MODIFIED MAGNETIC BEADS AND MEMBRANES FOR PROTEIN CAPTURE AND DIGESTION

By

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ABSTRACT

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Efforts to determine the temporal concentrations of the vast number of proteins in a cell (proteomics) are placing unprecedented demands on techniques for protein separation and analysis. Additionally, the development of recombinant proteins for both research and therapeutic applications requires efficient methods for protein purification. This dissertation describes the development of two kinds of materials for protein separation and analysis: polymer brush-modified magnetic nanoparticles and trypsin-modified membranes.

The growth of poly(2-hydroxyethyl methacrylate) brushes on magnetic nanoparticles and subsequent brush functionalization with nitrilotriacetate-Ni\(^{2+}\) yield magnetic beads that selectively capture polyhistidine-tagged (His-tagged) protein directly from cell extracts. Transmission electron microscopy, FT-IR spectroscopy, thermogravimetric analysis, and magnetization measurements confirm and quantify the formation of the brushes on magnetic particles, and multilayer protein adsorption to these brushes results in binding capacities (220 mg protein/g of beads) that are an order of magnitude higher than those of commercial magnetic beads. Moreover, the functionalized beads selectively capture His-tagged protein within 5 min. The high binding capacity, high protein purity, and short incubation time make brush-modified particles attractive for purification of recombinant proteins.

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 much higher than the 49% coverage obtained with in-solution digestion for 16 h or the 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.

I also present a preliminary investigation of how the number and size of peptide fragments vary with the protein residence time in the trypsin-containing membranes. We hope to use extremely short residence time to create large protein segments for potential applications in middle-down proteomics, where fragmentation of modest sized (3-20 kDa) protein pieces in the mass spectrometer facilitates protein sequencing and discovery of post-translational modifications.
ACKNOWLEDGMENTS

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I am also grateful to my fellow group members. They are really helpful to me on the research part. In addition, I had a lot of fun with them-picnics and bowling parties are all colorful parts of my years here. Without their presence, I would have had a much tougher time to finish my work. And I need to mention that I enjoyed working with Weihan Wang during our collaboration on a couple of projects.

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<th>Description</th>
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<tbody>
<tr>
<td>AIBN</td>
<td>2,2’-azoisobutyronitrile</td>
</tr>
<tr>
<td>ATRP</td>
<td>atom transfer radical polymerization</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>GOD</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>His-tagged</td>
<td>poly histidine tagged</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IDA</td>
<td>iminodiacetic acid</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>LBL</td>
<td>layer-by-layer</td>
</tr>
<tr>
<td>LCST</td>
<td>low critical solution temperature</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MNP</td>
<td>magnetic nanoparticle</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMP</td>
<td>nitrooxide-mediated polymerization</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetate</td>
</tr>
<tr>
<td>PAA</td>
<td>poly(acrylic acid)</td>
</tr>
<tr>
<td>PAH</td>
<td>polyallylamine</td>
</tr>
<tr>
<td>PAMAM</td>
<td>polyamidoamine</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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</tr>
<tr>
<td>PDADMAC</td>
<td>poly(diallyldimethylammonium chloride)</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PGMA</td>
<td>poly(glycidyl methacrylate)</td>
</tr>
<tr>
<td>PHEMA</td>
<td>poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>PMAA</td>
<td>poly(methacrylic acid)</td>
</tr>
<tr>
<td>PMES</td>
<td>poly(2-(methacryloyloxy)ethyl succinate)</td>
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<td>PMF</td>
<td>peptide mass fingerprinting</td>
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<td>PMPC</td>
<td>poly(2-methacryloyloxyethyl phosphorylcholine)</td>
</tr>
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<td>PNIPAM</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>PPEGMA</td>
<td>poly(poly(ethylene glycol) methacrylate)</td>
</tr>
<tr>
<td>PSS</td>
<td>poly(styrene sulfonate)</td>
</tr>
<tr>
<td>RAFT</td>
<td>reversible-addition fragmentation chain transfer</td>
</tr>
<tr>
<td>SA</td>
<td>succinic anhydride</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-tetramethyl-piperidin-1-oxyl radical</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
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Chapter 1 Introduction and background

This dissertation describes the development of two kinds of materials for protein separation and analysis: polymer brush-modified magnetic nanoparticles and trypsin-modified membranes. When properly functionalized, the magnetic nanoparticles selectively capture polyhistidine-tagged (His-tagged) proteins, whereas the trypsin-modified membranes facilitate the digestion of proteins prior to their analysis by mass spectrometry. To put this work in perspective, the first part of this introduction examines current strategies for protein separation and analysis. Because this dissertation builds on prior studies of magnetic particles and polymer brushes, subsequent sections discuss the synthesis and recent applications of magnetic nanoparticles, as well as the growth and functionalization of polymer brushes. Modification of membranes with trypsin occurs through layer-by-layer assembly, and the fourth section of this chapter covers prior work in this area. Lastly, I present an outline of my dissertation.

1.1 Protein separation and analysis

Efforts to determine the temporal concentrations of the vast number of proteins in a cell (proteomics) are placing unprecedented demands on techniques for protein separation and analysis. Additionally, the development of recombinant proteins for both research and therapeutic applications requires efficient methods for protein purification. In the following subsections, I briefly discuss the challenge and importance of protein separation and analysis, protein purification methods, and techniques for separation and sample preparation prior to protein analysis, especially peptide mapping by mass spectrometry.
1.1.1 The challenge of protein separation and analysis

In a living cell, proteins perform most activities,\textsuperscript{1-4} and thus they are the subject of intense research in the life sciences. Moreover, deliberate overexpression of protein provides new materials for therapeutic or research purposes. Although production of recombinant proteins is becoming routine, isolation of the overexpressed protein from the myriad of other cellular proteins is very difficult.

Separation and analysis of native cell proteins is perhaps an even greater challenge. Blackstock and Weir stated, “The proteome is the expressed protein complement of a genome and proteomics is functional genomics at the protein level.”\textsuperscript{5} Proteomics involves two stages, expressional proteomics—identifying, characterizing and correlating target proteins with biochemical pathways, and functional proteomics—studying intact protein tertiary structure or protein-protein interactions through the isolation of protein samples.\textsuperscript{2, 4, 5} Given that an organism contains thousands of proteins and that the level of proteins can range over several orders of magnitude, exquisite strategies for separation and analysis are vital for both stages of proteomics. Figure 1.1 outlines many of the currently available methods, but these strategies are complex and time consuming.
1.1.2 Purification of tagged proteins, especially His-tagged protein

Chromatographic techniques for isolating proteins or peptides include gel filtration (size exclusion), ion exchange, affinity chromatography, and reversed phase chromatography. Among these techniques, affinity chromatography is especially popular because of its high
selectivity. Typical affinity interactions in these separations include histidine tags binding to metal ions, glutathione S-transferase binding to glutathione, and biotin-avidin interactions.

This introduction focuses on the most common type of affinity chromatography, immobilized metal affinity chromatography (IMAC), which is the subject of my research with magnetic beads.

Jerker Porath and coworkers first introduced IMAC under the name of metal chelate chromatography in 1975, and this method is one of the most widely used techniques for separation of biomolecules. The purification employs chelated metal ions as affinity ligands that interact with specific amino acid residues, especially histidine due to the high affinity of the imidazole side chain for many transition metal ions (Figure 1.2). Immobilization of the metal ions occurs through complexation with anchored chelating agents such as iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), or tris(carboxymethyl) ethylenediamine, and the most common metal ions for His-tagged protein purification are Ni\(^{2+}\) and Co\(^{2+}\). The use of short histidine stretches, typically placed as affinity tags at either the N-terminus or C-terminus, enables the purification of the desired protein from the crude extract of the host cells in a single step. Single histidine residues do not interact strongly with Ni\(^{2+}\) and Co\(^{2+}\), so the immobilized metal complexes selectively bind proteins containing polyhistidine tags.
A number of chromatographic supports and strategies are available for IMAC, including column chromatography systems, membrane absorbers and modified beads.\textsuperscript{7,8} Moreover, IMAC has many advantages including low cost, simplicity of use, specific binding, mild elution conditions, robustness and the ability to control selectivity.\textsuperscript{12,13} In addition, the histidine tag can be removed as needed.\textsuperscript{11}

Although widely applicable, the use of his-tags and IMAC purification is not recommended for proteins containing metal ions. Similarly, naturally occurring cysteine-rich or histidine-rich regions may bind to immobilized metal ions and decrease the purity of the desired protein.\textsuperscript{17} In this case, the use of a low concentration of imidazole in the loading buffer may improve selectivity, but it could also decrease binding capacity.\textsuperscript{14}
1.1.3 Protein analysis by mass spectrometry (identification of proteins and post translational modifications)

Mass spectrometry has become an indispensable tool in identifying proteins and their post translational modifications (PTMs). Proteins extracted from biological samples can be analyzed by bottom-up or top-down methods (Figure 1.3). The bottom-up approach involves digesting the proteins prior to mass spectrometry, whereas the top-down technique includes fragmentation within the mass spectrometer.

![Figure 1.3](image_url)

Figure 1.3 Strategies for MS-based protein identification and characterization. (PMF is peptide mass fingerprinting.) Figure reproduced with permission from Han, X.; Aslanian, A.; Yates III, J. R. Curr. Opin. Chem. Biol. 2008, 12, 483-490.
Yates, Ruse and Nakorchevsky noted that the bottom-up approach is “the most popular method when tackling high-complexity samples for large-scale analyses”.\textsuperscript{18} In one expression of this method, proteins in complex mixtures are separated before enzymatic (or chemical) digestion and subsequent direct peptide mass fingerprint-based analysis. Further peptide separation by 1D SDS-PAGE, reversed-phase high performance liquid chromatography (RP-HPLC), or 2D chromatography on thin-layer cellulose (TLC) plates can also occur prior to tandem mass spectrometry. Alternatively, the protein mixture can be directly digested into a collection of peptides (‘shotgun’ approach), which are then separated by multidimensional chromatography before tandem mass spectrometric analysis. Peptide mapping is a powerful technique to analyze and compare the structure and composition of proteins. The most common applications of peptide mapping are comparing the primary structure of proteins suspected of being encoded by the same or related genes and determining the precise location of amino acid residues modified post-translationally.\textsuperscript{21}

Digestion of proteins into peptides can occur chemically or enzymatically. Strong acid (e.g. 6 M HCl) can cleave proteins, but this method is not sequence specific. Instead, sequence-specific proteolytic enzymes (proteases such as trypsin, chymotrypsin, pepsin, and Lys-C) and sequence-specific chemical cleavage (e.g. cyanogen bromide cleavage at methionine residues) are much more common than strong acid digestion.\textsuperscript{19, 22}

Most bottom-up approaches require database searching to identify peptide sequences or proteins.\textsuperscript{18} Typical computer algorithms use two different types of data generated by mass spectrometers to search sequence databases. After digestion with a site-specific protease, the molecular weights of the resulting collection of peptides, the mass map (or fingerprint), can be determined using mass spectrometry and compared with the possible peptides that result from in
silico digestion of the proteins in a database. A second algorithm employs the data created by tandem mass spectrometers. Tandem mass spectra contain highly specific sequence information in their fragmentation pattern. Again, this information is compared with the predicted, in silico–generated fragmentation patterns of the peptides under investigation. A major strength of using tandem mass spectra to search databases is the ability to identify proteins present in relatively complex mixtures. Yates et al. pointed out that “drawbacks of the bottom-up approach include limited protein sequence coverage by identified peptides, loss of labile PTMs, and ambiguity of the origin of redundant peptide sequences”.

In the top-down approach, proteins in complex mixtures are fractionated and separated into pure single proteins or less complex protein mixtures prior to off-line static infusion of sample into the mass spectrometer for intact protein mass measurement and intact protein fragmentation. An on-line LC–MS strategy can also be used for large-scale protein interrogation. Compared to the bottom-up approach, the top-down method affords higher sequence coverages of target proteins and better characterization of PTMs. However, it is currently limited by technical challenges such as intact protein separation, the need for high mass accuracy instruments, and difficulties in fragmentation of large proteins.

1.1.4 Proteolysis by immobilized trypsin

As mentioned in the last subsection, the bottom-up approach requires complete digestion prior to MS analysis. 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 autodigestion of the protease. However, the low concentration of proteases frequently requires long incubation times for complete digestion.
A number of reports show that immobilization of trypsin on a support can greatly decrease digestion time compared to in-solution digestion. The enhanced efficiency stems primarily from the high enzyme concentration at the substrate surface. In the following subsections, I discuss immobilization of trypsin to monoliths, membranes, microchips, and magnetic particles.

**1.1.4.1 Trypsin-modified monoliths for rapid proteolysis**

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. Epoxide-based monoliths are especially popular for immobilizing trypsin because of the versatility of the epoxy groups (Figure 1.4).
Figure 1.4 Schemes of enzyme immobilization chemistry via epoxy groups. Figure reprinted with permission from Ma, J.; Zhang, L.; Liang, Z.; Zhang, W.; Zhang, Y. J. Sep. Sci. 2007, 30, 3050-3059.
Krenkova and coworkers immobilized trypsin as well as the endoproteinase Lys-C) in hydrolyzed poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths (inner diameter: 100 μm). The reactor digested not only small model proteins (9-66 kDa) but also high-molecular weight proteins such as immunoglobulins G (>150 kDa). Typical residence times were 6 min.27 Similarly, Sproß and Sinz covalently immobilized trypsin in a poly(glycidyl methacrylate-co-acrylamide-co-ethylene glycol dimethacrylate) capillary monolith. With a residence time of ~70 s, the resulting reactor digested pure model proteins such as cytochrome C and BSA, or low abundance proteins in the presence of a 1000-fold excess of interfering protein.33

1.1.4.2 Trypsin-modified membranes for rapid proteolysis

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. 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. Lee’s group demonstrated protein digestion using PVDF membranes that contained trypsin adsorbed to membrane pores via hydrophobic interactions, and coupled these membranes with on-line ESI-MS.30 After optimization, the membrane reactor consumed ≤ 5 fmol of low-concentration samples (10⁻⁸ M).31 However, these membranes are inherently unstable (see Chapter 3).
1.1.4.3 Trypsin-modified microchips for rapid proteolysis

Microfluidic devices are popular tools for biological assays because of their high speed and low sample consumption.\textsuperscript{28, 34, 35} Most trypsin immobilization in microchips occurs through layer-by-layer (LBL) adsorption or by assembly of trypsin-modified magnetic nanoparticles.

Liu and coworkers adsorbed three bilayers of cationic chitosan and anionic hyaluronic acid in a poly(ethylene terephthalate) chip (100 μm-wide channel) prior to immobilization of a trypsin layer. The resulting chip digested model proteins, casein extracts from bovine milk, and hepatitis A vaccine, with a residence time of a few seconds.\textsuperscript{35} Ji et al. modified the microchips with nanozeolites using the LBL method, and then immobilized trypsin onto the nanozeolite assemblies through electrostatic interactions. The microchip had a surface coverage of 1 trypsin molecule/1.2 nm\textsuperscript{2} of channel area, and successfully digested cytochrome C at a concentration as low as 0.5 ng/μL to give a 47% sequence coverage in subsequent MS analysis. In addition, when coupled with MALDI-TOF-MS/MS or two-dimensional LC-ESI-MS/MS systems, the reactor identified 191 proteins from protein extracts from mouse macrophages.\textsuperscript{28}

Zhang’s group reported assembly of trypsin-modified magnetic particles in chip microchannels through use of a magnetic field.\textsuperscript{34} Immobilization of trypsin to the magnetic particles (~50 nm diameter) occurred through a reaction between the amine groups of trypsin and the aldehyde-functionalized particles. Subsequently, the particles were packed in glass microchannels through the application of a magnetic field. The authors utilized the reactor to digest a rat liver extract, and identified 6 proteins. If needed, simple replacement of the particles could regenerate the microchip.\textsuperscript{34}
1.1.4.4 Trypsin-modified magnetic particles for rapid proteolysis

One advantage of immobilizing trypsin on magnetic particles is that it allows heating in a microwave oven to facilitate proteolysis.\textsuperscript{29, 36, 37} Zhang’s group immobilized trypsin on epoxide-functionalized particles and mixed these particles with substrate protein solutions prior to exposure to microwave irradiation for 15 s. Subsequent analysis with MALDI-MS revealed four proteins (16 peptides) from a rat liver extract.\textsuperscript{29, 36}

1.2 Magnetic nanoparticles (MNPs)

Superparamagnetic nanoparticles (e.g. Fe\textsubscript{3}O\textsubscript{4} and γ–Fe\textsubscript{2}O\textsubscript{3}) are attractive for a host of applications because they can be dispersed in many solutions and subsequently collected in the presence of an external magnetic field. Potential areas for MNP application include magnetic storage, biosensing, bioseparations, targeted drug delivery, and magnetic resonance imaging (MRI, as contrast agents). The size distribution of the MNPs is critical to almost all of these applications because dispersion, distribution, and collection strongly depend on the dimension of the particles.\textsuperscript{38} The next two subsections discuss the syntheses and applications of MNPs.

1.2.1 Synthesis of magnetic iron oxide nanoparticles

Numerous methods have been developed to synthesize MNPs, including coprecipitation,\textsuperscript{39, 40} thermodecomposition,\textsuperscript{41, 42} microemulsion techniques,\textsuperscript{43} reactions in constrained environments,\textsuperscript{44} sol-gel reactions,\textsuperscript{45} polyl methods,\textsuperscript{46} flow injection syntheses,\textsuperscript{47} electrochemical methods,\textsuperscript{48} aerosol/vapor methods\textsuperscript{49} and sonolysis.\textsuperscript{50} In the following subsection, I will cover three of the most important methods for creating MNPs,
coprecipitation—a classic and simple technique, thermodecomposition—a method producing monodisperse MNPs, and microemulsion techniques—a strategy yielding ultrasmall particles. Subsequently, I discuss protection and stabilization of the resultant MNPs.

