme concentration at the substrate surface,[4–7] and previous materials employed for trypsin immobilization include polyvinylidene fluoride (PVDF) membranes,[4, 6] micro-particles,[8–10] monoliths,[5, 11, 12] and polymeric microfluidic channels.[8, 13] These bioreactors digest proteins in times ranging from seconds to minutes, and a recent study of digestion by trypsin entrapped in nanoporous particles suggests that large peptides generated in the initial proteolysis step undergo further proteolysis much faster than in typical in-solution digestion.[14] However, accelerating the transport of the substrate protein into the particle pores is critical for increasing the total digestion rate.[14] Thus, rapid digestion will likely require convective transport of proteins to immobilized proteases in systems such as monolithic columns, where flow-induced ingress of substrate protein into micron-size pores accelerates the encounter between the substrate protein and immobilized enzyme. In addition, monoliths are suitable for direct coupling with high performance liquid chromatography (HPLC)-MS analysis because of their relatively low back pressure (compared to packed bed columns) and the short radial diffusion distances that enhance digestion rates.[3, 11] All of the reported monolith-based fast digestion studies employ covalent linking for trypsin immobilization, which demands a high enzyme concentration in modification solutions[12] or reaction times as long as 19 hours.[5, 11, 12] The random covalent linking (via the N-terminus or lysine residues) of trypsin to the support may lead to a loss of enzyme activity.[15]

Commercially available polymer membranes are similar to monoliths because the numerous micron-size pores in the membrane lead to short radial diffusion distances (~1 μm) between the solution and the wall when the solution separates into many streams that pass through the membrane simultaneously (Scheme 1). Membranes have a much smaller thickness (usually about 100 μm) than monolithic columns, however, which results in very low transmembrane pressure drops that are especially desirable for simple syringe-based systems for protein digestion. In trypsin-modified membranes, the combination of convective transport, short radial diffusion distances, and a high localized trypsin concentration leads to efficient protein digestion in residence times as short as a few seconds.

Lee et al. demonstrated protein digestion using PVDF membranes that contain trypsin adsorbed to membrane pores via hydrophobic interactions.[4, 6] These modified membranes successfully digest single small proteins in minutes, but substrate proteins may displace trypsin by adsorbing to the PVDF support.[6] The presence of SDS and hydrophobic proteins might accelerate trypsin desorption and further shorten the effective lifetime of these materials. We observe a significant drop in digestion efficiency after passing several micrograms of even simple proteins through trypsin-modified PVDF membranes (see below).

As an alternative enzyme-immobilization strategy, electrostatic adsorption to a charged substrate is simple,[16] and the bound enzymes frequently show activities similar to or higher than those of enzymes in solution.[17–19] Liu et al. anchored trypsin in 100 μm-wide microfluidic channels using electrostatic layer-by-layer (LBL) adsorption with polyelectrolytes. Although this process is versatile and straight forward,[17, 18] the amount of accessible trypsin per solution volume in the 100 μm channels is much lower than in monolithic columns and membranes. Here we demonstrate trypsin immobilization through sequential adsorption of poly(styrene sulfonate) (PSS) and trypsin in the micron-size pores of a flat-sheet nylon membrane. This simple method yields trypsin-modified membranes that can be stored for several months with minimal loss in activity, and the immobilized enzyme is extremely effective in protein digestion.

Experimental Section

Materials

Trypsin (Type I, ~10,000 N_{α}-benzoyl-L-arginine ethyl ester hydrochloride units/mg protein, treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone), PSS (average molecular weight ~70,000), α-casein, BSA, 2,5-dihydroxybenzoic acid (DHB), dl-1,4-dithiothreitol (DTT), iodoacetamide (IAA), and SDS were purchased from Sigma-Aldrich. ZipTip SCX pipette tips, PVDF membranes (whole sheet, 0.45 μm pore size, 115 μm thickness), and nylon membrane discs (Hydrophilic, 0.45 μm pore size, 25 mm diameter, 170 μm thickness, HNWP02500) were obtained from Millipore. The low-pressure inline filter system (A-424) and Teflon tubing (I.D. 1/32" × 1/64") were acquired from Upchurch Scientific.

Modification of membranes with trypsin

Nylon membranes were modified with trypsin (nylon/PSS/trypsin) using a LBL method. After UV-ozone cleaning for 15 min, the nylon membrane was inserted in an Amicon cell (Model 8010, 10 mL, Millipore), and 10 mL of 0.02 M PSS in 0.5 M NaCl was pulled through the membrane at 3 mL/min using a peristaltic pump. Subsequently, the membrane was rinsed with 30 mL of water prior to circulation of 3 mL of 0.6 mg/mL trypsin in 2.7 mM HCl through the membrane at 3 mL/min for 1 h and rinsing with 30 mL of 1 mM HCl. For comparison, PVDF membranes were modified with trypsin (PVDF/trypsin) via hydrophobic adsorption using a literature procedure (see the supporting information (SI) for details).6

Protein digestion

Proteins were digested either directly in NH_4HCO_3 buffer without denaturation (only for α-casein) or after denaturation using urea or SDS.20 (See the SI for procedures.)

In-solution digestion—For in-solution digestion of protein, 10 μL of 0.5 mg/mL trypsin (prepared in 1 mM HCl) was added to a protein solution to achieve a 1:20 ratio of trypsin to substrate protein, and this solution was incubated at 37 °C for 16 h. The digestion was quenched by addition of 11 μL of acetic acid.