1.2.1.1 Coprecipitation

Coprecipitation of Fe(II) and Fe(III) in the presence of acid or base is the simplest technique to synthesize magnetic particles.\textsuperscript{38} The following equation expresses the chemical reaction under basic conditions.

\[ \text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O} \]

The formation of nanoparticles involves two stages: a short burst of nucleation that occurs when the concentration of the species reaches the critical supersaturation, and slow growth of the nuclei by diffusion of the solutes to the surface of the crystal.

Massart was one of the first scientists to prepare superparamagnetic iron oxide particles in a controlled way.\textsuperscript{38} He coprecipitated Fe(III) and Fe(II) upon addition of ammonia solution, and the average diameter of the particles was 12 nm, as determined by XRD. The particle size decreased when the pH and/or the initial Fe(III)/Fe(II) ratio increased.\textsuperscript{39}

Babes et al. synthesized iron oxide nanoparticles (diameter under 10 nm) in catheters. They used tetramethylammonium hydroxide (TMAOH) and ammonia as bases to coprecipitate the iron salts and systematically evaluated several experimental parameters including TMAOH/NH\textsubscript{3} ratio, injection fluxes, Fe\textsuperscript{2+}/Fe\textsuperscript{3+} ratio, TMAOH and Fe solution concentrations, and temperature. The Fe\textsuperscript{2+}/Fe\textsuperscript{3+} molar ratio was the most important factor in controlling the nanoparticle size. They found that Fe\textsuperscript{2+}/Fe\textsuperscript{3+} ratios between 0.4 and 0.6 produced stable colloidal solutions. The second most important factor was the iron concentration, and the optimum values are between 39-78 mM.\textsuperscript{40}
Increased ionic strengths and mixing rates result in decreased particle size because they lead to the formation of more nuclei.\textsuperscript{51, 52} However, elevated temperature increases the size of the nanoparticles because fewer nuclei form.\textsuperscript{40, 53} The removal of oxygen not only prevents the particle from being oxidized, but also reduces the particle size (6 nm) compared with no oxygen removal (8 nm).\textsuperscript{54}

The main advantage of the coprecipitation process is that it yields relatively large amounts of nanoparticles. Moreover, the process usually employs no organic solvents and is environmentally friendly. However, the particles size distribution is relatively wide because only kinetic factors control the growth of the crystal.\textsuperscript{38, 55}

1.2.1.2 Thermal decomposition

High-temperature decomposition of iron organic precursors, such as Fe(cup)\textsubscript{3} (cup = N-nitrosophenylhydroxylamine), Fe(acac)\textsubscript{3} (acac = acetylacetonate), or Fe(CO)\textsubscript{5} leads to high-quality monodispersed iron oxide particles.\textsuperscript{38, 55-57} However, this technique requires a high reaction temperature to induce nucleation and subsequent nanoparticle growth.\textsuperscript{58} The synthesis of nanoparticles is controlled by several experimental parameters, including the concentration and ratio of the starting reagents (organic precursors, surfactant, and solvent), as well as the reaction temperature and time.\textsuperscript{38, 55}

Yu and coworkers reported the synthesis of iron oxide (Fe\textsubscript{3}O\textsubscript{4}) nanocrystals with average diameters ranging from 6 to 30 nm and narrow size distributions (σ = 5–10%) by the pyrolysis of iron carboxylate salts (320-340 °C).\textsuperscript{41} Roca et al. synthesized MNPs (5-11 nm diameter) by thermal decomposition (above 250 °C) of iron acetylacetonate (acac) and iron oleate
complexes.\textsuperscript{42} The products of thermal decomposition of Fe(cup)\textsubscript{3} or Fe(CO)\textsubscript{5} are $\gamma$-Fe\textsubscript{2}O\textsubscript{3}.\textsuperscript{41,43,59} For example, Lee and coworkers demonstrated the synthesis of monodisperse $\gamma$-Fe\textsubscript{2}O\textsubscript{3} by heating the precursor Fe(CO)\textsubscript{5} at 90 °C for 1 h in a mixture of 1-$n$-octyl-3-methylimidazolium salts and DMF.\textsuperscript{58}

### 1.2.1.3 Formation of nanoparticles in microemulsions

Synthesis of nanoparticles in microemulsions provides small and uniform MNPs.\textsuperscript{60} A microemulsion is a stable dispersion of two immiscible liquids based on an interfacial film of surfactant. In water-in-oil microemulsions, the aqueous phase forms nanodroplets in a continuous oil phase. By mixing two water-in-oil emulsions containing different reactants that form nanoparticles, the droplets will collide and induce the formation of precipitates.\textsuperscript{43,55,60} The diameter of the resulting particles depends on the size of the water droplets.\textsuperscript{43}

Santra and coworkers synthesized ultrasmall (1-5 nm) Fe\textsubscript{3}O\textsubscript{4} nanoparticles with a water-in-oil microemulsion method. Both strong base (NaOH) and mild base (NH\textsubscript{3}) were used in the process, and the different basicity did not affect the morphology of the particles. They also explored different nonionic surfactants including Triton X-100, Igepal CO-520, and Brij-97, and observed that Igepal CO-520 with NH\textsubscript{3} produced less aggregated particles than the other two surfactants. The resulting nanoparticles were uniform (standard deviation less than 10%) and ultrasmall (1-2 nm).\textsuperscript{43}

### 1.2.1.4 Protection and stabilization of MNPs

Prevention of MNP aggregation is vital for most applications,\textsuperscript{38,55,59} and in most cases electrostatic or steric repulsive forces must be present between particles to achieve stable
suspensions.\textsuperscript{38} Citric acid (citrate) is one of the most common monomeric stabilizers that functions through electrostatic forces,\textsuperscript{38} whereas dextran is a widely used polymeric stabilizer that operates through steric repulsion.\textsuperscript{38, 61} Jain and coworkers recently reported a steric-based poly(acrylic acid)-\textit{b}-poly(acrylamide) copolymer stabilizer that stabilizes MNPs over a wide range of pH and ionic strengths.\textsuperscript{59}

Silica coating of MNPs provides an inorganic stabilizer that not only prevents the particles from aggregating, but also protects them against oxidation and provides sites for particle functionalization.\textsuperscript{38, 55} Typical silica coating occurs via a sol-gel process in which the silica precursor (e.g. tetraethylorthosilicate-TEOS) hydrolyzes and then condenses on the particles with ammonium as catalyst.\textsuperscript{62} The thickness of the silica layer varies with the ratio of TEOS/NH$_3$ and TEOS/MNPs as well as the reaction time.\textsuperscript{63} The negative charge on the resultant particles (at neutral pH) results in coulombic repulsion between particles, and the high density of hydroxyl groups on the surface allows further modification via silane coupling agents.\textsuperscript{40, 56, 64}

\textbf{1.2.2 Applications}

Properly functionalized magnetic particles have a number of promising applications in analytical and biological fields such as molecular imaging,\textsuperscript{65} bioseparations,\textsuperscript{8} drug delivery,\textsuperscript{66} and hyperthermia treatments.\textsuperscript{38} In the following sections, I will discuss two of the most important applications, bioseparations and drug delivery.

\textbf{1.2.2.1 Magnetic separation of proteins}

Magnetic collection of MNPs from a solution is usually a simple process (Figure 1.5). In a typical bioseparation, magnetic particles functionalized with immobilized affinity ligands or ion-
exchange groups are mixed with a sample containing target molecules. After incubation to allow the binding of the target molecule to the ligands, the MNPs along with bound biomolecules are collected from the sample with the help of an external magnetic field. Subsequently, the complex is washed to remove unbound species, and the isolated target molecules are eluted for analysis or subsequent use.8

Figure 1.5 A typical process for protein separation by magnetic particles.

Magnetic separation techniques have several advantages over standard separation procedures. As described above, the process is very simple, with only a few handling steps, all of which can take place in a single tube without any expensive liquid chromatography systems, pumps, centrifuges, filters or other equipment.38 In addition, the separation can be directly applied to crude samples, even in the presence of suspended solid materials and other biological fouling materials.8 Moreover, magnetic separation is very gentle to the target molecules. Hofmann and coworkers reported that large protein complexes that tended to dissociate during chromatography remained intact with magnetic separation.67 Finally, magnetic particles can be used for concentration of diluted protein solutions.8
Because of its many advantages, magnetic separation has been developing rapidly. MNPs functionalized with a variety of ligands as well as many magnetic separators (even automated separators) have been commercialized. Table 1.1 lists some of the commercially available magnetic particles for protein binding.

Table 1.1 Functionalized magnetic adsorbents suitable for binding proteins. Table reprinted with permission from Franzreb, M.; Siemann-Herzberg, M.; Hobley, T. J.; Thomas, O. R. T. Appl. Microbiol. Biotechnol. 2006, 70, 505-516.

<table>
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<th>Ligand</th>
<th>Target molecule</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>M(^{2+})-charged imino diacetic acid</td>
<td>His-tagged fusion proteins, proteins with surface-exposed His, Cys and Trp side chains</td>
<td>Chemagen(^b); Micromod(^c); Dynal(^e,h)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Glutathione-S-transferase (GST) fusion proteins</td>
<td>Promega(^g); Micromod(^e)</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>Biotinylated proteins</td>
<td>Bangs(^a); Chemagen(^b); Micromod(^c); Seradyn(^d); Dynal(^e); Promega(^g)</td>
</tr>
<tr>
<td>Biotin</td>
<td>Fusion proteins with streptavidin group or analog</td>
<td>Bangs(^a)</td>
</tr>
<tr>
<td>Protein A or G</td>
<td>Monoclonal antibodies</td>
<td>Bangs(^a); Micromod(^e); Dynal(^e)</td>
</tr>
<tr>
<td>-COOH</td>
<td>Molecules with positive (cationic) net charge</td>
<td>Bangs(^a); Chemagen(^b); Micromod(^c); Seradyn(^d); Dynal(^e)</td>
</tr>
<tr>
<td>-SO(_3)^-</td>
<td>Molecules with positive (cationic) net charge</td>
<td>Chemicell(^f)</td>
</tr>
<tr>
<td>-NH(_2)</td>
<td>Molecules with negative (anionic) net charge</td>
<td>Bangs(^a); Chemagen(^b); Micromod(^c); Dynal(^e)</td>
</tr>
<tr>
<td>-DEAE (Diethylaminoethyl)</td>
<td>Molecules with negative (anionic) net charge</td>
<td>Chemicell(^f)</td>
</tr>
<tr>
<td>-N(CH(_2)CH(_3))(_2)</td>
<td>Molecules with negative (anionic) net charge</td>
<td>Chemicell(^f)</td>
</tr>
</tbody>
</table>

\(^a\)Bangs Laboratories, Fishers, IN, USA; http://www.bangslabs.com

\(^b\)Chemagen Biopolymer Technology, Baesweiler, Germany; http://www.chemagen.de

\(^c\)Micromod Partikeltechnologie GmbH, Rostock, Germany; http://www.micromod.de

\(^d\)Seradyn, Indianapolis, IN, USA; http://www.seradyn.com
Dynal Biotech, Lake Success, NY, USA; [http://www.dynalbiotech.com](http://www.dynalbiotech.com)

Chemicell GmbH, Berlin, Germany; [http://www.chemicell.com](http://www.chemicell.com)

Promega, Madison, WI, USA;

Dynal offers magnetic adsorbent particles with TALON functionalisation for the purification of His-tagged proteins.

### 1.2.2.2 Drug delivery

Magnetic nanoparticles have been widely used as carriers in many therapeutic areas, including chemotherapy, radiotherapy, and biotherapeutics. After modification with proper “drugs” (chemotherapeutic compounds, therapeutic peptides, antibodies, DNA or RNA), the MNPs are guided to the target cell or tissues with the help of a magnetic field gradient.

A number of nanoparticle properties must be optimized to make magnetic drug delivery safe and efficient. For example, the potential for particle internalization depends greatly on its size. Particles should be small enough to bypass the in vivo barriers and yet provide enough surface area and be big enough to avoid extravasations and renal clearance. Thus, the optimal diameter range is 10-100 nm. In addition, strong magnetic response is vital to improve the localization of the MNPs to target tissue and minimize side effects. Finally, the particles should be stable and biocompatible.

Kohler and coworkers successfully immobilized methotrexate (MTX, a widely used drug for fighting various cancers) to MNPs. In their work, a bifunctional poly(ethylene glycol) (PEG) chain was anchored on Fe₃O₄ particles (diameter 10-15 nm) through silanization, followed by the immobilization of MTX through a peptide bond formed between the amine group on the PEG
chain and the carboxylic acid group of the MTX. The biocompatibility of the particles was confirmed by leucovorin (LV)-induced rescue analysis, in which LV (an MTX antidote) was used to rescue cells exposed to MTX-MNPs and the free MTX. Drugs could be released when the peptide bond was cleaved in vivo.66

Radionuclides (mostly $^{188}$Re radioactive isotopes) immobilized on MNPs allow site-selective radiotherapy as the $^{188}$Re isotopes decay in target cells and produce DNA-damaging free radicals.70 For example, radiolabeled amino acids are attractive MNP modifiers for radiotherapy because the amino acid uptake for protein synthesis is rapid in tumor tissues. Cao et al. reported functionalizing MNPs with $^{188}$Re-labeled histidine. First, histidine was immobilized to amine-functionalized MNPs (~20 nm in diameter) using glutaraldehyde as a linker, and then the particles were labeled with $[^{188}\text{Re(CO)}_3\text{(H}_2\text{O})_3]^+$ (labeling yield 91.4%) as the ion complexed with the imidazole groups in histidine. Such modified particles could potentially be used for delivering radiotherapeutic drugs.72

In addition to chemotherapy and radiotherapy, gene therapy has become a new approach for curing diseases. In this case, polycation-coated MNPs not only serve as carriers but can also prevent DNA (for therapeutic gene expression) or RNA (for silencing defective genes) from enzymatic degradation in vivo through the formation of a polycation-anionic nucleic acid complex.70 Huang’s group demonstrated this concept using dendrimer-modified MNPs to enhance gene delivery efficiency.68 MNPs (diameter 8 nm) were first modified with polyamidoamine (PAMAM) dendrimers and then mixed with antisense survivin oligodeoxynucleotide (asODN), which can bind the start location of mRNA translation inside cells, block translation of target RNA into target protein, and finally suppress cellular
proliferation. The asODN bound to immobilized PAMAM dendrimers through electrostatic interactions and had a long lifetime (>5 days). The resultant asODN-MNPs could cross cell membranes and inhibit tumor growth.

1.3 Polymer Brushes

Polymer brushes are long-chain polymer molecules with one end attached to a surface or interface. Typically, the polymers attach to a surface with a high graft density, so that chains are forced to stretch away from the interface. Part of this dissertation explores modification of magnetic nanoparticles with polymer brushes to enhance their capacity for binding biomolecules.

Two general strategies are utilized to prepare polymer brushes, grafting to (Figure 1.6 a) and grafting from (Figure 1.6 b) a surface. The grafting-to method involves a chemical reaction between polymer end groups and a complementary group on the substrate. Although many studies demonstrate the efficacy of the grafting-to technique, this method typically suffers from a low grafting density that limits the thickness of grafted films. Steric repulsions between the immobilized polymer chains and incoming polymers restrict the diffusion of further functionalized end-groups to the substrate and prohibit further grafting. In contrast, the grafting-from approach yields high-density polymer brushes because the polymerization is directly initiated from the surface, and the monomers can diffuse to the initiation sites much more easily than the presynthesized polymer chains.

In this section of the introduction, I will focus on brush-forming polymerization strategies, functionalization of polymer brush side chains, and the properties and applications of polymer brushes.
1.3.1 Polymerization strategies

Controlled radical-based polymerizations are the most frequently used methods for synthesis of brushes with control over brush thickness, composition, and architecture. A number of related controlled radical polymerization methods have been developed based on conversion of growing chains between dormant and radical forms (Scheme 1.1). These techniques vary in the mechanism and chemistry of the equilibration between dormant and active states, but the overall
result is the same: a low fraction of active chains at a given time and rapid conversion between dormant and active states leads to slow, controlled polymerization with relatively low polydispersity. Currently, atom transfer radical polymerization (ATRP), reversible-addition fragmentation chain transfer polymerization (RAFT), and nitroxide-mediated polymerization (NMP) can be applied to a host of monomers for creation of functional brushes. Herein I will first discuss the mechanisms of these polymerizations, and then compare their relative advantages and limitations.

\[
\begin{align*}
\text{ATRP: } & \text{Br-Cu}^{II}/L_r; Cu^{I}/L_r, \\
\text{NMP: } & \text{\cdotO-NR}_2 \\
\text{RAFT: } & \text{S=C(Z)-S-CH(R)-CH}_2\end{align*}
\]

1.3.1.1 Surface-initiated atom transfer radical polymerization

ATRP (Scheme 1.2) is probably the most common polymerization technique for producing polymer brushes because of its chemical versatility, good control over molecular weight and polydispersity, and robustness. This method is usually catalyzed by a transition metal complex (Mt\textsuperscript{n−}Y/Ligand, where Mt is the metal ion and Y is a counterion). The active carbon-centered radicals are generated through a reversible redox activation of a dormant alkyl halide-terminated
polymer chain end (R-X) with subtraction of a halogen atom X. At the same time, metal complex is oxidized to X-Mt\textsuperscript{n+1}-Y/Ligand. Polymer chains grow by the addition of the intermediate radicals to monomers similarly to a conventional radical polymerization, and termination reactions occur mainly through radical coupling and disproportionation. However, in a well-controlled ATRP, no more than a few percent of the polymer chains undergo termination (for surface initiated ATRP, this percentage may be larger because all the active radicals are immobilized in close proximity to the surface). Most importantly, the oxidized metal complexes X-Mt\textsuperscript{n+1} can reconver the growing active chain end to the corresponding halogen-capped dormant species and thereby minimize the contribution of termination. A successful ATRP will have not only a small contribution from terminated chains, but also a uniform growth of most of the chains, which is accomplished through fast initiation and rapid reversible deactivation.\textsuperscript{74, 79, 80} The reaction can be tuned by a number of parameters, including ligand to transition metal ratio, M\textsuperscript{n+1} to M\textsuperscript{n} ratio, type of ligand, counterion, solvent, or initiator.\textsuperscript{74}

\[
\begin{align*}
R-X + Mt^n-Y/Ligand &\overset{k_{act}}{\longrightarrow}\overset{k_{deact}}{\longleftarrow} R^* + X-Mt^{n+1}-Y/Ligand \\
&\text{monomer} \quad \text{termination}
\end{align*}
\]

In 1997, Huang and Wirth reported the first ATRP from a surface in the grafting of poly(acrylamide) brushes from a self-assembled benzyl chloride monolayer on silica gel. Since that time, many papers described the growth of polymer brushes via ATRP initiated from various surfaces, including silica (planar and particles), polymers, metal oxides, clay minerals, gold, metal and semiconductors, and carbon nanotubes.

This dissertation is particularly concerned with growth of brushes from silica particles. In most cases, initiator attachment on silica occurs through silanization with trichlorosilane (-SiCl₃), triethoxysilane (-Si(OEt)₃), trimethoxysilane (-Si(OMe)₃), or monochlorosilane (-SiMe₂Cl) molecules. Bromide moieties can be immobilized to the organosilane molecules prior to the silanization, or alternatively, amino-functionalized organosilane reagents are anchored to the particles followed by the reaction of 2-bromoisobutyryl bromide with the amine groups. Additionally, Armes’s group developed a cationic microinitiator, which could adsorb to negatively charged silica particles in protic media and later initiate synthesis of poly(2-hydroxyethyl methacrylate) and poly (2-(dimethylamino)ethyl methacrylate).

1.3.1.2 Surface-initiated reversible-addition fragmentation chain transfer

In ATRP, the equilibrium is between the dormant and active states, and chain propagation is based on reversible termination, whereas for RAFT, the controlled growth of the chain is based on reversible chain transfer. Conventional radical polymerization can be converted to RAFT fairly easily by adding a RAFT agent (such as a dithioester, dithiocarbamate, or trithiocarbonate.
compound), without changing other reaction parameters (monomer, initiator, solvent, and temperature).  

In RAFT polymerization, initiation and radical-radical termination occur in the same way as in conventional polymerization (Scheme 1.3). In the early stages of the RAFT polymerization, a propagating radical ($P_{n}^{•}$) can be added to the thiocarbonylthio compound 1 (RAFT agent). Then the fragmentation of the intermediate radical 2 gives back the propagating chain and 1 or yields a polymeric thiocarbonylthio compound 3 (the dormant species) and a new radical ($R^{•}$). Reaction of the radical ($R^{•}$) with monomer forms a new propagating radical ($P_{m}^{•}$). A rapid equilibrium between the active propagating radicals ($P_{n}^{•}$ and $P_{m}^{•}$) and the dormant polymeric thiocarbonylthio compound 3 provides equal probability for all chains to grow and allows the production of narrow dispersity polymers.  

74, 89, 90
Scheme 1.3 Mechanism of RAFT.\textsuperscript{78, 89-91}

Surface initiated RAFT can occur via two strategies, either using conventional free radical initiators (Scheme 1.4A) or surface-immobilized RAFT agents (Scheme 1.4 B and C).\textsuperscript{74} The Z-group approach is similar to the \textit{grafting to} method, and the brush grafting density might be low because of the accessibility of the anchored RAFT agents.\textsuperscript{74, 92} In addition, there would be free polymer chains in the polymerization solution.\textsuperscript{93} Similar to ATRP, RAFT has been performed on a wide range of surfaces.\textsuperscript{93, 94}
Scheme 1.4 SI-RAFT polymerization: (A) bimolecular process for the preparation of poly(methyl methacrylate) brushes from azo-functionalized silicon wafers; (B) R-group approach to grow poly(n-butyl acrylate) brushes from dithiobenzoate modified silica; (C) Z-group approach for the grafting of poly(methyl acrylate) brushes from silica particles supported trithiocarbonate derivative. (Acronyms: RT-room temperature; THF-tetrahydrofuran; AIBN-2,2'-azoisobutyronitrile.) Scheme reprinted with permission from Barbey, R.; Lavanant, L.; Paripovic, D.; Schuwer, N.; Sugnaux, C.; Tugulu, S.; Klok, H.-A. *Chem. Rev.* **2009**, *109*, 5437-5527.
1.3.1.3 Surface-initiated nitroxide-mediated polymerization

NMP is based on reversible activation/deactivation of growing polymer chains by a nitroxide radical (Scheme 1.5).\cite{74} The key step is a reversible thermal C-O bond cleavage of an alkoxyamine 6, generating the corresponding radical 7 and a nitroxide 8. Addition of monomer to the reactive radical 7 forms 9, and the subsequent reversible nitroxide (such as 2,2,6,6-tetramethyl-piperidin-1-oxyl radical TEMPO, 8) trapping of 9 leads to the chain-extended alkoxyamine 10. Compound 10 is the dormant species which can again thermally cleave to propagate the polymerization.\cite{76,95}

Two procedures are generally used for the conduction of NMP on surfaces. In the first case, initiation of the polymerization can occur from an alkoxyamine initiator attached to the surface.\cite{96} Alternatively, a non-alkoxyamine based radical initiator can be anchored to the surface, and the polymerization is then performed in the presence of a nitroxide, which allows in situ alkoxyamine formation.\cite{97} However, this latter method should lead to significant polymerization in solution because the initiation step leads to both a surface-anchored radical and a radical in solution.
1.3.1.4 Comparison of controlled radical polymerization techniques

Table 1.2 compares the relative advantages and limitations of ATRP, NMP, and RAFT in terms of monomers, conditions, end groups and additives. One of the advantages for NMP and RAFT is the absence of any metal, e.g. Cu, that might affect polymer function. ATRP is especially well suited for the synthesis of low molar mass functional polymers and block copolymers, whereas RAFT is successful for the polymerization of many less reactive monomers (e.g. vinyl acetate) and the preparation for high molecular weight.
Table 1.2 Comparison of NMP, ATRP and RAFT. Table reproduced with permission from Matyjaszewski, K., Ed. *Controlled/Living Radical Polymerization Progress in ATRP, MNP, and RAFT*; The American Chemical Society, 2000.  

<table>
<thead>
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<th>Feature</th>
<th>Systems</th>
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<tr>
<td></td>
<td>NMP</td>
</tr>
<tr>
<td>Monomers</td>
<td>♦ Styrenes for TEMPO</td>
</tr>
<tr>
<td></td>
<td>♦ Acrylates &amp; acrylamides for new nitroxides</td>
</tr>
<tr>
<td></td>
<td>♦ NO methacrylates</td>
</tr>
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<td></td>
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<td>Conditions</td>
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<td>♦ Requires radical chemistry for transformations</td>
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<td>♦ Thermally unstable</td>
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</tr>
<tr>
<td>Additives</td>
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</tr>
<tr>
<td></td>
<td>♦ NMP may be accelerated with acyl compounds</td>
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</table>

1.3.2 Functionalization of polymer brushes

Radical polymerization is attractive for its high tolerance of functional groups, however, there are still many functional groups that cannot be introduced to the brushes directly via
surface-initiated polymerization of the corresponding monomer. For example, chelating functional groups may be incompatible with ATRP because they will change the properties of the catalyst. In the following sections, I will discuss the functionalization of polymer brushes that contain hydroxyl, carboxylic acid, carboxylic ester, and epoxide groups in their side chains. Functionalization of brushes after their formation also allows development of one polymerization condition for the production of a variety of functional surfaces.

1.3.2.1 Functionalization of hydroxyl-containing polymer brushes

Poly(2-hydroxyethyl methacrylate) (PHEMA) and poly(poly(ethylene glycol) methacrylate) (PPEGMA) are the two most widely synthesized polymer brushes containing hydroxyl groups, and a number of post-polymerization reactions of hydroxyl-functionalized polymer brushes have been reported. (PPEGMA contains a hydroxyl group at the termini of its side chains.) Acid chlorides react extensively with the hydroxyl groups of PHEMA brushes to dramatically change film properties (Scheme 1.6a). Jennings’ group reported the derivatization of PHEMA through reaction with with C$_7$F$_{15}$COCl to change the film’s wettability and electrochemical barrier properties. The same group also studied the reaction of PHEMA films with perfluoroalkyl (C$_3$F$_7$ and C$_7$F$_{15}$) and perfluoroaryl (C$_6$F$_5$) acid chlorides to yield partially fluorinated films with lowered surface tension. Sun and coworkers derivatized hydroxyl groups with octanoyl chloride, pentadecafluoroocanoyl chloride, or palmitoyl chloride side chains to alter the hydrophobicity of the polymer brushes on membranes for selective pervaporation of organic solvents from water.
Scheme 1.6 Functionalization of hydroxyl-containing polymer brushes.
Reaction of hydroxyl-containing side chains with succinic anhydride (Scheme 1.6b) in the presence of a base such as pyridine or 4-dimethylaminopyridine converts the hydroxyl-terminated side chains in polymer brushes to carboxylic acid-terminated side chains. Because of the wide range of reactions for coupling to carboxylic acid groups, this conversion step allows further chemical derivatization for generation of a variety of functional polymer brushes.101-103

Halogen moieties (especially Br) are interesting targets for reactions with hydroxyl groups because they allow the formation of comb-shaped polymer brushes as well as other derivatization reactions. Esterification of –OH groups with 2-bromoisobutyryl bromide effectively introduces ATRP initiation sites (Scheme 1.6c) into polymer brushes.88, 104 Reaction of –OH groups with SOCl₂ can introduce chloroalkyl functional groups for further modification using nucleophilic substitution reactions.105

Several strategies have been developed to derivatize hydroxyl-containing polymer brushes with immobilized peptides and proteins.74, 75 Activation of hydroxyl groups with p-nitrophenyl chloroformate yields carbonate intermediates which readily react with the amine groups of peptides and proteins (Scheme 1.6d).106-109 Disuccinimidyl carbonate can also serve as a coupling reagent to activate hydroxyl groups for covalent protein binding (Scheme 1.6e).110 Alternatively, PHEMA or PPEGMA brushes can first react with succinic anhydride prior to coupling with nitrilotriacetate (NTA)-metal ion complexes that bind certain peptides or proteins (Scheme 1.6f).101, 111-113
1.3.2.2 Functionalization of carboxylic acid-containing polymer brushes

Poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA) and poly(2-(methacryloyloxy)ethyl succinate) (PMES) are three examples of carboxylic acid-containing polymer brushes.\textsuperscript{74} Jain reported the polymerization of MES to be the first direct surface initiated ATRP of a protonated acidic monomer.\textsuperscript{114} Most other carboxylic acid-functionalized polymer brushes were obtained by hydrolysis of the protecting group of a polymer precursor (e.g. deprotection of \textit{tert}-butyl methacrylate to methacrylic acid)\textsuperscript{115-117} or by derivatization of other functional groups (e.g. reaction of –OH groups with succinic anhydride as described in previous section).

Carboxylic acid-functionalized polymer brushes are especially attractive for immobilization of biomacromolecules. In one example, activation of –COOH groups through reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) precedes reaction with the amine groups from the peptides or proteins.\textsuperscript{118-121} Alternatively, the activated –COOH groups can react with aminobutyl NTA and then bind certain metal ions to capture peptides or proteins through metal ion–affinity interactions.\textsuperscript{116, 120, 122}

Carboxylic ester-containing polymer brushes are often synthesized to provide –COOH groups after deprotection.\textsuperscript{74} The ester-protected monomers avoid the complexities inherent in polymerization of acidic monomers such as poisoning or altering of transition metal catalysts.\textsuperscript{74, 79} Poly(\textit{tert}-butyl methacrylate) and poly(\textit{tert}-butyl acrylate) are the most common examples of carboxylic ester-functionalized brushes, and two methods have been reported to deprotect the brushes, acidic hydrolysis and pyrolysis.\textsuperscript{74} Acidic hydrolysis with HCl or methanesulfonic acid
is widely used, but might cleave brushes which are tethered to a surface by an ester bond. Exposure to 10% trifluoroacetic acid resulted in a slower hydrolysis as well as less chain degrafting compared with use of the strong acid HCl. Pyrolysis presents an alternative strategy to deprotect poly(tert-butyl methacrylate) brushes. Usually the brushes were kept at 190-200 °C for 30 min to quantitatively remove the protecting tert-butyl groups and reduce the risk of degrafting the brushes.

1.3.2.3 Functionalization of epoxide-containing polymer brushes

Epoxide groups in poly(glycidyl methacrylate) (PGMA) brushes undergo ring opening reactions with many compounds and provide various options for brush functionalization. Huck and coworkers reported that cross-linking of PGMA brushes with octylamine was complete after 4 h at 60 °C and gave heavily cross-linked networks (Scheme 1.7a). The same group also cross-linked PGMA films with 1,4-phenylenediamine (60 °C, 30 min) to enhance the film’s etch-resist properties. Kim’s group first selectively opened the epoxide ring of the brushes with sodium bisulfite and then further cross-linked the sulfonated brushes with sulfosuccinic acid via an esterification reaction (Scheme 1.7b).

PGMA also provides a substrate for synthesis of macroinitiators. Xu and coworkers reacted the epoxide pendant groups of PGMA with 2-chloropropionic acid, and the resultant polymer surface could initiate new film growth (Scheme 1.7c). ATRP initiators can also be attached to PGMA films by reaction of the epoxy groups with 2-bromo-2-methylpropionic acid. Armes and coworkers reported the synthesis of an anionic macroinitiator from PGMA. They first esterified 36 mol% of the hydroxyl groups with 2-bromoisobutyryl bromide, and then esterified 72% of the remaining –OH groups with excess 2-sulfobenzoic acid cyclic anhydride.
Scheme 1.7 Functionalization of epoxide-containing polymer brushes.
Similar to polymer brushes containing –OH and –COOH groups, PGMA is widely used to immobilize biomolecules through several strategies: direct covalent coupling by epoxide ring opening, or reaction with amine-containing metal affinity ligands (e.g. IDA) followed by protein binding. Immobilization of proteins like ferritin, glucose oxidase, anti-estrogen antibody, and penicillin G acylase to PGMA brushes occurs through the reaction of the amine groups on proteins with the epoxide groups when stirred at room temperature (Scheme 1.7d). IDA can open the epoxide rings of PGMA during incubation at 60 °C for 12 h, and subsequent formation of the PGMA-IDA-Cu\(^{2+}\) complex yields a polymer that binds bovine hemoglobin (Scheme 1.7e). Abu-Saied and coworkers bound Cu\(^{2+}\) ions to sulfonated PGMA films to create surfaces that adsorb β-galactosidase.

Several other methods are also available for derivatization of PGMA. For example, azido groups can be introduced to PGMA brushes upon reaction with sodium azide. This step affords a platform for postmodification reactions of the polymer brushes by click chemistry. PGMA brushes can also react with \(N\)-(1-pyrenylsulfonyl) ethylenediamine to generate a nitrite-selective fluorescent sensor.

### 1.3.2.4 Functionalization of other side chains of polymer brushes

In addition to the previously discussed reactions of hydroxyl-, carboxylic acid-, carboxylic ester-, and epoxide-contained polymer brushes, a number of other reactions have been employed for brush modification. Here, I will briefly cover two of them: tertiary amine quaternization and styrene sulfonation. Quaternization of poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) with bromoethane, bromohexane, bromooctane, and bromododecane has been reported to yield antibacterial surfaces. In addition, quaternization of PDMAEMA with methyl iodide is a
critical step for the synthesis of cationic macroinitiator. Poly(styrene sulfonate) (PSS) can be used to make proton-conducting membranes for potential fuel cell applications. Sulfonation of the polystyrene films with chlorosulfonic acid, fuming sulfuric acid, or acetyl sulfate is one of the ways to yield PSS brushes.

1.3.3 Applications of polymer brushes

Polymer brushes have potential applications for creating responsive, nonbiofouling, cell adhesive, and low friction surfaces, enhancing the capacity of chromatography supports, and immobilizing proteins. In this section, I will discuss polymer brushes as responsive and antibiofouling surfaces, as well as their application to protein analysis and purification.

1.3.3.1 Responsive surfaces

Stimuli-responsive polymer brushes exhibit conformational and structural changes in the presence of external triggers, such as solvent composition, temperature, pH, ionic strength, light or mechanical stress. Solvent-responsive polymer brushes are often based on conformation changes in diblock copolymer chains upon exposure to a different solvent (Figure 1.4). The brushes attempt to maximize the polymer-solvent contacts with a “good” solvent while minimizing the brush contact with a “bad” solvent.
Figure 1.7 Structural changes in a diblock copolymer brush upon variations in solvent quality; solvent B is a good solvent for both blocks, while solvent A is a good solvent for the blue block but a nonsolvent for the red block. Reproduced with permission from Barbey, R.; Lavanant, L.; Paripovic, D.; Schuwer, N.; Sugnaux, C.; Tugulu, S.; Klok, H.-A. *Chem. Rev.* **2009**, *109*, 5437-5527.

Most of the reported temperature responsive brushes have a low critical solution temperature (LCST), above which the polymer undergoes a phase transition from a soluble state (hydrophilic) to an insoluble (hydrophobic) state. Poly(N-isopropylacrylamide) (PNIPAM) is the most widely studied temperature responsive polymer because it has a LCST between 30-35 ºC in solution. The LCST value depends upon the detailed macromolecular structure as well as the salt concentration. When grafted on a surface, the LCST of PNIPAM is broader than in solution and depends on brush thickness and grafting density.