Membrane digestion—To evaluate the completeness of membrane digestion, a 25-mm-diameter nylon/PSS/trypsin membrane (big membrane in Scheme 2) was employed for digesting several mL of α-casein solution containing 10 mM NH_4HCO_3, and the digests were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, see SI for detailed procedures).

For smaller samples (10's of μL), a 4-mm disc was cut from a 25-mm-diameter trypsin-modified membrane and inserted in an Upchurch low-pressure inline filter system, with a frit between the membrane and the downstream tubing (Scheme 2). The effective filtration area was ~0.02 cm^2, as determined by exposure to dye in the holder and measurement of the diameter of the stained spot. Typically, prior to digestion the 0.5 mg/mL protein samples were diluted to 0.1 mg/mL with either water or 10 mM NH_4HCO_3 to achieve a final NH_4HCO_3 concentration of ~10 mM. Digestion occurred during passage of the solution through the miniaturized system at 0.1 mL/h using a syringe pump (Scheme 2). Some of the above protein digests were subjected to SDS removal or phosphopeptide enrichment21 (see the SI for procedures) prior to MS analysis.

MALDI-MS, collision-induced dissociation (CID)-MS/MS and data analysis

Prior to MALDI-MS analysis, protein digests were diluted (if needed) or reconstituted (after drying) to 0.1 mg/mL using either water or 10 mM NH_4HCO_3 to achieve a final NH_4HCO_3

concentration of ~10 mM (the only exception is the 0.02 mg/mL α-casein digest which was reconstituted as 0.1 mg/mL in water with a final NH_4HCO_3 concentration of 50 mM). One drop (0.5 μL) of these solutions was added to the MALDI plate and allowed to dry prior to addition of 0.3 μL of 40 mg/mL DHB (prepared in 0.1% TFA in 50% acetonitrile) and crystallization. Positive-ion mode MS was performed on an LTQ XL ion trap mass spectrometer equipped with a vMALDI source (Thermo Fisher Scientific, San Jose, CA). Tryptic peptides were subjected to CID-MS/MS analysis followed by a MASCOT search to confirm/identify peptide sequences (see the SI for details).

Results and discussion

Membrane modification with trypsin

Trypsin immobilization relies on simple electrostatic adsorption in a nylon membrane modified with a layer of adsorbed PSS. The porous membrane provides a large surface area-to-volume ratio, and the strong polyanion PSS likely adsorbs to the alkyl backbone of nylon via hydrophobic interactions to give negatively charged pores.[22, 23] With a pI of ~10.5,[24] trypsin is positively charged in acidic solutions and readily binds to the previously deposited PSS layer. Compared to covalent linking (e.g. epoxy ring opening reactions) to magnetic particles or monoliths,[5, 9] the electrostatic immobilization method, which simply involves passing solutions through the membrane, is easier and faster. Immobilization of the trypsin under acidic conditions (pH 3) where the enzyme is reversibly inactivated (trypsin is most active at pH 7–9) avoids autolysis,[25] and the high positive charge density of trypsin at pH 3 should enhance electrostatic adsorption to the PSS layer.

To estimate the amount of trypsin immobilized, we measured the UV absorbance of the trypsin solution before and after passage through the membrane. Based on a 0.10 mg/mL decrease in the concentration of the 3.00 mL trypsin loading solution after circulating through the membrane, the binding capacity is 0.097 mg of trypsin/cm^2 of membrane, which equals 11 mg of trypsin per cm^3 of membrane pores. (This calculation assumes a membrane thickness of 170 μm and a porosity of 50%. The effective external filtration area is 3.1 cm^2.) The amount of trypsin per pore volume is 450-fold greater than the trypsin concentration in a typical in-solution digestion (0.025 mg/mL). In a control experiment with a bare nylon membrane (no PSS layer) no detectable trypsin binding occurred, confirming that the enzyme immobilization takes place via electrostatic interaction with the PSS layer.

Efficiency of α-casein digestion as a function of residence time in the membrane and protein concentration

α-Casein (without denaturation) served as the initial model protein for examining membrane-based digestion efficiency at different flow rates and protein concentrations. The electropherogram in Figure 1a shows no detectable undigested protein after passing a 0.1 mg/mL α-casein solution through a 25-mm-diameter nylon/PSS/trypsin membrane at flow rates ranging from 0.02 to 2.0 mL/min (residence times of 79–0.79 s). The detection limit of SDS-PAGE with Coomassie blue staining is 0.1–0.5 μg,[26] and Lanes 3–6 were loaded with 7 μg of membrane-digested protein, so the electropherogram suggests at least 93% digestion. Figure S1 (figure and table numbers beginning with “S” refer to the SI) shows the gel electropherogram of membrane–digested α-casein in solutions with initial protein concentrations from 0.020–2.0 mg/mL. With a 16 s membrane residence time, the absence of detectable protein in the electropherogram again suggests greater than 93% digestion.

When using tryptic digestion for identification of post-translational modifications by MS, high sequence coverages are vital, and sequence coverage is a function of both the completeness of the protein digestion and the detection efficiency for the various tryptic

peptides. Membrane digestion of α-casein using a wide range of residence times (Figure 1b) or protein concentrations (Figure S2) and subsequent MALDI-MS give a relatively constant sequence coverage of ~53% for the α–S1 chain (22.9 kDa). The maximum sequence coverage of 43% for the α–S2 chain (24.3 kDa) occurs with a 3.2 s residence time (0.5 mL/min flow rate) and a 0.1 mg/mL protein concentration, but the sequence coverage for this chain is more variable than for the α–S1 chain. For comparison, the sequence coverages of α-S1 and α-S2 chains after in-solution digestion of 0.5 mg/mL α-casein in 50 mM NH_4HCO_3 for 16 h are 46% and 26%, respectively (see Figure S3). Thus, the membrane-based digestion affords sequence coverages equal to or better than those from solution digestion, and digestion is much faster in the membrane.

Protein digestion in a miniaturized nylon/PSS/trypsin membrane

In many cases, the volume of biological samples is very limited, and the Swinnex membrane holder used in the above studies has a large volume (~1.5 mL), which is only suitable for samples with more than 100 μg of proteins. To digest smaller amounts of protein, we cut the 25-mm trypsin-modified membrane into 4-mm-diameter discs and inserted a small disc inside a low pressure inline filter system, with a frit between the membrane and the downstream Teflon tubing. With an effective filtration area of 2 mm^2 and a ~4 μL dead volume, the miniaturized setup (Scheme 2) can process a few μL of protein sample.

Figure S3 shows the mass spectra of non-denatured α-casein digested both in solution and using the small membrane. The MALDI-MS sequence coverages of the α–S1 and α–S2 chains after digestion in the small membrane are 55% and 33%, respectively, and these values are slightly higher than those after in-solution digestion (46% for α–S1 and 26% for α–S2). With a flow rate of 0.1 mL/h (residence time of 6.4 s), digesting 1 μg of protein in a 0.1 mg/mL solution of protein takes 6 min.

BSA (66.4 kDa) is the substrate protein most widely used to examine digestion performance. Figure 2 shows the MALDI-mass spectra of urea-denatured BSA digested with the small membrane (6.4 s residence time) or in solution (16 h, 37 °C). Remarkably, the sequence coverage with membrane digestion (84%) is much higher than with in-solution digestion (49%) of 0.5 mg BSA/mL. However, the concentrations of protein, urea and buffer for in-solution digestion (Figure 2b), though typical for biological samples, are 5 times higher than for membrane digestion. These high salt and urea concentrations might result in decreased trypsin activity in solution. (The solution-digested samples were diluted 5-fold with water before analysis, so the lower sequence coverage is not due to extra urea or salt on the MALDI plate.) A five-fold dilution of the denatured-BSA solution with water prior to in-solution digestion leads to an increase in BSA sequence coverage to 71%, which is still lower than after membrane digestion. (The trypsin to protein ratio was 1:20 for all in-solution digestions.) Moreover, the membrane digestion results in detectable signals for 52 peptides whereas the dilute in-solution digestion leads to signals for only 37 peptides (Table 1). Of the extra 15 peptides, 4 contain no missed cleavage sites (Table S2).

Table 1 compares MS analyses of BSA digested by a number of different methods, including in-solution digestion, membrane digestion, and other techniques that employ immobilized trypsin on different substrates. Although the comparison is complicated by varying sample preparation conditions, digestion of BSA using the trypsin-modified nylon membrane allows detection of nearly twice as many peptides as most other techniques and gives a sequence coverage that is 24% higher than the next best method using immobilized trypsin (allowing 1 missed cleavage). The unusually high sequence coverage with the trypsin-modified membrane, even compared to other methods with immobilized trypsin, likely stems from (1) a high localized trypsin concentration, (2) short diffusion distances to

immobilized trypsin in the highly porous membrane, and (3) a high activity of the immobilized enzyme due to the electrostatic immobilization.

Digestion of SDS-denatured proteins by nylon/PSS/trypsin membranes

The anionic surfactant SDS is frequently present in protein samples as a denaturation agent,[29–31] a detergent for water-insoluble proteins,[32, 33] or the eluent for removal of immunoprecipitated antigens from antibody beads.[20, 34, 35] Despite its various applications, however, SDS often hinders protein digestion[36] and MS analysis,[33, 37, 38] and more effective methods are needed for protein digestion in the presence of SDS. We separately denatured α-casein and BSA (both 2.0 mg/mL) in 1 wt% SDS and diluted these solutions to 0.1 mg protein/mL with 10 mM NH_4HCO_3 for membrane digestion (final SDS concentration of 0.05%). Comparative in-solution digestions occurred with a 0.1 mg/mL protein solution containing 0.05% SDS, as well as with 0.5 mg/mL protein and 0.25% SDS. The latter protein concentration is more typical for in-solution digestion, and with both solutions we employ a 1:20 ratio of trypsin to substrate protein.

Figure 3a shows the MALDI mass spectrum of α-casein digested in the membrane in the presence of 0.05% SDS. Analysis of the spectrum yields sequence coverages of 46% for α-S1 (11 peptides) and 9% for α-S2 (3 peptides) chains. In contrast, the in-solution digestions with either 0.1 or 0.5 mg/mL α-casein containing 0.05% or 0.25% SDS, respectively, give rise to MALDI mass spectra that contain only surfactant- or matrix-related clusters (Figures 3b and 3c). Because SDS may suppress the ionization of peptides in addition to preventing digestion, we also collected the mass spectrum of a sample containing pre-digested α-casein (in-solution digestion without SDS) after mixing with SDS in 100 mM pH 7.4 tris-HCl buffer, and incubating in a boiling water bath for 5 min. This mass spectrum (Figure 3d) shows that the peptides from α-casein are readily detectable in the presence of 0.05% SDS, although a few SDS-related clusters appear in the low-mass region of the spectrum, and background signals are higher than without addition of SDS. This control experiment indicates that the absence of signals after in-solution digestion in the presence of SDS (Figures 3b and 3c) stems from inefficient digestion. In contrast, the immobilized trypsin shows significant tolerance for SDS.