Polyelectrolytes are well known for their responses to pH or ionic strength. The charge states of weak polyelectrolytes strongly depend on the environmental pH value and ionic strength because of the protonation or deprotonation of the charged groups. Jiang’s group reported pH-responsive polymer brushes for non-fouling surfaces. The copolymerization of [2-(acryloyloxy)ethyl] trimethyl ammonium chloride and 2-carboxy ethyl acrylate yields a surface
that is not charged (bacteria-resistant) under neutral or basic conditions and positively charged (bacteria-adhesive) in acid solutions.\textsuperscript{155}

Photoresponsive brushes can switch color and wettability using light as the external stimulus. Under illumination with the appropriate wavelength, conformational changes or bond-breaking/forming in the brushes will occur to create a change in physical properties such as dipole moment or refractive index.\textsuperscript{156-158} Samanta and Locklin reported the polymerization of spiropyran-functionalized norbornyl derivatives via surface-initiated ring-opening metathesis polymerization. The relatively nonpolar spiropyran in the resultant polymer film can be reversibly switched to a polar, zwitterionic merocyanine isomer (with a larger dipole moment) using light of the appropriate wavelength.\textsuperscript{158}

Mechanosensitive brushes actively respond to an external mechanical stress.\textsuperscript{151, 159} For example, Sottos’ group demonstrated the transmission of mechanical stress from polymer backbones (poly(methacrylate) or poly(methyl methacrylate)) to mechanoresponsive spiropyran groups embedded within the chains. The polymers exhibit a color change under loading of mechanical forces. Such materials can potentially serve as stress-sensors or damage-reporting polymers.\textsuperscript{159}

1.3.3.2 Nonbiofouling surfaces

Surfaces that are resistant to nonspecific adsorption of biological species (e.g. proteins, cells, etc) are termed nonbiofouling surfaces. Such surfaces are especially interesting for applications in drug delivery, contact lenses, biosensors, and even the coating of the ship hulls.\textsuperscript{74} Polymer brushes, especially those grafted from the surface via controlled/living polymerization to achieve well-defined thickness, composition, and architecture, are attractive candidates for nonbiofouling
coatings. According to the charge states of the polymer chains, there are two main categories of nonbiofouling polymeric coatings, neutral ones and zwitterionic brushes. The hydrated ultrathin polymer films of both categories present enthalpic and entropic barriers to nonspecific adsorption of proteins and cells.\textsuperscript{74, 160}

Several neutral nonbiofouling polymer brushes have been investigated, and among them, PPEGMA is the most attractive, because of its similarity to the biocompatible and nonbiofouling poly(ethylene glycol).\textsuperscript{74, 161} Yao et al. reported a PPEGMA-\textit{b}-PDMAEMA-modified surface showing nonfouling effects for bacteria due to the hydrophilic nature of the PPEGMA block.\textsuperscript{162}

Zwitterionic brushes, including polyphosphobetaine, polysulfobetaine and polycarboxybetaine are also effective nonbiofouling polymers. Zwitterionic surfaces likely form an interfacial hydration layer via both electrostatic interactions and hydrogen bonding, and this layer enhances resistance to nonspecific protein or cell adsorption.\textsuperscript{163-165} As mentioned previously, quaternization of PDMAEMA has been reported to yield antibacterial surfaces.\textsuperscript{166} Liu and coworkers reported grafting of three zwitterionic polymers, poly(\textit{N},\textit{N}-dimethyl-\textit{N}-(\textit{p}-vinylbenzyl)-\textit{N}-(3-sulfopropyl) ammonium) (PDMVSA), poly(2-(methacryloyloxyethyl) ethyl-dimethyl- (3-sulfopropyl)- ammonium) (PDMMSA), and poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) (Scheme 1.8). All the three polymers exhibit resistance to nonspecific protein adsorption (tested with platelet poor plasma from blood) and platelet adhesion (tested with platelet rich plasma from blood).\textsuperscript{163}
Scheme 1.8 Structures of zwitterionic polymers for nonbiofouling surfaces.

**1.3.3.3 Protein binding and immobilization**

Polymer brushes have been used to bind proteins because their three-dimensional structure should allow for greatly enhanced protein loading relative to monolayers.\textsuperscript{74, 75} In addition, as mentioned previously, swollen hydrophilic polymers can minimize nonspecific protein adsorption, which makes them attractive for applications as substrates for protein microarrays.\textsuperscript{74, 75}

Covalent linking of proteins to polymers can take place with the formation of peptide and disulfide bonds, as well as the ring-opening of epoxides as described in sections 1.3.2.1, 1.3.2.2, and 1.3.2.3. Yao and coworkers reported the immobilization of horseradish peroxidase and chicken immunoglobulin by reaction with NHS-ester functionalized brushes.\textsuperscript{167} Iwasaki et al. demonstrated the introduction of disulfide bonds to PMPC-\text/em:co\text/em:PGMA brushes, and the specific immobilization of antibodies to the polymer via a thiol-disulfide interchange reaction (Scheme 1.9).\textsuperscript{168} Huang and coworkers successfully immobilized penicillin G to PGMA brushes during a simple 24-h incubation.\textsuperscript{137}
Scheme 1.9 Immobilization of antibody fragments to PMPC-co-PGMA brushes via disulfide bonds.

In addition to covalent linkages, protein can also adsorb noncovalently to polymer brushes via hydrophobic or electrostatic interactions, binding to metal-ion complexes, and avidin-biotin interactions, but hydrophobic and electrostatic interactions are nonspecific. Alexander’s groups demonstrated the temperature-controlled binding of BSA to PNIPAM films through hydrophobic interactions.\textsuperscript{169} Several groups showed binding of lysozyme to PAA and PMES brushes by electrostatic interactions.\textsuperscript{116, 122, 170} After chelation of the appropriate metal ions (Ni\textsuperscript{2+} or Fe\textsuperscript{3+}), NTA-modified brushes can selectively bind His-tagged proteins or phosphopeptides.\textsuperscript{111, 112} Dong and coworkers covalently attached avidin to PAA brushes, and then immobilized biotin-tagged proteins via avidin-biotin interaction.\textsuperscript{119}

1.4 Layer-by-layer assembly

Layer-by-layer (LBL) assembly involves alternating adsorption of interacting materials. It is a simple, inexpensive and versatile method to prepare controlled layered structures (although there is often overlap of layers),\textsuperscript{171, 172} and in this work, we use the LBL method to immobilize
trypsin in porous membranes. Below, I discuss this technique and some of its applications in bioscience.

1.4.1 Methods of layer-by-layer assembly

LBL assembly is one of the most widely used strategies to form films with controlled structure. Figure 1.8 illustrates the example of LBL assembly of polyelectrolytes and colloids on a flat surface. Upon exposure of a negatively charged substrate to a solution containing a cationic polyelectrolyte, a thin layer of polycation adsorbs to the surface via multiple polyelectrolyte-surface interactions. The excess adsorption leads to the overcompensation of surface charge to provide a positively charged surface. After rinsing to remove the excess polyelectrolytes, immersion in a solution containing anionic colloids results in colloid adsorption, and the surface charge reverses again to allow for polycation adsorption after rinsing. The process can be repeated until the desired number of bilayers is formed.

Figure 1.8 LBL assembly of polyelectrolytes and colloids via electrostatic interactions on a flat surface. Figure reprinted with permission from K. Ariga et al., Phys. Chem. Chem. Phys. 2007, 9, 2319.
Because of the versatility of LBL assembly, films can form from a number of charged species such as proteins, nucleic acids, organic polymers, colloids, and zeolites. The integration of different poly electrolytes and control over molecular architecture may lead to various applications. (I should note that layers are frequently intertwined, precluding a highly defined layer structure.) In the following section, I will focus on applications in biology.

1.4.2 Applications in bioscience

Novel bio-composite films fabricated by LBL assembly are being examined in the fields of enzyme catalysis, sensing, drug release, drug delivery, and cell technology. In the following sections, I will cover the first two applications.

1.4.2.1 Enzyme reactors formed by the LBL process

Using LBL methods, enzymes have been immobilized to various substrates, including polymeric microchips, silicon microchannels, fused silica capillaries, and polymeric membranes. Ji and coworkers reported the immobilization of trypsin on polyethylene terephthalate microchip channel walls to enhance protein digestion. The authors sequentially deposited poly(diallyldimethylammonium chloride) (PDADMAC) and zeolite nanocrystals, and then positively charged trypsin was bound to the anionic zeolite via electrostatic interactions. The resulting reactor shows the ability to digest diluted proteins (low femtomole range for cytochrome C) as well as complex protein extracts from mouse macrophages to facilitate protein identification.

Palmer’s group demonstrated the immobilization of urease to the walls of silicon microchannels. They examined the LBL immobilization of the negatively charged urease with three polycations, polyethylenimine (PEI), PDADMAC, and polyallylamine (PAH), and found
that PEI provided the highest retention of urease activity. The immobilized urease showed significant activity for at least two weeks, confirming that the LBL method can help preserve the active state of enzymes.\textsuperscript{177}

Tang, Wang and Kang developed acetylcholinesterase-based capillary reactors through sequential adsorption of PDADMAC and enzyme on fused silica capillary walls, and the capillary reactor was used to screen acetylcholinesterase inhibitors in enzyme activity assays. The electrostatic interaction was strong enough for the enzyme to withstand 100 consecutive assays. The authors also pointed out that the reactor can be easily renewed by simply flushing the capillary with 1 M NaCl to remove the old enzyme.\textsuperscript{175}

Datta, Cecil and Bhattacharyya investigated the activity of glucose oxidase immobilized in membranes using the LBL method. After formation of three (PSS/PAH) bilayers in anhydride-activated nylon membrane pores, glucose oxidase was electrostatically adsorbed to the PSS/PAH film. The immobilized glucose oxidase showed a wide range of operating pH values and presented higher stability than free enzyme. The authors also pointed out that the technique could also be applied to other enzyme-substrate systems.\textsuperscript{173}

\subsection*{1.4.2.2 Biosensing}

The above examples demonstrate the versatility of LBL deposition for forming enzymatic reactors. These enzymes can also perform sensing functions. A recent study by Chen and coworkers demonstrated the construction of a bienzyme (horseradish peroxidase—HRP and glucose oxidase—GOD) electrode for glucose biosensing (Figure 1.9). A layer of 3-mercapto-1-propanesulfonic acid was deposited on a bare gold electrode followed by a layer of positively charged (PAH-coated carbon nanotubes) (PAH-MCNTs) and a layer of anionic HRP. Subsequently, layers of concanavalin A (ConA) and a layer of GOD were sequentially deposited
via the biospecific affinity between the lectin (ConA) and sugar residues from HRP and GOD, which are known glycoproteins. As a biosensor, this film exhibited a fast (5 s) response for detecting glucose with a detection limit as low as $2.5 \times 10^{-7}$ M.\textsuperscript{178}

Figure 1.9 Schematic illustration of the construction of a bienzyme electrode based on functionalized CNTs and sugar–lectin biospecific interactions. Figure reprinted with permission from Chen, H.; Xi, F.; Gao, X.; Chen, Z.; Lin, X. Anal. Biochem. \textbf{2010}, \textit{403}, 36-42.

1.5 Outline of dissertation

Chapter 2 of this dissertation describes the growth of PHEMA brushes on magnetic nanoparticles and subsequent brush functionalization with NTA-Ni\textsuperscript{2+} to provide magnetic beads that selectively capture His-tagged protein directly from cell extracts. Transmission electron
microscopy, FT-IR spectroscopy, thermogravimetric analysis, and magnetization measurements confirm and quantify the formation of the brushes on magnetic particles, and multilayer protein adsorption to these brushes results in binding capacities (220 mg protein/g of beads) that are an order of magnitude higher than those of commercial magnetic beads. Moreover, the functionalized beads selectively capture His-tagged protein within 5 min. The high binding capacity, high protein purity, and short incubation time make brush-modified particles attractive for purification of recombinant proteins.

In Chapter 3, 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 residences 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 much higher than the 49% coverage obtained with in-solution digestion for 16 h or the 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, 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.

Chapter 4 expands the work in chapter 3 by presenting a preliminary investigation of how the number and size of peptide fragments varies with the protein residence time in the membrane. We hope to use extremely short residence times to create large protein segments for potential
applications in middle-down proteomics, where fragmentation of modest-sized (3-20 kDa) protein pieces in the mass spectrometer facilitates protein sequencing and discovery of post-translational modifications.

In the last chapter, I will present the conclusions of my research and propose some future work.
1.6 References


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Chapter 2 Polymer Brush-Modified Magnetic Nanoparticles for His-Tagged Protein Purification

This chapter describes the growth of poly(2-hydroxyethyl methacrylate) brushes on magnetic nanoparticles and subsequent brush functionalization with nitrilotriacetate-Ni$^{2+}$ to yield magnetic beads that selectively capture polyhistidine-tagged (His-tagged) protein directly from cell extracts. Transmission electron microscopy, FT-IR spectroscopy, thermogravimetric analysis, and magnetization measurements confirm and quantify the formation of the brushes on magnetic particles, and multilayer protein adsorption to these brushes results in binding capacities (220 mg protein/g of beads) that are an order of magnitude higher than those of commercial magnetic beads. Moreover, the functionalized beads selectively capture His-tagged protein within 5 min. The high binding capacity, high protein purity, and short incubation time make brush-modified particles attractive for purification of recombinant proteins.

2.1 Introduction

Many protein purification methods employ a separation step based on specific interactions between immobilized ligands and affinity tags on biomolecules,\(^1\)\(^-\)\(^3\) and the most common affinity tag is polyhistidine, which binds to immobilized Ni$^{2+}$ or Co$^{2+}$ complexes. Isolation of His-tagged protein typically occurs in columns containing resins modified with Ni$^{2+}$ complexes, often Ni$^{2+}$-nitrilotriacetate (Ni$^{2+}$-NTA), and these methods are attractive for their high protein loading, mild elution conditions, and easy regeneration of the metal complexes on the resin.\(^4\)\(^-\)\(^6\) However, slow intra-particle diffusion of biomolecules to binding sites in porous micron-sized beads can lead to relatively long processing times,\(^7\) which can be problematic when
purifying unstable proteins. As pointed out by Kaufmann, shortening purification time is vital for keeping the bioactivity of unstable proteins during the separation.

Functionalized magnetic particles are attractive for small-scale purification and analysis because they can be readily isolated from solutions using a strong magnetic field, in some cases with commercially available magnetic separators or automated magnetic bead processors. Specific modification of the particles allows isolation of protein or DNA, as well as magnetic cell separation. However, most commercial magnetic beads are micron sized and thus have a limited surface area and binding capacity (typically <25 mg protein/g of beads). The use of highly porous beads can increase capacity, but as mentioned above, slow diffusion in pores will limit binding rates. Several recent studies examined the use of modified magnetic nanoparticles for protein binding because these small particles have a large surface area to volume ratio and hence a high binding capacity of 70 - 200 mg BSA/g beads. In some instances, high binding capacities may increase the binding efficiencies for low-abundance protein. Xu and coworkers reported preliminary capacities of 2-3 g of protein per g of bead when using extremely small (diameters less than 3 nm) FePt nanoparticles modified with NTA, but such particles may be difficult to collect. Another recent studies also suggested capacities as high as 2 g protein/ per g of bead for 20 nm beads, but in all cases only a monolayer of binding ligand was present on the particle surface.

This work reports the modification of magnetic nanoparticles (MNPs) through growth of polymer brushes by atom transfer radical polymerization (ATRP) from immobilized initiators. Several recent studies show that such brushes can increase protein binding capacities in porous membranes, and prior research on ATRP from silica demonstrates that this polymerization
technique allows controlled brush growth without particle aggregation.\textsuperscript{27-33} Control over brush thickness is vital for negotiating the tradeoff between enhancing binding capacity and decreasing the magnetization of MNPs. ATRP of glycidyl methacrylate from magnetic beads permits covalent immobilization of proteins, but the highest reported binding capacity of such systems is 50 mg protein per g of beads.\textsuperscript{34,35} This work shows that MNPs with ~50 nm-thick brushes can be collected with a permanent magnet and that multilayer binding of tagged protein (see Figure 2.1) leads to a binding capacity of 220 mg protein/g of beads. Moreover, protein isolation can occur directly from cell extracts in a few minutes.

Figure 2.1 Schematic diagram of brush-modified beads and protein binding to these beads.

2.2 Experimental section

2.2.1 Materials

N,N-dimethylformamide (DMF, anhydrous, 99.8%), CuCl (99.999%), CuBr\textsubscript{2} (99%), 2,2’-bipyridyl (bpy, 99%), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 4-dimethylaminopyridine (DMAP), imidazole (99%), 2-hydroxyethyl methacrylate (HEMA, 97%), TWEEN-20 surfactant, and bovine serum albumin (BSA) were obtained from Sigma Aldrich. HEMA was passed through a column of activated basic alumina (Aldrich) prior to use. NiSO\textsubscript{4}·5H\textsubscript{2}O (Columbus Chemical), CuSO\textsubscript{4}·5H\textsubscript{2}O (CCI),
NaH₂PO₄ (CCI), Na₂HPO₄ (Aldrich), tetraethyl orthosilicate (TEOS, Fluka), Nα,Nα-bis(carboxymethyl)-L-lysine hydrate (aminobutyl NTA, Fluka), succinic anhydride (SA, Matheson Coleman & Bell), Coomassie protein assay reagent (Pierce), and all other chemicals were of analytical grade, and used as received.