The digestion of BSA, a much larger and more hydrophobic protein than α-casein, in the presence of SDS followed by MALDI-MS analysis, however, shows different results. Neither the membrane digestion nor the in-solution digestion lead to MS signals from BSA peptides. Moreover, the mixture of pre-digested BSA and SDS does not generate BSA peptide signals either, suggesting that the SDS greatly suppresses BSA peptide ionization. The interaction of SDS with the more hydrophobic peptides of BSA is likely stronger than that of SDS with α-casein peptides.

To examine whether trypsin-modified membranes can digest BSA in the presence of SDS, we removed SDS from the digested samples using SCX ZipTips to improve the peptide ionization. After SDS removal the mass spectrum of the membrane-digested BSA reveals 26 tryptic peptides (Figure S4a), which cover 57% of the protein sequence. In contrast, the in-solution digestion gives no BSA tryptic peptide signals even after SDS removal (Figures S4b and S4c).

The sequence coverages of α-casein and BSA after membrane digestion in the presence of SDS are lower than for similar digestion without SDS (α-S1 chain: 46% vs. 55%; α-S2 chain: 9% vs. 33%; BSA: 57% vs. 84%), and some autolysis of trypsin also occurs (e.g. peaks appear at m/z=2163 and 2289). This suggests that the SDS decreases the stability or activity of the immobilized trypsin to some extent. However, the immobilized trypsin shows much higher activity than free trypsin in the presence of SDS, and the 57% sequence

coverage of BSA with membrane digestion in SDS-containing solutions is still higher than the sequence coverages after digestion using most other reported techniques without SDS (Table 1). We should note, however, that when the SDS concentration is 0.25%, membrane digestion yields no peptide signals even after removal of SDS prior to analysis.

Durability and storability

To determine how much protein a small nylon/PSS/trypsin membrane (with ~2 μg of immobilized trypsin) can digest before losing its activity, we continuously passed a 0.1 mg/mL BSA solution (denatured with urea) through the miniaturized membrane reactor for 33 h (a total of 330 μg protein) at 0.1 mL/h while occasionally collecting effluent aliquots (20 μL). Analysis of these aliquots by MALDI-MS shows no drastic change in the peptide pattern (Figures S5a–S5c), and the sequence coverage is above 80% over the full 33 h (Figure 4). The S/N for BSA peptides also remains relatively constant for 33 h, and such a high durability for protein digestion suggests that the electrostatic deposition strategy produces a robust enzyme reactor.

In contrast a similar test with a PVDF/trypsin membrane shows a rapid sequence coverage decline from 78% to 19% after passing 390 μL of solution (39 μg of BSA) through the membrane (3.9 h) (Figure 4). Additionally, the S/N ratios of BSA peptides in MALDI-mass spectra (Figures S5d and S5e) drop dramatically with the amount of protein passed through the membrane. The much lower durability compared with the nylon/PSS/trypsin reactor suggests that the hydrophobic interactions between PVDF and trypsin are not as strong as the electrostatic interactions between trypsin and PSS. The substrate protein likely replaces the immobilized trypsin in the PVDF membrane.6

The nylon/PSS/trypsin membranes are also quite stable in long-term storage. At designated time intervals, a small piece was taken from a membrane (stored in a desiccator) and used for digestion of BSA in the miniaturized setup. The sequence coverage was 73% after 7 months (Figure S6), demonstrating good stability after long-term storage in a dry environment. Although the number of detectable BSA peptides decreased from 52 to 46 when using a 7-month old membrane, the S/N ratios in the MALDI-mass spectra were essentially constant.

Digestion of protein mixtures

In mixtures containing both low- and high-abundance proteins, effective analysis requires efficient digestion without respect to concentration. One example of this situation is the analysis of phosphorylated proteins in a large excess of non-phosphorylated protein.39, 40 We examined digestion (both in-solution and by nylon/PSS/trypsin membranes) of α-casein (a target phosphorylated protein) in a 10-fold excess of BSA (a non-phosphorylated protein) using enrichment to facilitate the MALDI-MS analysis of phosphopeptides. Simply analyzing the membrane-digested sample by MALDI-MS without phosphopeptide enrichment generates a complex spectrum that precludes determination of how well α-casein is digested and how many phosphorylation sites can be detected. However, after phosphopeptide enrichment on a MALDI plate modified with polymer-oxotitanium, most of the non-phosphorylated peptides are removed and phosphopeptides from the target protein α-casein dominate the spectrum (Figure 5). The spectra of the species enriched from both in-solution and membrane digests show somewhat similar patterns with comparable S/N for phosphorylated peptides. Both membrane and in-solution digestion reveal 9 phosphorylation sites for the α-S1 chain. However, for the α-S2 chain, membrane digestion reveals 8 phosphorylation sites, whereas in-solution digestion reveals only 5. This is consistent with the results described above, where in the absence of BSA, membrane digestion gives a slightly higher sequence coverage for α-casein than in-solution digestion. The membrane

digestion also takes much less time than in-solution digestion and could potentially be included in an online system.

Conclusions

Sequential adsorption of PSS and trypsin in nylon supports yields a high concentration of trypsin in membrane pores and allows proteolysis to occur in residence times of a few seconds. The MALDI-MS protein sequence coverage for membrane-digested α-casein is similar to or higher than that from in-solution digestion. Moreover, compared to in-solution or other digestion techniques including those with immobilized trypsin in monoliths, digestion in membranes leads to at least 1.4 times as many MS-detectable peptides for BSA. Miniaturized membranes consume only a few μL of sample, and processing of a 10 μL solution takes about 6 min. (This time may decrease significantly with optimization of conditions.) The immobilized trypsin also allows digestion in solutions containing 0.05% SDS, whereas solution digestion under similar conditions yields no peptide signals in MALDI-MS, even after SDS removal by cation-exchange prior to analysis. Due to the above advantages, as well as their long-term stability and simple fabrication, the trypsin-modified membranes can potentially simplify protein analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the US National Institutes of Health (GM080511) and the National Science Foundation (CHE-0616795) for funding this work.

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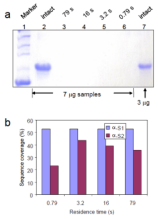


Figure 1.

Membrane digestion efficiency for 0.1 mg/mL non-denatured α-casein solutions in 10 mM NH_4HCO_3. (a) SDS-PAGE of intact α-casein (Lanes 2 and 7) and α-casein after membrane digestion with different residence times (Lanes 3–6); (b) α-casein sequence coverages (revealed by MALDI-MS) obtained after membrane digestion.

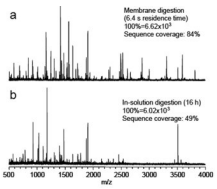


Figure 2.

MALDI-mass spectra of urea-denatured BSA (a) after membrane-digestion (miniaturized membrane reactor with an effective filtration area of 2 mm^2, residence time of 13 s, BSA concentration of 0.1 mg/mL for both digestion and MS analysis) and (b) after in-solution digestion for 16 h (BSA concentration of 0.5 mg/mL for digestion and 0.1 mg/mL for MS analysis).

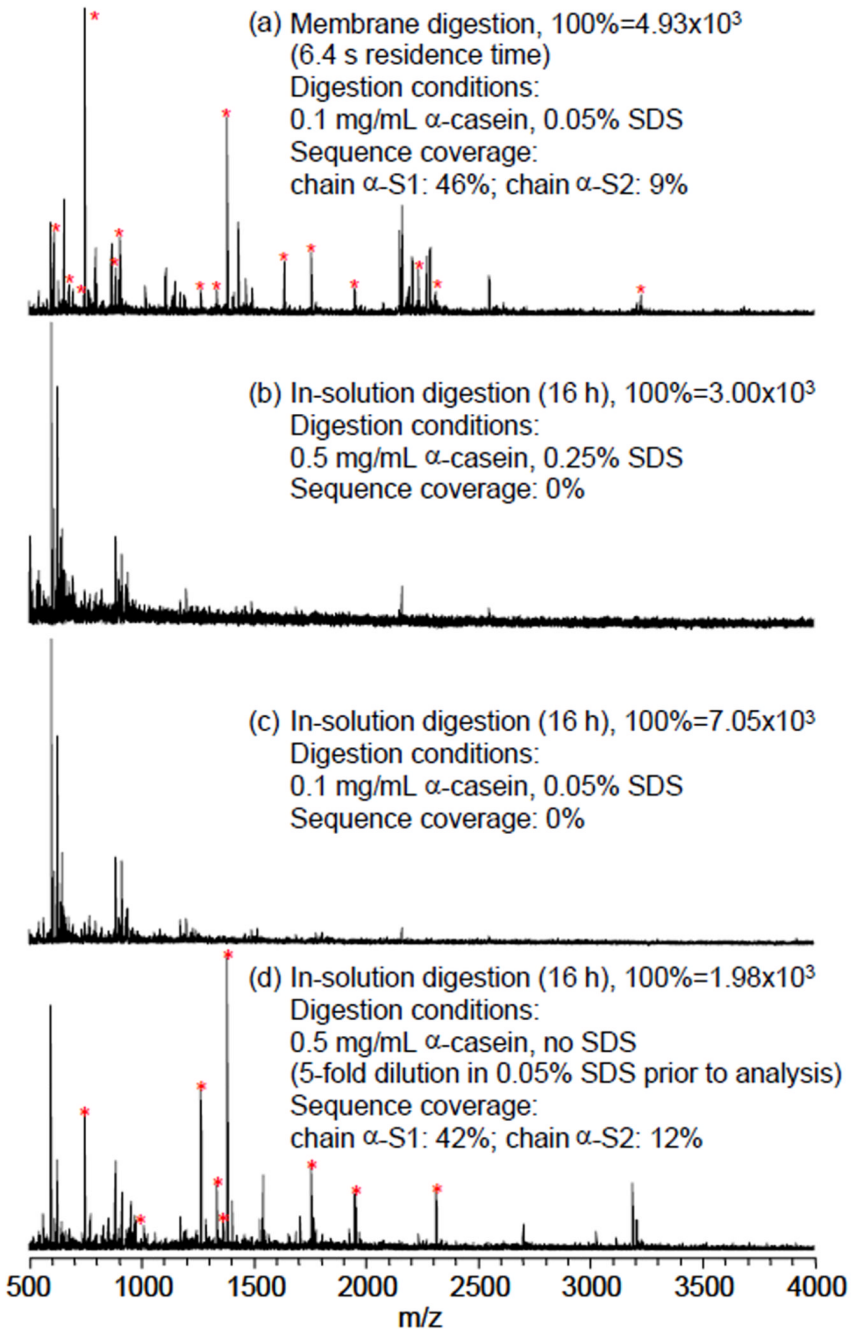


Figure 3.