2.2.2 Synthesis of silica-coated magnetic nanoparticles

EMG 308 (1 mL, Ferrotec), a solution of Fe₃O₄ nanoparticles, was diluted with water (13.6 mL) prior to addition of isopropanol (80 mL) to precipitate the magnetite nanoparticles and remove excess surfactant. The supernatant was decanted, and the magnetically collected nanoparticles were then dispersed in 80% (v:v) ethanol (EtOH) in water (200 mL). After sonication of this solution for 15 min, concentrated ammonia (5 mL) and TEOS (2 mL) were added to the solution, and the mixture was mechanically stirred for 3 h. HCl (1 mL, 2M) was added to stop the reaction, and the silica-coated MNPs were magnetically collected and washed twice with EtOH (50 mL, the same volume of solvent was used for all the washing steps), twice with water, and again with EtOH before drying under vacuum for 6 h.

2.2.3 Initiator attachment

SiO₂-MNPs were dried at 120 °C overnight and then dispersed in toluene through sonication (0.2 g beads in 150 mL). Trichlorosilane initiator, (11-(2-bromo-2-methyl)propionyloxy)undecyltrichlorosilane, was added (0.2 mL), and the mixture was stirred at 65 °C overnight while bubbling with N₂. The initiator-modified beads were washed twice with toluene (50 mL) and twice with THF (50 mL) prior to drying in vacuum for 4 h.
2.2.4 Growth of poly(2-hydroxyethyl methacrylate) (PHEMA) on beads

Initiator-modified beads (0.1 g) were dispersed by sonication in purified HEMA (10 mL), and the mixture was degassed via 3 freeze-pump-thaw cycles. In another Schlenk flask, HEMA (10 mL) was mixed with water (10 mL) and bpy (366 mg). After 3 degassing cycles, CuCl (82.5 mg) and CuBr₂ (54 mg) were added and another 2 degassing cycles were performed. In a glove bag, the two degassed HEMA mixtures were combined and magnetically stirred for 1 h. Subsequently, air was bubbled through the polymerization solution until its color turned from dark brown to dark green, and the beads were then washed sequentially with DMF (20 mL, same volume of solvent was used for all the washing steps), EtOH (twice), water, and EtOH, and dried in vacuum.

2.2.5 Derivatization

DMF (20 mL) containing SA (200 mg) and DMAP (200 mg) was mixed with PHEMA-MNPs (200 mg) and stirred at 55 ºC overnight. The SA-MNPs were washed with DMF (20 mL, same volume of solvent was used for all the washing steps), EtOH (twice) and then water (twice). Subsequently, an aqueous solution (20 mL) containing EDC (0.1 M) and NHS (0.1 M) was mixed with the SA-MNPs for 30 min followed by washing with EtOH three times. Then, the EDC/NHS-activated beads were exposed to a pH 10, aminobutyl-NTA (0.1 M) aqueous solution for 1 h with magnetic stirring and washed with water three times. Finally, the NTA-SA-PHEMA-MNPs were immersed in CuSO₄ (0.05 M) for 1 h, washed with water three times and EtOH twice and dried under vacuum for 5 h.
2.2.6 Characterization methods

Thermogravimetric analysis (TGA) was run under air flow with a 3-step program: heating from 30 °C to 120 °C at a rate of 10 °C/min, holding at 120 °C for 30 min, and finally ramping the temperature to 800 °C at 10 °C min⁻¹. Transmission electron microscopy (TEM) images were obtained on a JEOL 100CX microscope. All the samples were prepared by a drop-dry method on carbon-coated copper grids. For dynamic light scattering (DLS), particles were dispersed in water and the size distribution was determined on a ZetaPALS instrument (Brookhaven, Holtsville, NY) with a 35 mW 660 nm excitation.

2.3 Results and discussion

2.3.1 Preparation and derivatization of magnetic nanoparticles

Scheme 2.1 shows the procedure for creating magnetic particles coated with functional polymer brushes that bind proteins. The first step is the formation of a silica coating on the magnetic nanoparticles to prevent oxidation and dissolution of the Fe₃O₄. The high density of −OH groups on the silica also allows further modification of the particles through silanization. To coat Fe₃O₄ nanoparticles with silica, we employ standard sol gel methods with some important modifications.⁶,⁷ Among these modifications, precipitation of the commercial Fe₃O₄ nanoparticles in an isopropanol/water mixture (prior to their resuspension in an ethanol/water solution) presumably removes excess surfactant to minimize particle aggregation during deposition of silica through hydrolysis of TEOS and condensation on the Fe₃O₄ seeds. After formation of the encapsulated Fe₃O₄, rapid neutralization of the silica-coating solution by addition of HCl is critical to avoid the formation of large clusters during collection of the
particles with a magnet. TEM images show unaggregated particles with a dark Fe$_3$O$_4$ core, which may consist of several Fe$_3$O$_4$ nanoparticles, inside a silica shell (Figure 2.2a).

Growth of polymer brushes from silica-coated nanoparticles requires immobilization of polymerization initiators on the surface via silanization (Scheme 2.1, step 2). However, this reaction releases HCl, which may dissolve the Fe$_3$O$_4$ core even after coating with silica because of the high reaction temperature (65 ºC) and relatively long time (~15 h). In initial studies, TEM images revealed white spots in the center of some of the nanoparticles after silanization as well as an absence of dark cores, suggesting the dissolution of the Fe$_3$O$_4$ (see Figure 2.3a). To overcome this problem, we sparge the silane solution with N$_2$ during initiator attachment to both remove HCl and prevent the introduction of water vapor. With this procedure, all the initiator-modified nanoparticles in TEM images show dark Fe$_3$O$_4$ cores (Figure 2.3b).
Scheme 2.1 Synthesis and derivatization of polymer brush-modified magnetic nanoparticles.
Figure 2.2 TEM images of (a) SiO$_2$-Fe$_3$O$_4$, (b) PHEMA-SiO$_2$-Fe$_3$O$_4$ nanoparticles, and (c) NTA-SA-PHEMA-SiO$_2$-Fe$_3$O$_4$ (the inset showing the image of an NTA-SA-PHEMA-SiO$_2$-Fe$_3$O$_4$ particle in high resolution).
Figure 2.3 TEM images of initiator-modified SiO$_2$-Fe$_3$O$_4$ particles prepared (a) without and (b) with nitrogen bubbling during initiator attachment.

Subsequent polymerization of HEMA from the immobilized initiators gives MNPs modified with polymer brushes, and quenching the polymerization with air prior to collecting the brush-modified nanoparticles helps to avoid particle aggregation. The ultimate goal of this work is to create readily collectible nanoparticles that exhibit high protein-binding capacities, and binding capacity should increase with the length of polymer chains. However, long brushes also decrease the magnetization of the MNPs and make their collection difficult, so we limit the polymerization time to 1 h. ATRP is attractive for modifying MNPs because it affords control over the brush molecular weight and film thickness through variation of polymerization time. Additionally, surface-initiated ATRP minimizes solution polymerization that could result in physisorbed polymers that cause particle aggregation.

Derivatization of the polymer brushes includes reaction with succinic anhydride (SA), activation of the resulting –COOH groups with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), and subsequent
reaction with aminobutyl NTA (Scheme 2.1). Visible changes in the reaction suspensions qualitatively demonstrate the success of the derivatization procedure. For example, SA-modified MNPs disperse well in water because the side chains on the polymer brushes end in carboxylic acids, but after activation with EDC/NHS mixtures, the reacted particles tend to aggregate because they are more hydrophobic. In addition, the aggregation makes beads more collectable.

2.3.2 Characterization of particle size and the extent of polymerization and derivatization

TEM images confirm nanoparticle modification and provide estimates of particle diameters. Bare Fe$_3$O$_4$ particles have diameters of 5-15 nm, and after encasing these seeds in silica, some particles contain multiple Fe$_3$O$_4$ nuclei because of iron oxide aggregation prior to coating (Figure 2.2a). The size distribution for 290 silica-coated particles in TEM images (Figure 2.4) shows an average particle diameter of 43 ± 8 nm, and dynamic light scattering data (Figure 2.5, top) suggest that 90% of the particles have diameters between 43 and 57 nm. However, the light scattering data also show that about 10% of the particles are between 151 nm and 216 nm in diameter, which could be due to some particle aggregation.

After growth of PHEMA from the silica-coated nanoparticles, TEM images such as that in Figure 2.2b show the formation of a 10 nm-thick polymer layer. In contrast, light scattering measurements show that 90% of the PHEMA-modified particles have diameters between 134 and 170 nm, whereas the other 10% of particles are between 289 nm and 365 nm in diameter (Figure 2.5, bottom). The ~100 nm increase in diameter after polymerization suggests that the polymer layer is ~50 nm thick. Light-scattering measurements occur in water, which likely swells the film to increase thickness. Such swelling of polymer brushes is vital for binding of multilayers of protein to modified beads. The particle diameter in TEM images increases to ~100 nm after modification with NTA, showing a ~30 nm thick polymer layer on the SiO$_2$-
Fe$_3$O$_4$ core (Figure 2.2c). The contrast between the polymer and the underlying nanoparticle is relatively low, but the inset suggests a ~30 nm film on the outside of the particle. Derivatization of the polymer with NTA triples the molecular weight of the polymer repeat unit, so the large increase in thickness is not surprising. Notably, the particles are not aggregated after the derivatization process, presumably because of the high negative charge of NTA.

![Graph showing size distribution of SiO$_2$-Fe$_3$O$_4$ particles in TEM images.](image)

Figure 2.4 Size distribution of SiO$_2$-Fe$_3$O$_4$ particles in TEM images.
Figure 2.5 Nanoparticle size distribution determined from light scattering data, SiO$_2$-Fe$_3$O$_4$ (top) and PHEMA-SiO$_2$-Fe$_3$O$_4$ (bottom).
TGA plots of weight loss versus temperature (Figure 2.6) provide an estimate of the quantity of polymer grown from the nanoparticles. The coated particles consist of both thermally stable compounds that remain in the residue (silica, magnetite, and metal ions bound to the polymer) and decomposable polymer brushes and initiators that contribute to weight loss. Even with just silica-coated Fe$_3$O$_4$, however, a small amount of water and possibly residual ethoxy groups from the TEOS give rise to an 8% total weight loss. After growth of PHEMA from the particle, TGA shows a total weight loss of 71%, which corresponds to a PHEMA thickness of \( \sim 14 \) nm (see the section 2.6.2 for details of the thickness calculations from TGA data). This thickness value agrees reasonably well with the TEM thickness of \( \sim 10 \) nm. Reaction of PHEMA with SA leads to a total weight loss of 81%, which is consistent with essentially complete reaction between SA and PHEMA (see section 2.6.1). After derivatization of SA-PHEMA with NTA and NTA-Cu$^{2+}$, the percent mass loss of the particles during TGA does not increase significantly, probably because sodium and copper remain in the residue.
Figure 2.6 Weight losses obtained from TGA of SiO$_2$-Fe$_3$O$_4$ (black), Initiator-SiO$_2$-Fe$_3$O$_4$ (pink), PHEMA-Initiator-SiO$_2$-Fe$_3$O$_4$ (blue), SA-PHEMA-Initiator-SiO$_2$-Fe$_3$O$_4$ (brown), NTA-SA-PHEMA-Initiator-SiO$_2$-Fe$_3$O$_4$ (red), and Cu$^{2+}$-NTA-SA-PHEMA-Initiator-SiO$_2$-Fe$_3$O$_4$ (green).

The FTIR spectra in Figure 2.7 also confirm the polymerization and derivatization reactions in Scheme 2.1. After growth of PHEMA on the SiO$_2$-Fe$_3$O$_4$ particles, an absorption maximum due to carbonyl groups in the polymer appears at $\sim 1740$ cm$^{-1}$ (spectrum 3b). Reaction of the PHEMA with SA results in a greatly increased absorbance at $\sim 1740$ cm$^{-1}$ (spectrum 3c) because of the conversion of -OH groups to esters and the presence of carboxylic acid groups. Peaks due to formation of the succinimide ester (1817 and 1786 cm$^{-1}$, spectrum 3d) appear upon activation of carboxylic acids with EDC/NHS, and these peaks disappear after reaction with
NTA (spectrum 3e). A strong absorbance at 1680 cm\(^{-1}\) in spectrum 3e also suggests NTA immobilization and is probably due to the carboxylate groups from NTA as well as the amide bond that immobilizes the NTA.\(^{39}\)

![Normalized Absorbance](image)

Figure 2.7 KBr FTIR spectra of (a) SiO\(_2\)-Fe\(_3\)O\(_4\), (b) PHEMA-SiO\(_2\)-Fe\(_3\)O\(_4\), (c) SA-PHEMA-SiO\(_2\)-Fe\(_3\)O\(_4\), (d) NHS-SA-PHEMA-SiO\(_2\)-Fe\(_3\)O\(_4\), and (e) NTA-SA-PHEMA-SiO\(_2\)-Fe\(_3\)O\(_4\). The spectra are normalized with respect to the absorbance at 1099 cm\(^{-1}\), where most of the absorption should be due to silica.

### 2.3.3 Nanoparticle Magnetization

Brush-coated nanoparticles must be highly magnetic for collection with a magnet after protein binding. Figure 2.8 shows magnetization curves obtained with a vibrating sample magnetometer for Fe\(_3\)O\(_4\) particles before and after different modification steps. As expected, the
saturation magnetism per g of these nanoparticles decreases after silica coating (from 61.3 emu g\(^{-1}\) to 7.1 emu g\(^{-1}\)) and then decreases further to 2.5 emu g\(^{-1}\) after growth of polymer brushes. Derivatization with NTA and loading of Cu\(^{2+}\) reduces the magnetization to 0.94 emu g\(^{-1}\) because of the additional increase in mass of the particles. However, even after coating with Cu\(^{2+}\)-NTA-SA-PHEMA, a rare earth NdFeB magnet (2”x2”x0.5”) can collect particles suspended in an Eppendorf tube in 5 min, which simplifies modification and washing steps. (Centrifugation is not effective for collecting SiO\(_2\)-Fe\(_3\)O\(_4\) particles, but it is possible to collect modified particles using a bench-top centrifuge.) Optimization of the collection geometry should further decrease the time required for magnetic collection of the particles.

![Magnetization curves](image)

Figure 2.8 Magnetization curves for Fe\(_3\)O\(_4\) (Black), SiO\(_2\)-Fe\(_3\)O\(_4\) (red), PHEMA-SiO\(_2\)-Fe\(_3\)O\(_4\) (blue), and Cu\(^{2+}\)-NTA-SA-PHEMA-SiO\(_2\)-Fe\(_3\)O\(_4\) (purple) nanoparticles.
2.3.4 Binding of bovine serum albumin (BSA) to beads modified with Cu$^{2+}$-NTA-SA-PHEMA

In our initial examination of protein binding to brush-modified MNPs, we mixed 0.45 mg of Cu$^{2+}$-NTA-SA-PHEMA-modified nanoparticles in 50 μL of water with a 0.25-mL solution containing 1.2 mg BSA per mL of 20 mM pH 7.2 phosphate buffer. In this case, the protein binding likely occurs through interaction of BSA histidine residues with NTA-Cu$^{2+}$ complexes. In a control experiment, NTA-SA-PHEMA-modified MNPs without Cu$^{2+}$ showed no detectable binding of BSA, suggesting the selective binding of the histidine residues in BSA to Cu$^{2+}$. After a 30-min incubation with shaking, we isolated the beads with a magnet and determined the protein concentration in the supernatant with a Bradford assay. Based on a 33% decrease in the amount of protein in solution after mixing with the beads, the binding capacity is 220 ± 10 mg BSA per g of beads, which corresponds to approximately $1.8 \times 10^{-16}$ g BSA per modified SiO$_2$-Fe$_3$O$_4$ particle (a Cu$^{2+}$-NTA-SA-PHEMA-modified particle has a mass of $8.2 \times 10^{-16}$ g, see section 2.6.4). This mass of BSA is the equivalent of ~8 monolayers on the bare SiO$_2$-Fe$_3$O$_4$ nanoparticle, which has an average diameter of 43 nm, and the presence of the BSA alone would increase the diameter of the base SiO$_2$-Fe$_3$O$_4$ particle to ~75 nm. The total particle size after protein adsorption is of course much larger than this because of the polymer. (See section 2.6.3 for details of the calculation.) The mass of adsorbed BSA is about 25% of the mass of the Cu$^{2+}$-NTA-SA-PHEMA brush on the beads.
2.3.5 Purification of His-tagged protein from cell lysate

To demonstrate the utility of these beads for protein purification, we isolated over-expressed, His-tagged cellular retinaldehyde-binding protein (CRALBP) from a cell lysate obtained as described previously. The procedure simply involves mixing 100 μL of 20 mg/mL Ni$^{2+}$-NTA-SA-PHEMA-modified beads with 400 μL crude cell lysate, incubating for 30 min on a shaker table, collecting the beads with a magnet, washing them three times with 150 μL washing buffer (pH 7.2, 20 mM phosphate buffer containing 45 mM imidazole and 0.15 M NaCl), and eluting the protein by exposing the beads for 15 min (with shaking) to 40 μL of elution buffer (pH 7.2, 20 mM phosphate buffer containing 0.5 M imidazole and 0.5 M NaCl). Figure 2.9 shows the gel electropherogram of both the cell lysate and the bead eluate and demonstrates that the Ni$^{2+}$-NTA-SA-PHEMA nanoparticles are highly selective. Remarkably, the eluate electropherogram shows no impurity bands.