MALDI-mass spectra of α-casein digested using both solution and membrane methods: (a–c) digestion in the presence of SDS; (d) addition of SDS only after digestion. (* shows identified peptides from α-casein.)

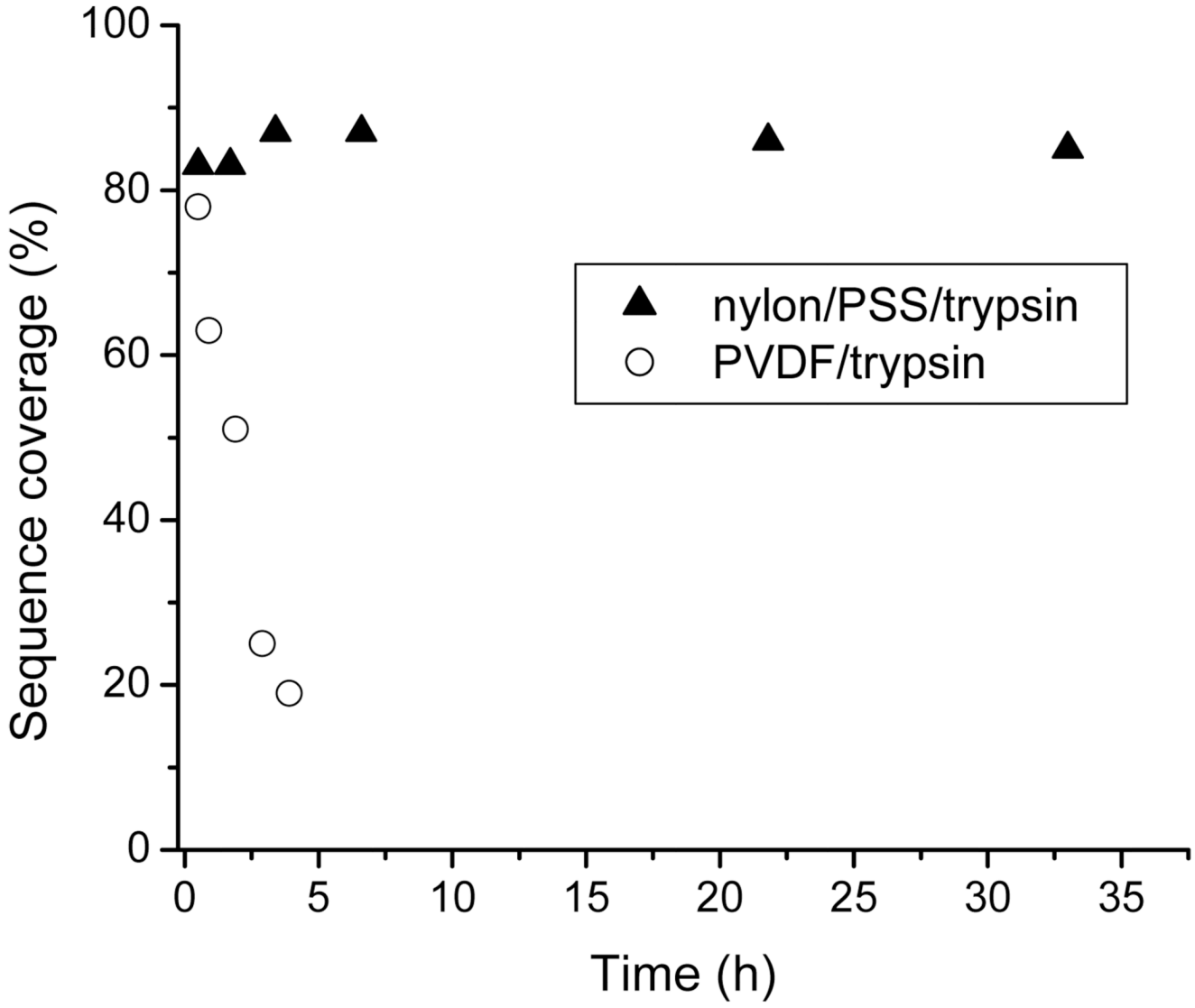


Figure 4.
MALDI-MS sequence coverages of membrane-digested BSA as a function of the time employed for continuously passing urea-denatured BSA (0.1 mg/mL, 0.1 mL/h) through miniaturized nylon/PSS/trypsin and PVDF/trypsin membranes.

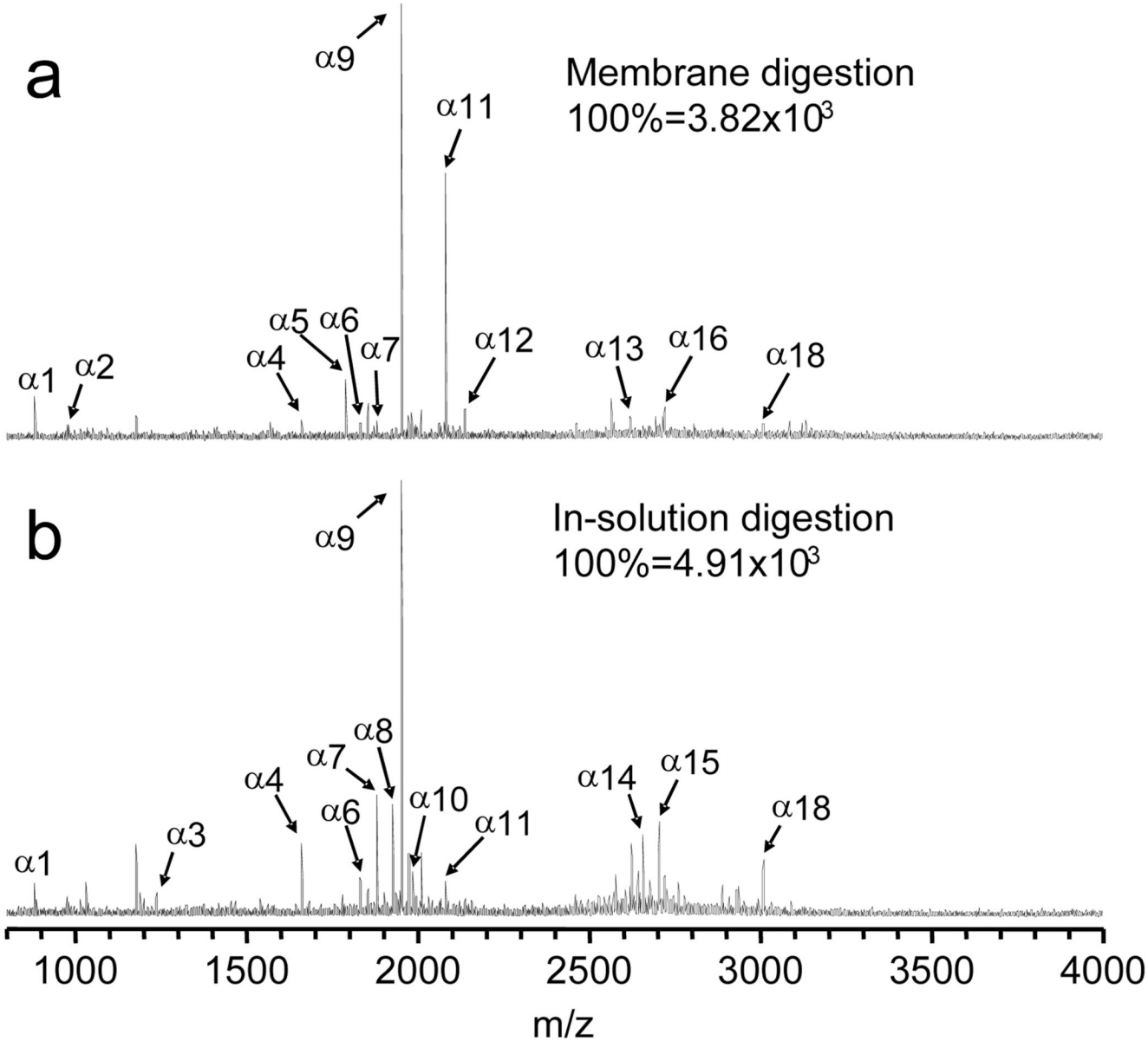
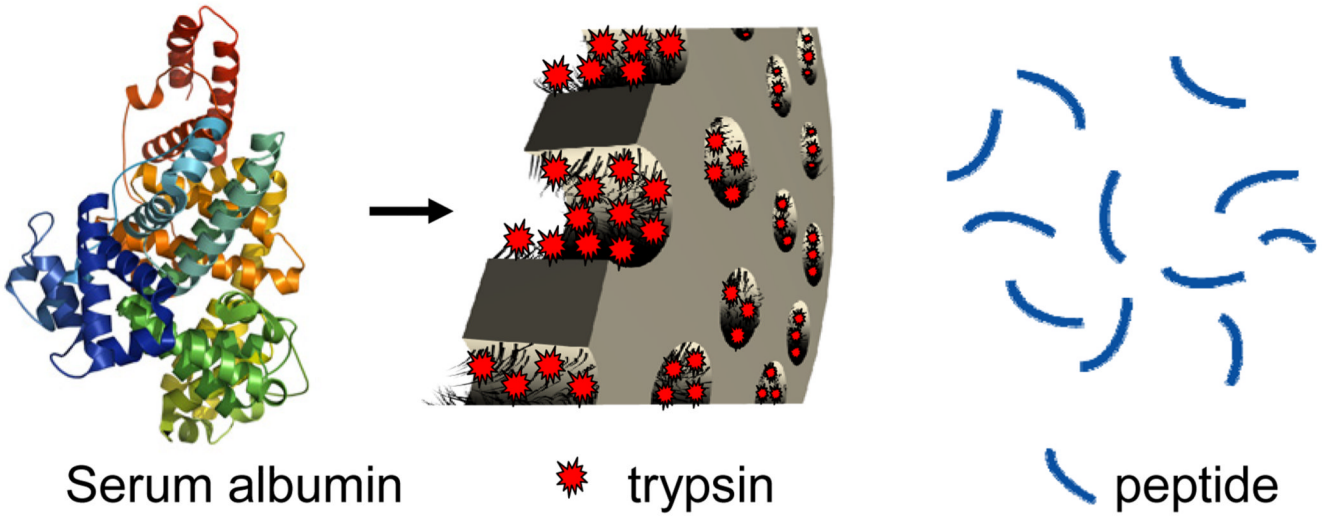
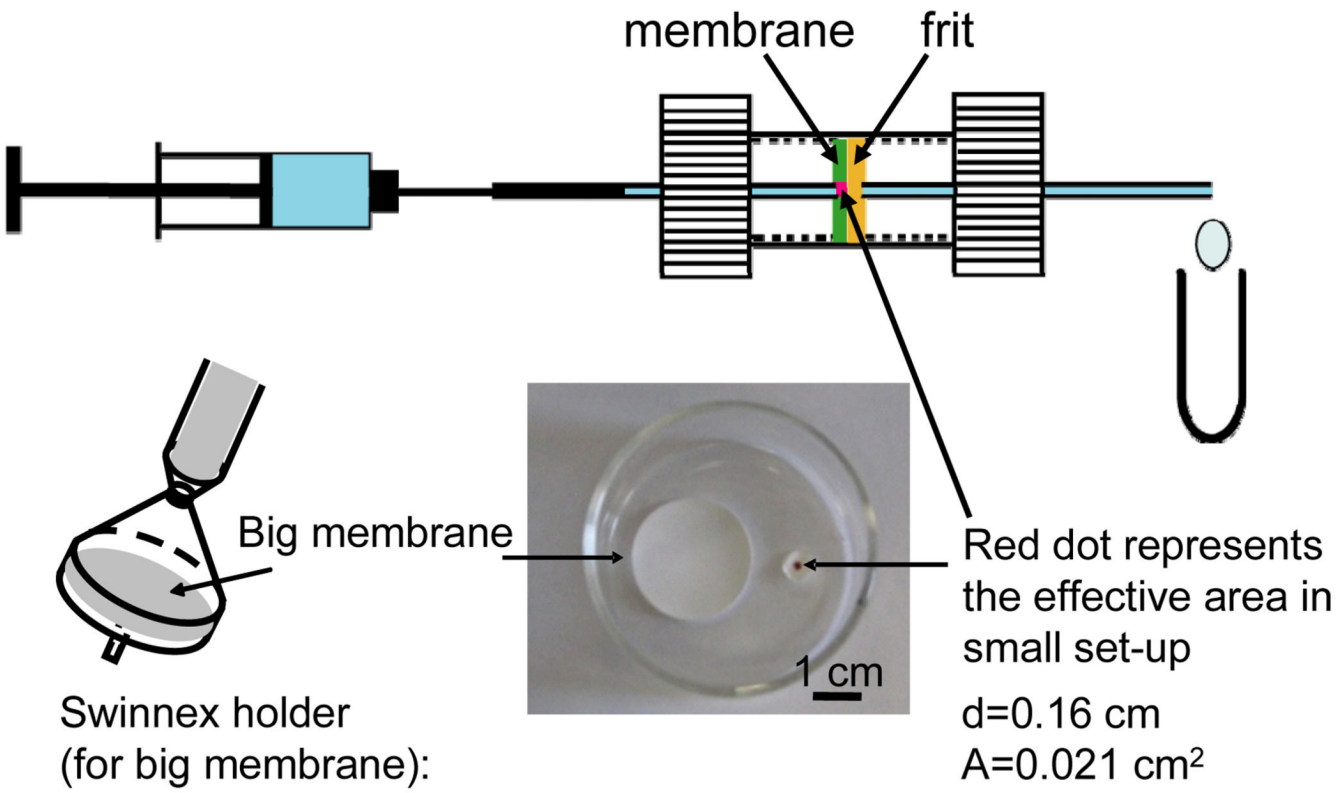