![SDS-PAGE analysis](image)

Figure 2.9 SDS-PAGE analysis (Coomassie staining) of a cell lysate containing overexpressed His-tagged CRALBP before (lane 2) and after (lane 3) purification using Ni$^{2+}$-NTA-SA-PHEMA-SiO$_2$-Fe$_3$O$_4$ beads. Lane 1 shows a standard protein ladder.
One potential advantage of brush-modified nanoparticles over traditional porous microbeads is the elimination of mass-transport limitations due to diffusion within the bead. The short processing time is critical for successful purification of unstable proteins. To examine capture rates, we mixed 300 μL of an aqueous suspension containing 20 mg of Ni\(^{2+}\)-NTA-modified beads per mL with 1.2 mL of ice-cold cell lysate and collected 350-μL aliquots of the mixture after 2, 5, 15, and 30 min. (The mixture was stirred with a vortex mixer for ~1 min at the beginning of the experiment, immediately before collection of an aliquot, and every 3-4 minutes during the incubation.) We centrifuged each aliquot, washed them four times, eluted the protein, and estimated protein concentration using a Bradford assay. (Collection of the modified beads via centrifugation facilitates kinetics experiments because it is more rapid (1 min) than magnetic collection, which takes ~5 min.) Importantly, the amount of eluted protein did not vary significantly with incubation times of 5 min, 15 min or 30 min, and beads reached 80-90% of their saturation capacities within 2 min of incubation (Figure 2.10a). Thus, 5 min is sufficient time for the beads to bind protein. Gel electropherograms (Figure 2.10b) confirm that the amount of protein binding does not vary greatly with incubation time. The fast capture presumably occurs because the swollen polymer brushes are ~50 nm thick, so diffusion into the polymer is rapid. Typical procedures for purifications using agarose beads suggest either 30-min incubation times\(^{41-43}\) or only 2-5 min for incubation with beads of very limited capacity (1-5 mg protein/ mL beads).\(^{44,45}\)
Figure 2.10 (a) Bradford assay analysis of the amount of His-CRALBP eluted from Ni\textsuperscript{2+}-NTA-SA-PHEMA-SiO\textsubscript{2}-Fe\textsubscript{3}O\textsubscript{4} beads after incubation of the beads in a cell lysate for various times. (The beads were washed prior to elution.) The amounts are normalized to the concentration with the 15-min incubation, and the error bars are the standard deviations of three measurements of the concentration in a single experiment.

(b) SDS-PAGE analysis (Coomassie staining) of a cell lysate containing overexpressed His-tagged CRALBP before (lane 2) and after (lane 3-6) purification through adsorption on Ni\textsuperscript{2+}-NTA-SA-PHEMA-SiO\textsubscript{2}-Fe\textsubscript{3}O\textsubscript{4} beads with various incubation times (lane 3: 2 min; lane 4: 5 min; lane 5: 15 min; lane 6: 30 min). Prior to analysis, the purified protein was eluted from washed beads using 0.5 M imidazole in buffer. Lane 1 shows a standard protein ladder.
To test the reusability of Ni\textsuperscript{2+}-NTA-SA-PHEMA-modified beads, after elution of His-tagged CRALBP we extracted the Ni\textsuperscript{2+} from the beads with an ethylenediaminetetraacetic acid solution, reloaded the Ni\textsuperscript{2+}, and again purified His-tagged CRALBP from a sample of cell lysate. Gel electropherograms (compare Figure 2.9 and Figure 2.11) are similar for the first and second purifications.

Figure 2.11 SDS-PAGE analysis (Coomassie staining) of a cell lysate containing overexpressed His-tagged CRALBP before (lane 2) and after (lane 3) purification using Ni\textsuperscript{2+}(reloaded)-NTA-SA-PHEMA-SiO\textsubscript{2}-Fe\textsubscript{3}O\textsubscript{4} beads. Prior to analysis, the purified protein was eluted from washed beads using 0.5 M imidazole in buffer. Lane 1 shows a standard protein ladder.

2.3.6 Comparison with other magnetic beads

Table 2.1 summarizes some of the properties of commercially available magnetic beads used for isolation of His-tagged proteins. Most beads are micron sized and have binding capacities that are a factor of 5.5-200 lower than the 220 mg/g of brush-modified beads found here. In 2004, Xu and coworkers\textsuperscript{15} suggested that modified particles with diameters of \(\sim\)2 nm bind His-tagged green fluorescent protein with a capacity of 2-3 mg per g of beads, but as mentioned in
the introduction such particles may be difficult to collect. As far as we are aware, these particles or similar nanoparticle systems with high capacities were not further developed.

Table 2.1 Properties of commercially available beads that bind His-tagged proteins.

<table>
<thead>
<tr>
<th>Beads</th>
<th>Company</th>
<th>Size</th>
<th>Incubation time (on ice)</th>
<th>Capacity</th>
<th>Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>PopCulture His•Mag\textsuperscript{TM} Purification Kit\textsuperscript{44}</td>
<td>Novagen</td>
<td>3 µm</td>
<td>5 min</td>
<td>5 mg/mL</td>
<td>Agarose</td>
</tr>
<tr>
<td>MagneHis\textsuperscript{TM} Ni-Particles\textsuperscript{45}</td>
<td>Promega</td>
<td>2 min</td>
<td>1 mg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni-NTA Magnetic Agarose Beads\textsuperscript{41}</td>
<td>QIAGEN</td>
<td>20–70 µm</td>
<td>30 min</td>
<td>0.25–1 mg/mL</td>
<td>Agarose</td>
</tr>
<tr>
<td>Dynabeads TALON\textsuperscript{TM}\textsuperscript{46}</td>
<td>Dynal Biotech</td>
<td>1.1 µm</td>
<td>10 min</td>
<td>40 mg/g</td>
<td></td>
</tr>
<tr>
<td>HIS-Select Nickel Magnetic Beads\textsuperscript{43}</td>
<td>Sigma</td>
<td>20–75 µm</td>
<td>30 min</td>
<td>≥10 mg/mL</td>
<td>Agarose</td>
</tr>
<tr>
<td>µMACS His Isolation Kit\textsuperscript{42}</td>
<td>Miltenyi Biotec</td>
<td>50 nm</td>
<td>30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni\textsuperscript{2+}-NTA-SA-PHEMA-Initiator-SiO\textsubscript{2}-Fe\textsubscript{3}O\textsubscript{4} (this work)</td>
<td>N/A</td>
<td>150 nm</td>
<td>5 min</td>
<td>220 mg/mL</td>
<td>Polymer</td>
</tr>
</tbody>
</table>

2.4 Conclusions

Growth of polymer brushes on silica-coated Fe\textsubscript{3}O\textsubscript{4} yields stable MNPs that bind an order of magnitude more protein than typical commercial magnetic microparticles. These particles can rapidly isolate multilayers of His-tagged protein directly from a cell extract with high purity, and the ∼50 nm thickness of the polymer brushes reduces diffusion limitations to allow purification in as little as 5 min. After protein elution, the beads can be reused if desired.
2.5 Appendices

2.5.1 Estimation of the Extent of Reaction of PHEMA with SA

To estimate the percent yield of the reaction of PHEMA with SA, suppose we start from 1.00 g of initiator-Silica-MNPs for all samples. TGA analysis shows that such a sample yields approximately 0.89 g of residue. Added polymer and its SA derivatives should completely decompose, so the weight of residue should be constant at 0.89 g for all the samples. To convert the initial mass for a sample of arbitrary mass to a normalized initial mass based on starting from 1.00 g of initiator-Silica-MNPs, we simply divide 0.89 g by the weight fraction remaining after TGA. Subtraction of the 1.00 g of initiator-Silica-MNPs from the normalized initial mass gives the mass of added polymer. Table 2.1 shows the masses of PHEMA, and PHEMA-SA attached to initiator-Silica-MNPs. Based on the molecular masses of PHEMA (130 g/mol) and PHEMA-SA (230 g/mol), the PHEMA and PHEMA-SA masses determined from TGA suggest that the reaction with SA occurs in 105% yield. Thus, the reaction proceeds essentially to completion.

Table 2.2 Calculated Masses (based on TGA) of PHEMA and PHEMA-SA Formed on Initiator-Silica-MNPs. The masses are normalized to samples starting with 1.00 g of initiator-Silica-MNPs with 0.89 g residue remaining.

<table>
<thead>
<tr>
<th>sample</th>
<th>% mass of residue from TGA</th>
<th>Initial Mass of sample/g</th>
<th>Mass of polymer/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiator-Silica-MNPs</td>
<td>89</td>
<td>1.00</td>
<td>0</td>
</tr>
<tr>
<td>PHEMA-MNPs</td>
<td>29</td>
<td>3.1</td>
<td>2.1</td>
</tr>
<tr>
<td>SA-PHEMA-MNPs</td>
<td>18</td>
<td>4.9</td>
<td>3.9</td>
</tr>
</tbody>
</table>

2.5.2 Estimation of PHEMA thickness from TGA data.

Figure 2.12 illustrates the composition of a PHEMA-SiO$_2$-Fe$_3$O$_4$ bead. The thickness of the polymer brushes can be calculated from the mass of PHEMA as follows.
Figure 2.12 Structure of PHEMA-SiO$_2$-Fe$_3$O$_4$.

Roughly:

Mass(PHEMA)/Mass(initiator+SiO$_2$+Fe$_3$O$_4$) = 2.1 from TGA data in Table 2.1

Density of polymer ($\rho_{\text{PHEMA}}$): 1.2 mg/cm$^3$; thickness: $x$ nm

Density of silica ($\rho_{\text{SiO}_2}$): 2 mg/cm$^3$; thickness (d): 16.5 nm

Density of magnetite ($\rho_{\text{Fe}_3\text{O}_4}$): 5 mg/cm$^3$; radius (r): 5 nm

Noting that mass equals the product of density, $\rho$, and volume, $V$,

$$\frac{\rho_{\text{PHEMA}} \times V_{\text{PHEMA}}}{\rho_{\text{silica}} \times V_{\text{silica}} + \rho_{\text{Fe}_3\text{O}_4} \times V_{\text{Fe}_3\text{O}_4}} = 2.1$$

Using the formulae for the volumes of spherical shells,

$$\frac{\rho_{\text{PHEMA}} \times \frac{4}{3} \times \pi \times [(x + d + r)^3 - (d + r)^3]}{\rho_{\text{SiO}_2} \times \frac{4}{3} \times \pi \times [(d + r)^3 - r^3] + \rho_{\text{Fe}_3\text{O}_4} \times \frac{4}{3} \times \pi \times r^3} = 2.1$$

$$\frac{1.2 \times \frac{4}{3} \times \pi \times [(x + 16.5 + 5)^3 - (16.5 + 5)^3]}{2 \times \frac{4}{3} \times \pi \times [(16.5 + 5)^3 - 5^3] + 5 \times \frac{4}{3} \times \pi \times 5^3} = 2.1$$
Solving the simple equation, \( x = 14 \) nm. This calculation neglects the thickness of the initiator (less than 0.5 nm based on TGA data).

2.5.3 Calculation of the Mass of Protein in a BSA monolayer on a SiO\(_2\)-Fe\(_3\)O\(_4\) Particle with a Diameter of 43 nm

To calculate the mass of a BSA monolayer on a SiO\(_2\)-Fe\(_3\)O\(_4\) Particle (43 nm in diameter), we assume that a 4 nm thick (\( d_{\text{BSA}} \)) BSA monolayer\(^{47} \) forms on the outside of a single bead with a surface area of \( S_{\text{bead}} \). (The assumption of a monolayer thickness of 4 nm and a film density of 1 g/cm\(^3\) may be a slight overestimation because of incomplete packing.)

\[
m_{\text{bead}} = m_{\text{silica}} + m_{\text{Fe}_3\text{O}_4} = \rho_{\text{SiO}_2} \times V_{\text{SiO}_2} + \rho_{\text{Fe}_3\text{O}_4} \times V_{\text{Fe}_3\text{O}_4}
\]

\[
= \frac{4}{3} \times \pi \times \{ \rho_{\text{SiO}_2} \times [(d + r)^3 - (r)^3] + \rho_{\text{Fe}_3\text{O}_4} \times r^3 \}
\]

\[
= \frac{4}{3} \times \pi \times \{2 \times [(21.5e - 7)^3 - (5e - 7)^3] + 5 \times (5e - 7)^3 \}
\]

\[
= (8.5e - 17)g
\]

\[
m_{\text{monolayerBSA/ bead}} = \rho_{\text{BSA}} \times d_{\text{BSA}} \times S_{\text{bead}} = \rho_{\text{BSA}} \times d_{\text{BSA}} \times 4\pi \times (d + r)^2
\]

\[
= 1 \times (4e - 7) \times 4 \times \pi \times (21.5e - 7)^2
\]

\[
= (2.3e - 17)g
\]

2.5.4 Calculation of the mass of a fully modified bead

The above calculations show that the mass of a single SiO\(_2\)-Fe\(_3\)O\(_4\) particle is 8.5e-17 g. The TGA data in Table 2.1 show that there are 2.1 g of polymer per g of initiator-modified SiO\(_2\)-Fe\(_3\)O\(_4\). Assuming complete derivatization of each PHEMA repeat unit with SA and aminobutyl NTA-Cu\(^{2+}\), the molar mass of the repeat unit will increase from 130 to 538 g/mol. Thus, after modification there will be 8.7 g of Cu\(^{2+}\)-NTA-SA-PHEMA per g of initiator-SiO\(_2\)-Fe\(_3\)O\(_4\). Hence,
neglecting the initiator mass, which should be negligible, a single, fully modified bead will have a mass of $8.2e-16$ g. (Note that we neglect the initiator in part because its TGA data are difficult to interpret as the the silane will become part of the residue.)
2.6 References


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Chapter 3 Facile Trypsin Immobilization in Polymeric Membranes for Rapid, Efficient Protein Digestion

(This research was performed in collaboration with Weihan Wang.)

This chapter describes the use of sequential adsorption of poly(styrene sulfonate) and trypsin in nylon membranes as 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 much higher than the 49% coverage obtained with in-solution digestion for 16 h or the 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.

3.1 Introduction

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.\textsuperscript{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).\textsuperscript{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 nearly twice 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.\textsuperscript{3} The enhanced efficiency stems primarily from the high enzyme concentration at the substrate surface,\textsuperscript{4-7} and previous materials employed for trypsin immobilization include polyvinylidene fluoride (PVDF) membranes,\textsuperscript{4, 6} micro-particles,\textsuperscript{8-10} monoliths,\textsuperscript{5, 11, 12} and polymeric microfluidic channels.\textsuperscript{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.\textsuperscript{14} However, accelerating the transport of the substrate protein into the particle pores is critical for increasing the total digestion rate.\textsuperscript{14} Thus, rapid protein 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.\textsuperscript{3, 11} Unfortunately, preparation of monolithic columns is somewhat complex (normally this takes several days), and the trypsin immobilization occurs via covalent linking, which demands a high enzyme concentration in modification solutions\textsuperscript{12} or reaction times as long as 19 hours.\textsuperscript{5, 11, 12} Moreover, the random covalent linking (via the N-terminus or lysine residues) of trypsin to the support might lead to the loss of enzyme activity.\textsuperscript{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 \( \mu \text{m} \)) between the solution and the wall when the solution separates into many streams that pass through the membrane simultaneously (Scheme 3.1). Membranes have a much smaller thickness (usually about 100 \( \mu \text{m} \)) 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.

Scheme 3.1 Conceptual representation of a membrane reactor for tryptic digestion.

Lee et al. demonstrated protein digestion using PVDF membranes that contain trypsin adsorbed to membrane pores via hydrophobic interactions. These modified membranes successfully digest single small proteins in minutes, but substrate proteins may displace trypsin by adsorbing to the PVDF support. 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, and the bound enzymes frequently show activities similar to those of enzymes in solution. 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 straightforward, 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.

3.2 Experimental Section

3.2.1 Materials

Trypsin (Type I, ~10,000 $N_\alpha$-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, 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.

3.2.2 Modification of membranes with trypsin

3.2.2.1 Modification of nylon 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.

3.2.2.2 Modification of PVDF membranes with trypsin

For comparison, PVDF membranes were modified with trypsin (PVDF/trypsin) via hydrophobic adsorption following a literature. Briefly, A 25-mm-diameter disc was cut from a PVDF membrane sheet and inserted in a Swinnex holder (Millipore, SX0002500). NH₄HCO₃ solution (10 mM) was passed through the membrane at 10 mL/h for 1 h using a syringe pump to wet the pores prior to passage of the trypsin solution (0.6 mg/mL in 10 mM NH₄HCO₃) through the membrane at a flow rate of 5 mL/h for 1 h. The membrane was flushed with 10 mM NH₄HCO₃ solution (10 mL/h) for another 20 min to remove any unbound trypsin before drying with nitrogen.

3.2.3 Protein denaturation

Protein solutions were prepared in water (10 mg/mL), separated into aliquots containing 100 μg of protein, dried (SpeedVac), and stored at -20 °C until use.

3.2.3.1 Chaotropic denaturation

Briefly, 100 μg of BSA or BSA/α-casein mixture (10:1 mass ratio) was dissolved in 20 μL of 6 M urea containing 50 mM NH₄HCO₃, mixed with 5 μL of 10 mM DTT, and heated in a water bath at 56 °C for 1 h to cleave the disulfide bonds. After diluting with 160 μL of 50 mM NH₄HCO₃, 10 μL of 100 mM iodoacetamide was added, and the cysteine residues were
carbamidomethylated in the dark for 1 h (final protein concentration of 0.5 mg/mL). The protein samples were either digested immediately or stored at 4 ºC.

3.2.3.2 SDS denaturation

Protein (100 µg) was dissolved in a 50 µL solution containing 100 mM tris-HCl, 10 mM DTT and 1 wt% SDS, and this solution was incubated in a boiling water bath (5 min for α-casein and 30 min for BSA) followed by addition of 10 µL of 100 mM iodoacetamide to alkylate cysteine residues (1 h in dark). The mixture was finally diluted with 10 mM NH₄HCO₃ to 0.5 mg protein/mL (SDS concentration of 0.25 wt%), and digested immediately (in some cases after dilution).

3.2.4 Protein digestion

Proteins were digested either directly in NH₄HCO₃ buffer without denaturation (only for α-casein) or after denaturation using urea or SDS.¹⁸

3.2.4.1 In-solution digestion

Unless otherwise stated, in-solution digestion of proteins was performed with 0.5 mg substrate protein/mL by adding 10 µL of 0.5 mg/mL trypsin (prepared in 1 mM HCl) to 200 µL of the protein solution and incubating at 37 ºC for 16 h. (The protein solutions contained ~50 mM NH₄HCO₃ for non-denatured and urea-denatured proteins, and ~10 mM NH₄HCO₃ for SDS-denatured proteins. The trypsin/substrate protein mass ratio is always 1/20.) The digestion was quenched by addition of 11 µL of acetic acid.

3.2.4.2 Membrane digestion

To evaluate the completeness of membrane digestion, a 25-mm-diameter nylon/PSS/trypsin
membrane (big membrane in Scheme 3.2) was employed for digesting several mL of α-casein in ~10 mM NH₄HCO₃, and the digests were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 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 3.2). The effective filtration area was ~0.02 cm², as determined by exposure to dye in the holder and measurement of the diameter of the stained spot. Typically, the aforementioned 0.5 mg/mL protein samples were diluted to 0.1 mg/mL with either water or 10 mM NH₄HCO₃ to achieve a final NH₄HCO₃ 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 3.2).

Scheme 3.2 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.
3.2.5 SDS-PAGE evaluation of membrane digestion

To evaluate membrane digestion efficiency, a 25-mm-diameter nylon/PSS/trypsin membrane was inserted in a Swinnex holder (Scheme 3.2); ten mL of 10 mM NH₄HCO₃ was passed through the membrane for conditioning; and non-denatured α-casein solutions (0.020-2.0 mg/mL in 10 mM NH₄HCO₃) were passed through the membrane using a syringe pump at specific flow rates (0.02-2.0 mL/min) and collected. The permeates were dried and re-dissolved in gel loading buffer¹⁹ to achieve a final protein concentration of 1 mg/mL. Seven-μL aliquots of each protein digest as well as intact α-casein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (gel prepared as a 4% stacking gel and a 12% separation gel) with Coomassie blue staining to reveal any undigested α-casein.

3.2.6 Removal of SDS from protein digests

Removal of SDS from tryptic digests of SDS-denatured BSA was performed with ZipTip SCX pipette tips following the manufacturer’s protocol. Briefly, the protein digest was diluted to 0.1 mg/mL (if needed), and a 13.5-μL aliquot was acidified by mixing with 1.5 μL TFA. The SCX tip was equilibrated with 0.1% TFA in 30% methanol, loaded with the acidified digest sample, and rinsed with 0.1% TFA in 30% methanol. The peptides bound to the tip were finally eluted with 4 μL of 5% NH₄OH in 30% methanol, and 1 μL of eluate was subjected to MALDI-MS analysis.

3.2.7 Phosphopeptide enrichment

Tryptic digests of urea-denatured BSA/α-casein mixtures were dried and reconstituted to 0.3 mg/mL using 1% TFA. One μL of each of the digests was spotted on a
polymer-oxotitanium-modified MALDI plate, incubated for 30 min, rinsed with 1% TFA in 50% acetonitrile, and dried with nitrogen. Elution of phosphopeptides occurred via adding 1 μL of 1% H₃PO₄ on the enrichment spot followed by addition of 0.25 μL of 40 mg/mL DHB (prepared in 0.1% TFA in 50% acetonitrile). MALDI-MS and collision-induced dissociation (CID)-MS/MS were performed after allowing the matrix solution to dry.

3.2.8 MALDI-MS, 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 NH₄HCO₃ to achieve a final NH₄HCO₃ 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 NH₄HCO₃ 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.

3.2.9 Peptide sequence confirmation/identification

The mono-isotopic mass of each peptide detected in the mass spectrum was first compared with the theoretical mono-isotopic mass (obtained using the ProteinProspector MS-Digest program and allowing up to 2 missed cleavages, cysteine alkylation (for BSA only), variable methionine oxidation, and variable phosphorylation on Ser and Thr (for α-casein only)). Ions with S/N>3 and m/z values that matched theoretical values (allowing up to 0.5 m/z mass
were subjected to ion trap CID-MS/MS followed by a MASCOT MS/MS search against the SwissProt database to confirm the sequence of the peptide (cut-off score > 5). Searching parameters for BSA peptides were: tryptic digestion with up to 2 missed cleavages, metazoan taxonomy, fixed carboxylamidomethylation of Cys, variable modification by oxidation of methionine, an allowed mass tolerance of 0.5 Da for both peptide ions and fragment ions, a peptide charge state of 1+, and an ESI-Trap instrument. Parameters for α-casein peptides (without phosphopeptide enrichment) were the same as for BSA except that no fixed modification was assigned and variable phosphorylation of Ser and Thr was included.

For phosphopeptide identification after on-plate phosphopeptide enrichment, all the signals present in the mass spectra (S/N>3) were subjected to CID-MS/MS. To map as many phosphorylation sites as possible, we expanded the MASCOT search parameters by choosing semiTrypsin as the enzyme and allowing more variable modifications: carboxymethylamidation of Glu and Asp (+57), carbamylation of the N-terminus and Lys (+43), dethiomethylation of Met (-48), and formation of pyroglutamic acid due to cyclization of a N-terminal glutamine (Pyro-glu from Gln, -17). Each mass spectrum shown is a representative of experiments using at least three membrane discs (except for the durability test).

3.3 Results and discussion

3.3.1 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 adsorbs to nylon via hydrophobic
interactions to give negatively charged pores.\textsuperscript{21, 22} With a pI of \textasciitilde{}10.5,\textsuperscript{23} 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,\textsuperscript{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,\textsuperscript{24} 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\textsuperscript{2} of membrane, which equals 11 mg of trypsin per cm\textsuperscript{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\textsuperscript{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.
3.3.2 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 3.1 (top) 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 150-1.5 s). The detection limit of SDS-PAGE with Coomassie blue staining is 0.1-0.5 μg,\textsuperscript{25} and lanes 3-6 were loaded with 7 μg of membrane-digested protein, so the electropherogram suggests at least 93% digestion. Figure 3.1 (bottom) shows the gel electropherogram of membrane-digested α-casein in solutions containing initial protein concentrations ranging from 0.020-2.0 mg/mL. With a 30 s membrane residence time, the absence of detectable protein in the electropherogram again suggests greater than 93% digestion.
Figure 3.1 SDS-PAGE analysis of intact $\alpha$-casein (lanes 2 and 7) and $\alpha$-casein after membrane digestion (lanes 3-6) with different residence times (top) and different protein concentrations during digestion (bottom). For the upper electropherogram, the $\alpha$-casein concentration prior to digestion was 0.1 mg/mL, and for the bottom electropherogram the residence time was 30 s. The protein was not denatured prior to digestion.
Figure 3.2 MALDI-MS sequence coverages obtained after membrane digestion of non-denatured α-casein solutions using different residence times (top) and protein concentrations (bottom). For the upper graph the α-casein concentration prior to digestion was 0.1 mg/mL, and for the bottom graph the residence time was 30 s.

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.
digestion of $\alpha$-casein using a wide range of residence times (Figure 3.2 top) or protein concentrations (Figure 3.2 bottom) and subsequent MALDI-MS give a relatively constant sequence coverage of $\sim$53% for the $\alpha$−S1 chain (22.9 kDa). The maximum sequence coverage of 43% for the $\alpha$−S2 chain (24.3 kDa) occurs with a 6 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 $\alpha$−S1 chain. For comparison, the sequence coverages of $\alpha$-S1 and $\alpha$-S2 chains after in-solution digestion for 16 h are 46% and 26%, respectively. 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.

3.3.3 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 $\mu$g 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 $2 \text{ mm}^2$ and a $\sim 4 \mu \text{L}$ dead volume, the miniaturized setup (Scheme 3.2) can process a few $\mu\text{L}$ of protein sample.

Figure 3.3 shows the mass spectra of non-denatured $\alpha$-casein digested both in solution and using the small membrane. The MALDI-MS sequence coverages of the $\alpha$–S1 and $\alpha$–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 $\alpha$–S1 and 26% for $\alpha$–S2). With a
flow rate of 0.1 mL/h (residence time of 13 s), digesting 1 μg of protein in a 0.1 mg/mL solution of protein takes 6 min.

**Membrane digestion**
(13 s residence time)
100% = 1.03x10^4
Sequence coverage:
chain α-S1: 55%; chain α-S2: 33%

**In-solution digestion (16 h)**
100% = 1.41x10^4
Sequence coverage:
chain α-S1: 46%; chain α-S2: 26%

Figure 3.3 MALDI-mass spectra of α-casein after membrane digestion (top spectrum, miniaturized membrane reactor with an effective filtration area of 2 mm^2, 0.1 mg protein/mL for both digestion and analysis) and in-solution digestion (bottom spectrum, 0.5 mg protein/mL for digestion, dilution to 0.1 mg/mL for analysis).

BSA (66.4 kDa) is the substrate protein most widely used to examine digestion performance. Figure 3.4 shows the MALDI-mass spectra of urea-denatured BSA digested with the small membrane (13 s residence time) or in solution (16 h). Remarkably, the sequence coverage with
membrane digestion (84%) is much higher than with in-solution digestion (49%), showing that
the trypsin-modified membrane digests this protein more efficiently than in-solution digestion in
terms of both sequence coverage and digestion time.

<table>
<thead>
<tr>
<th>Method</th>
<th>Residence Time</th>
<th>Sequence Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane digestion</td>
<td>13 s</td>
<td>84%</td>
</tr>
<tr>
<td>In-solution digestion</td>
<td>16 h</td>
<td>49%</td>
</tr>
</tbody>
</table>

500 1000 1500 2000 2500 3000 3500 4000
m/z

Figure 3.4 MALDI-mass spectra of urea-denatured BSA after membrane-digestion (top, miniaturized membrane reactor with an effective filtration area of 2 mm², residence time of 13 s, 0.1 mg protein/mL for both digestion and analysis) and after in-solution digestion for 16 h (bottom, 0.5 mg protein/mL for digestion, dilution to 0.1 mg/mL for analysis).

Table 3.1 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. Digestion of BSA using the trypsin-modified nylon
membrane allows detection of nearly twice as many peptides as 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 0.45 μm membrane, and (3) a high activity of the immobilized enzyme due to the electrostatic immobilization.

Table 3.1 Comparison of MS sequence coverages for tryptic digestion of BSA by different techniques (all the results were obtained using MALDI-MS).

<table>
<thead>
<tr>
<th></th>
<th>Sequence coverage</th>
<th>Peptides found</th>
<th># of missed cleavages allowed</th>
<th>Residence time</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-solution</td>
<td>49%</td>
<td>28</td>
<td>2</td>
<td>16 h</td>
</tr>
<tr>
<td>Fe₃O₄/carbon nanotube</td>
<td>46%</td>
<td>28</td>
<td>1</td>
<td>5 min</td>
</tr>
<tr>
<td>Magnetic particle on chip-1²⁷</td>
<td>21%</td>
<td>13</td>
<td>n/a</td>
<td>5 min</td>
</tr>
<tr>
<td>Magnetic particle on chip-2²⁸</td>
<td>43%</td>
<td>30</td>
<td>n/a</td>
<td>10 s</td>
</tr>
<tr>
<td>Microchip (zeolite)¹³</td>
<td>44%</td>
<td>19</td>
<td>n/a</td>
<td>&lt; 5 s</td>
</tr>
<tr>
<td>Monolith-1⁵</td>
<td>44%</td>
<td>n/a</td>
<td>n/a</td>
<td>4.5 min</td>
</tr>
<tr>
<td>Monolith-2¹¹</td>
<td>26%</td>
<td>12</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nylon membrane</td>
<td>70%</td>
<td>44</td>
<td>1</td>
<td>13 s</td>
</tr>
<tr>
<td></td>
<td>84%</td>
<td>52</td>
<td>2</td>
<td>13 s</td>
</tr>
</tbody>
</table>

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

The anionic surfactant SDS is frequently present in protein samples as a denaturation agent,²⁸-³⁰ a detergent for water-insoluble proteins,³¹, ³² or the eluent for removal of immunoprecipitated antigens from antibody beads.¹⁸, ³³, ³⁴ Despite its various applications, however, SDS often hinders protein digestion³⁵ and MS analysis,³², ³⁶, ³⁷ 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 NH₄HCO₃ for membrane digestion (final SDS concentration of 0.05%). Comparative in-solution digestions occurred with 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 3.5a 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). 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 3.5b and 3.5c). 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. The mass spectrum of this sample (Figure 3.5d) 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 3.5b and 3.5c) stems from inefficient digestion. In contrast, the immobilized trypsin shows significant tolerance for SDS.
Membrane digestion, 100%=4.93x10³
(13 s residence time)
Digestion conditions:
0.1 mg/mL α-casein, 0.05% SDS
Sequence coverage:
chain α-S1: 46%; chain α-S2: 9%

In-solution digestion (16 h), 100%=7.05x10³
Digestion conditions:
0.1 mg/mL α-casein, 0.05% SDS
Sequence coverage: 0%

In-solution digestion (16 h), 100%=3.00x10³
Digestion conditions:
0.5 mg/mL α-casein, 0.25% SDS
Sequence coverage: 0%

(d) In-solution digestion (16 h), 100%=1.98x10³
Digestion conditions:
0.5 mg/mL α-casein, no SDS
(5-fold dilution in 0.05% SDS prior to analysis)
Sequence coverage:
chain α-S1: 42%; chain α-S2: 12%

Figure 3.5 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.)
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 α-casein peptides and SDS.

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 membrane-digested BSA reveals 26 tryptic peptides (Figure 3.6a), which cover 57% of the protein sequence. In contrast, the in-solution digestion gives no BSA tryptic peptide signals even after SDS removal (Figures 3.6b and 3.6c).
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 in-solution digestion and other reported digestion techniques without SDS (Table 3.1). 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.

3.3.5 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 3.7a-3.7c), and the sequence coverage is above 80% over the full 33 h (Figure 3.8). 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.
Figure 3.7 MALDI-mass spectra of membrane-digested BSA as a function of the time employed for continuously passing 0.1 mg/mL urea-denatured BSA solution through the miniaturized trypsin-modified membranes (0.1 mL/h). The urea concentration in the diluted digest is 0.12 M.
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 (time of 3.9 h) (Figure 3.8). Additionally, the S/N ratios of BSA peptides in MALDI-mass spectra (Figures 3.7d and 3.7e) 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 interaction between trypsin and PSS. The substrate protein likely replaces the immobilized trypsin in the PVDF membrane.\textsuperscript{6}

![Figure 3.8](image)

Figure 3.8 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, flow rate of 0.1 mL/h) through miniaturized nylon/PSS/trypsin and PVDF/trypsin membranes.
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, 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 (Figure 3.9).

![MALDI-mass spectra](image)

Figure 3.9 MALDI-mass spectra of 0.1 mg/mL, urea-denatured BSA after digestion with a freshly prepared membrane (top spectrum) and a membrane stored for 7 months (bottom spectrum). These experiments employed the miniaturized membrane reactor with an effective filtration area of 2 mm².
3.3.6 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.\textsuperscript{38, 39} 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 3.10). 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 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.
Figure 3.10 Mass spectra of phosphopeptides enriched from a mixture containing urea-denatured BSA and α-casein (mass ratio of 10:1) digested with a membrane (top, 0.1 mg/mL total protein during digestion) and in solution (bottom, 0.5 mg/mL total protein during digestion). The phosphopeptides 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).  

3.4 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 nearly twice 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. Due to the above advantages, as well as their long-term stability and simple fabrication, the trypsin-modified membranes can potentially simplify protein analyses.
3.5 Appendices

Table 3.2 Peptide ions observed in MALDI-MS spectra of α-casein digested in membranes and in solution. m/z shows the theoretical mono-isotopic m/z of the [M+H]+ ion, “+” means the peptide was detected in the sample, “In-sln” represents in-solution digestion of non-denatured protein, “Mem” represents membrane digestion of non-denatured protein by nylon/PSS/trypsin, and “SDS” represents membrane digestion of α-casein in the presence of SDS.

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* The sequence numbers correspond to the α-S1-casein (P02662) and α-S2-casein (P02663) sequences provided by Swiss-Prot database, which both include the signal peptide segment (sequence number 1-15). However, the signal peptides do not exist in either α-S1 or α-S2 chains of our α-casein and are not counted in the sequence coverage calculation.
Table 3.3 Peptides observed in the MALDI-MS spectra of denatured BSA after digestion in membranes and in solution. “In-sln” represents in-solution digestion of urea-denatured BSA, “Mem (Nylon)” represents digestion of urea-denatured BSA by nylon/PSS/trypsin, “Mem (PVDF)” represents digestion of urea-denatured BSA by PVDF/trypsin. “SDS” represents digestion of SDS-denatured BSA by nylon/PSS/trypsin.

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<td>2355.1</td>
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<td>3511.7</td>
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# of peaks detected

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* The sequence numbers correspond to the BSA (P02769) sequence provided by the Swiss-Prot database including the signal peptide segment (sequence number 1-18). However, the signal peptide does not exist in our BSA, and is not counted in the sequence coverage calculation.
Table 3.4 α-Casein phosphopeptides observed in the MALDI-MS spectra of a urea-denatured mixture of BSA and α-Casein. Mass spectra were obtained after membrane digestion and enrichment on MALDI plates modified with polymer-oxotitanium. The phosphopeptide sequences, numbers of phosphoryl groups, and theoretical mono-isotopic m/z values of [M+H]^+ are listed.