Figure 5.

Mass spectra of the peptides enriched from a mixture containing urea-denatured BSA and α-casein (mass ratio of 10:1) digested with a membrane (a, 0.1 mg/mL total protein during digestion) and in solution (b, 0.5 mg/mL total protein during digestion). The phosphopeptides, which are labeled, were enriched on a MALDI plate modified with polymer-oxotitanium (1 μL of 0.3 mg/mL protein digest was spotted on the MALDI plate for enrichment).21



Scheme 1.
Conceptual representation of a membrane reactor for tryptic digestion.



Scheme 2.
Conceptual drawing of the miniaturized membrane holder (top) and the Swinnex holder (bottom left), and a photograph (bottom right) of the 25-mm membrane disk and a miniaturized membrane that was exposed to dye while in the holder.

Table 1

Comparison of MS sequence coverages for tryptic digestion of BSA by different techniques (all the results were obtained using MALDI-MS). *

	Sequence coverage	Peptides found	# of missed cleavages allowed	Residence time
In-solution (0.5 mg/mL)	49%	28	2	16 h
In-solution (0.1 mg/mL)	71%	37	2	16 h
Fe_3O_4/carbon nanotube27	46%	28	1	5 min
Magnetic particle on chip-128	21%	13	n/a	5 min
Magnetic particle on chip-28	43%	30	n/a	10 s
Microchip (zeolite)13	44%	19	n/a	< 5 s
Monolith-15	44%	n/a	n/a	4.5 min
Monolith-211	26%	12	n/a	n/a
Nylon membrane	70%	44	1	6.4 s
	84%	52	2	6.4 s

* The literature studies employed α-cyano-4-hydroxycinamic acid as a MALDI matrix, whereas the samples from in-solution and membrane digestion were mixed with DHB prior to MALDI-MS. Denaturation conditions and digestion temperatures vary widely in the literature studies.