<table>
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<tr>
<th>Phospho-peptide</th>
<th>Phosphopeptide sequence*</th>
<th>Sequence number***</th>
<th># of −PO3H2</th>
<th>m/z MH+</th>
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<tr>
<td>α1</td>
<td>V.PNpSVEQ.K.Q</td>
<td>S1 88-94</td>
<td>1</td>
<td>881.4</td>
</tr>
<tr>
<td>α2</td>
<td>I.VPNpSAEER.L</td>
<td>S1 127-134</td>
<td>1</td>
<td>981.4</td>
</tr>
<tr>
<td>α3</td>
<td>K.TVDMEpSTEV.F.T</td>
<td>S2 153-162</td>
<td>1</td>
<td>1237.5</td>
</tr>
<tr>
<td>α4</td>
<td>K.VPQLEIVPnSAEER.L</td>
<td>S1 121-134</td>
<td>1</td>
<td>1660.8</td>
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<tr>
<td>α5</td>
<td>Y.KVPQLEIVPnSAEER.L</td>
<td>S1 120-134</td>
<td>1</td>
<td>1788.9</td>
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<tr>
<td>α6**</td>
<td>YLGEYLIVPnSAEER</td>
<td>S1 1</td>
<td>1</td>
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<tr>
<td>α7</td>
<td>T.DIGpSEPSTEDQAMEDIK.</td>
<td>S1 58-73</td>
<td>2</td>
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<td></td>
<td>QM(cam)-105</td>
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<td>α8</td>
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<tr>
<td>α9</td>
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<td>S1 119-134</td>
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<tr>
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<td>Q</td>
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<td></td>
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<td></td>
<td>D(cam)</td>
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<td>α11</td>
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<td>QETYK.Q</td>
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<tr>
<td>α14</td>
<td>K.QMEAEpSlpSpSPEEVNpSVEQ.K.H</td>
<td>S1 74-94</td>
<td>5</td>
<td>2655.9</td>
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<tr>
<td></td>
<td>Pyro-glu from Q, M(cam)-105</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>α15</td>
<td>K.QMEAEpSlpSpSPEEVNpSVEQ.K.H</td>
<td>S1 74-94</td>
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Table 3.4 continues.

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<tr>
<th>α17</th>
<th>R.NANEEYSIGpSpSpSEEp</th>
<th>S2 61-85</th>
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<td></td>
<td>SAEVATEEVK.I</td>
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</tbody>
</table>

* Most of these sequences were identified by Mascot, and all of the sequences were manually verified. Some phosphopeptides share the same sequence, but are differently modified during protein denaturation, alkylation, or digestion.

** This is a new sequence variant of part of the α-S1 chain, which was manually verified.39

*** The sequence numbers correspond to the α-S1-casein (P02662) and α-S2-casein (P02663) sequences provided by Swiss-Prot database, which both include the signal peptide segment (sequence number 1-15). However, both α-S1 and α-S2 chains in our α-casein sample do not contain the signal peptide part.
3.6 References


(20) Wang, W. H.; Palumbo, A. M.; Tan, Y. L.; Reid, G. E.; Tepe, J. J.; Bruening, M. L. *J.*
Proteome Res. 2010, 9, 3005-3015.


(27) Li, Y.; Xu, X.; Yan, B.; Deng, C.; Yu, W.; Yang, P.; Zhang, X. J. Proteome Res. 2007, 6, 2367-2375.


Chapter 4 Quantitative Study of Peptide Patterns after Trypsin-Modified Membrane Digestion with Various Residence Times

(This research was performed in collaboration with Weihan Wang.)

The previous chapter shows that a high concentration of immobilized trypsin in the pores of nylon membranes leads to much more rapid protein digestion than in-solution digestion as well as increased sequence coverages. This chapter presents a preliminary investigation of how the number and size of peptide fragments varies with the protein residence time in the membrane. We hope to use extremely short residence time to create large protein segments for potential applications in middle-down proteomics, where fragmentation of modest sized (3-20 kDa) protein pieces in the mass spectrometer facilitates protein sequencing and discovery of post-translational modifications.

4.1 Introduction

Currently there are two popular strategies for mass spectrometry (MS)-based proteomics. The dominant bottom-up method is well developed and involves complete digestion of substrate proteins prior to analysis with mass spectrometry. However, this technique suffers from a few drawbacks, such as limited protein sequence coverage by identified peptides, loss of labile posttranslational modifications (PTMs), and ambiguity of the origin of redundant peptide sequences. The other strategy is the fast developing top-down approach, which includes fragmentation and analysis of intact proteins within the mass spectrometer. This method affords high sequence coverages of target proteins and efficient characterization of PTMs, but technical challenges such as intact protein separation, the need for high mass accuracy.
instruments, and difficulties in fragmentation of large proteins limit this strategy. An alternative “middle-down” approach (Figure 4.1) uses limited digestion to generate large peptides (3-20 kDa) and combines some of the advantages from both bottom-up and top-down strategies. For example, the long peptides can cover extensive regions of the protein, increase the sequence coverage, and retain multiple PTMs.

**Figure 4.1** Flow chart of middle-down protein analysis. Figure reprinted with permission from Garcia, B. A. *J. Am. Soc. Mass. Spectrom.* 2010, 21, 193-202.
Under certain conditions, enzymatic or chemical digestion can generate long peptides for middle-down protein analyses.\textsuperscript{4,8} Proteases such as Glu-C, Asp-N and Lys-C are used instead of trypsin, because they target less abundant amino acids, which leads to longer peptides.\textsuperscript{4,9} Garcia and coworkers reported middle-down MS analysis of histones (an alkaline protein that binds to DNA in eukaryotic cell nuclei) to characterize their PTMs.\textsuperscript{5} They incubated the histones with Glu-C, which mainly cleaves peptide bonds at the C-terminus of glutamic acid, at a protease to substrate ratio of 1:10 or 1:15 for 4 h (37 °C) to generate large peptides (5.4 kDa).\textsuperscript{10,11} Zhang, Liu and Katta digested recombinant monoclonal antibodies (~150 kDa) with Lys-C at a protein/enzyme weight ratio of 200:1 for 20 min at room temperature to get long peptides (~25 kDa).\textsuperscript{12}

Fenselau’s group developed acid cleavage of ribosome proteins using microwave heating of protein mixtures in 12.5% acetic acid at 140 ± 5 °C for 20 min.\textsuperscript{13,14} The microwave-accelerated proteolysis by acetic acid occurs specifically on either or both sides of aspartic acid residues. The human ribosomal protein database shows that 3.81% of the total protein residues are Asp, while the percentages for Arg, Lys, and Arg-plus-Lys are 9.30, 12.36, and 21.65%, respectively.\textsuperscript{13} Based on the abundances of the amino acid residues, Fenselau predicted the distribution of peptide products (masses >0.5 kDa with 0 or 1 missed cleavage) by length in 84 proteins from human ribosomes (Figure 4.2). Tryptic digestion gives 3397 peptides (Figure 4.2a), Lys-C generates fewer peptides (2406, Figure 4.2b), and acid digestion shows the smallest number of peptides (991, Figure 4.2c) as well as the longest peptides. The authors then experimentally identified 28% of the peptides predicted for acid digestion (Figure 4.2d).\textsuperscript{13}
Figure 4.2 Distribution of peptide digestion products by length as predicted for the 84 proteins in the human ribosome when cleaved by (a) trypsin, (b) Lys-C, and (c) Asp-C acid cleavage. Graph (d) shows the distribution of Asp-C peptides identified experimentally after an acid cleavage digestion. Figure reprinted with permission from Cannon, J.; Lohnes, K.; Wynne, C.; Wang, Y.; Edwards, N.; Fenselau, C. J. Proteome Res. 2010, 9, 3886-3890.

Although enzymatic and chemical digestions have proven successful for applications in middle-down proteomics, the proteases (Glu-C and Lys-C) are much more expensive than trypsin, and coupling acid digestion with on-line separation and analysis can be extremely difficult. The ultimate aim of this work is development of an inexpensive, on-line digestion method that uses short residence times in trypsin-modified membranes to generate long peptides. The minimal thicknesses of membranes make them uniquely suited for fine control over the time
of exposure to trypsin. Before moving on to our ultimate goal, the first step is to quantitatively compare the peptides that result from digestion with different residence times.

4.2 Experimental section

4.2.1 Materials

Trypsin (Type I, ~10,000 $N\alpha$-benzoyl-L-arginine ethyl ester hydrochloride units/mg protein, treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone), poly(styrene sulfonate) (PSS, average molecular weight ~70,000), $\alpha$-casein, propionic anhydride, and propionic anhydride-D$_{10}$ were purchased from Sigma-Aldrich. Nylon membrane discs (Hydrophilic, 0.45 $\mu$m pore size, 25 mm diameter, 170 $\mu$m 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.

4.2.2 Protein digestion with trypsin-modified membranes

Nylon/PSS/trypsin membranes were prepared as described in chapter 3, and membrane digestion was carried out with the miniaturized setup that has an external surface area of ~0.02 cm$^2$. Non-denatured $\alpha$-casein (0.1 mg/mL) in 10 mM NH$_4$HCO$_3$ was passed through the trypsin-modified membrane at different flow rates, 12 mL/h, 1.2 mL/h and 0.1 mL/h, which resulted in residence times of 0.1 s, 1 s and 13 s, respectively. After passing through the membrane, the digest was collected and quenched with glacial acetic acid to avoid any possibility of continuing digestion by leached trypsin.

4.2.3 Peptide modification with propionic anhydride.

Protein digests were dried using a speed vac, dissolved in 5 $\mu$L of 10 mM NH$_4$HCO$_3$, and mixed with 10 $\mu$L of propionic anhydride/MeOH (1/9 v/v). After a 12-h reaction time, the solution was again dried with a speed vac. The sample was reconstituted in 0.1% TFA to achieve
a final protein concentration of 1 μM. All digests were derivatized by normal propionic anhydride, and a second sample digested with a 13-s residence time was also derivatized with D$_{10}$-propionic anhydride.

**4.2.4 Analysis by matrix assisted laser desorption/ionization (MALDI)-MS**

Each of the digest samples (0.1-s, 1-s, and 13-s residence times labeled with H$_5$-acetyl groups) was spotted on the MALDI plate followed by adding the same amount of 13-s residence-time digest that was labeled with D$_5$-acetyl groups. The solution was 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). The ratio of relative peptide abundances for each digestion time were calculated by dividing the MALDI-MS intensity of the H$_5$-labeled peptide by the intensity of the D$_5$-labeled peptide (13-s digestion).

**4.3 Results and discussion**

**4.3.1 Reaction of peptides with propionic anhydride and propionic anhydride-D$_{10}$**

Peak intensities in MALDI mass spectra do not directly correlate to analyte abundances because of different ionization efficiencies and inhomogeneous crystallization.$^{15}$ However, labeling of two samples of digested peptides with isotopic compounds allows quantitative determination of the peptide ratios in the samples.$^{16-18}$ Here we reacted the peptide mixtures with propionic anhydride or propionic anhydride-D$_{10}$ (Figure 4.3)$^{17}$ These anhydrides react with amine groups at the peptide N-terminus or in Lys residues.
Figure 4.4 shows the mass spectra of peptides before and after labeling. The base peak in the unlabeled peptides (Figure 4.4a) has an m/z value of 1267.8, and MS/MS spectra show that this signal stems from the peptide R.YLGYLEQLLR.L, which has only 1 amine group (N-terminus) and no Lys residues for N-propionylation. After labeling with H₅-acetyl groups (Figure 4.4b) or D₅-acetyl groups (Figure 4.4c), the monoisotopic mass increases to 1324.0 or 1329.0, respectively, suggesting reaction with only the terminal amine group. The mass difference between the H₅- and D₅-peptides is 5.0 as expected. In other cases, multiple amine groups react with propionic anhydride. For example, the signal with m/z 1953.1 (Figure 4.4a) corresponds to K.YKVPQLEIVPnSAEER.L (2 sites for N-propionylation), and after modification with H₅-acetyl (Figure 4.4b) or D₅-acetyl groups (Figure 4.4c), the m/z values are 2065.2 and 2075.2, respectively, with a mass difference of 10.0. The mass spectra confirm that the labeling is successful, but the noise in the spectra increases after labeling, probably due in part to different extents of reaction with propionic anhydride.
Figure 4.4 MALDI-mass spectra of α-casein digested using a trypsin-modified membrane (13 s residence time) prior to (a) no labeling, (b) labeling with H$_5$-acetyl groups, and (c) labeling with D$_5$-acetyl groups.
4.3.2 Quantification of α-S1-casein peptide patterns after membrane digestion with different residence times

To compare peptide patterns that result from membrane digestion using various residence times, we labeled a 13-s digest by reaction with D₁₀-propionic anhydride and then mixed this sample 1:1 with membrane-digested samples reacted with H₁₀-propionic anhydride. Finally, we performed MALDI-MS with these mixtures and compared the intensity ratios of the same peptides labeled with D₅-acetyl and H₅-acetyl groups. Figure 4.5a shows the results of a control experiment, where both H₅-acetyl and D₅-acetyl labeled peptides were digested in residence times of 13 s. We identified six peptides (P₁, P₂, …, and P₆; Table 4.1) from α-S1-casein and calculated the (H₅-acetyl-Pᵢ)/(D₅-acetyl-Pᵢ) intensity ratios. In principle, all the ratios should be 1.0, but there were small variations (<30%) (Figure 4.6a).

To take these small variations into account, when calculating the (H₅-acetyl-Pᵢ)/(D₅-acetyl-Pᵢ) intensity ratios for 1-s and 0.1-s residence-time samples mixed with the D₅-acetyl labeled peptides (Figure 4.5b and 4.5c), we divided the intensity ratio by the corresponding (H₅-acetyl-Pᵢ)/(D₅-acetyl-Pᵢ) value where both peptides were digested in 13s. Figure 4.6b and 4.6c show the corrected ratios. Generally, the six peptides can be divided into two groups. For samples with residence times of 1 s or 0.1 s, (H₅-acetyl-Pᵢ)/(D₅-acetyl-Pᵢ) ratios for peptides 2, 3 and 4 are bigger than (or close to) 1, meaning these peptides are equally or more abundant than in the slow digestion sample (13-s residence time). In contrast, the corrected (H₅-acetyl-Pᵢ)/(D₅-acetyl-Pᵢ) ratios for peptides 1, 5 and 6 are all less than 0.3 for both 1 s and 0.1 digestion times, showing that formation of these peptides requires longer digestion times. In addition, the
difference between P₂, P₃, and P₄ (H₅-acetyl-Pᵢ)/(D₅-acetyl-Pᵢ) ratios and similar ratios for P₁, P₅, and P₆ is larger for the 0.1-s digestion than for the 1-second digestion (compare Figures 4.6b and 4.6c).

Figure 4.5 MALDI-mass spectra of membrane-digested α-casein (13 s residence time) labeled with D₅-acetyl groups and mixed with H₅-acetyl-labeled digests prepared using membrane residence times of (a) 13 s, (b) 1 s, and (c) 0.1 s.
Table 4.1 Peptides observed in the MALDI-MS spectra of α-casein after digestion in membranes. (m/z shows the theoretical mono-isotopic mass of the [M+H]^+ ion.)

<table>
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<tr>
<th>Peptide number</th>
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<tr>
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<td>S1 106-115</td>
<td>0</td>
<td>1267.8</td>
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<tr>
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<td>K.HIQKEDVPSER.Y</td>
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<td>4</td>
<td>K.HQGLPQEVLNLLL.R.F</td>
<td>S1 23-37</td>
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<tr>
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<td>S1 119-134</td>
<td>1</td>
<td>1952.3</td>
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<tr>
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<td>K.KYKVPQLEIVPnSAEER.L</td>
<td>S1 118-134</td>
<td>2</td>
<td>2080.0</td>
</tr>
</tbody>
</table>

* The sequence numbers correspond to the α-S1-casein (P02662) sequence provided by the Swiss-Prot database, which includes the signal peptide segment (sequence number 1-15).

However, the signal peptides do not exist in the α-S1 chain of our α-casein.

The high (H_5-acetyl-P_i)/(D_5-acetyl-P_i) ratios of peptides 2, 3 and 4 show that these peptides can be cleaved from the protein in less than 0.1 s; whereas the other three peptides require longer times for effective cleavage. Figure 4.7 shows the position of the cleaved peptides in α-casein. Peptides 2 and 3 are connected and their cleavage from α-casein essentially divides the protein chain into two parts (not including peptide 2 and 3). Subsequent cleavage of peptide 4 from the protein should divide the bottom part of the peptide into two parts. Peptides 1 and 6 (the difference between peptides 5 and 6 is a terminal Lys residue, so we only discuss peptides 1 and 6 here) are in the top region, and their absence in MALDI mass spectra after 0.1 s digestion may indicate that short residence times will lead to a large fragment containing the top of the protein.
Figure 4.6 \((\text{H}_5\text{-acetyl-Pi})/(\text{D}_5\text{-acetyl-Pi})\) MALDI-MS signal intensity ratios for mixtures where \(\alpha\)-casein was digested in a membrane for (a) 13 s, (b) 1 s, or (c) 0.1 s, labeled with \(\text{H}_5\)-acetyl groups, and mixed with membrane-digested \(\alpha\)-casein (13-s residence time) that was labeled with \(\text{D}_5\)-acetyl groups. (For 1-s and 0.1-s residence time samples, the ratios were obtained from three mass spectra.)
Figure 4.7 Position of peptides 1-6 in α-S1-casein (from the crystal structure\textsuperscript{19}).

Overall, short residence times in membrane-based digestion result in increased intensities of some peptides that likely reside in areas of the protein that are readily accessible to trypsin. Conversely, inaccessible peptides require longer times for cleavage from the protein. The selective cleavage sites at short digestion times may provide relatively large peptides for middle-down proteomics. Examination of whether larger peptides are present will require analysis of digests using a MALDI-time of flight (TOF), electrospray ionization (ESI)-TOF, or ESI-ion trap instrument to increase the detectable m/z range.

4.4 Conclusions

We successfully digested α-casein in different residence times in a trypsin-modified membrane and labeled the digests with H\textsubscript{5}-acetyl and D\textsubscript{5}-acetyl groups to compare peptide intensities. Quantitative examination of the peptides patterns in MALDI-mass spectra of the
digests reveals selective cleavage at highly accessible sites on the protein. Large protein fragments may also be present in the digest, but their analysis will require further studies with MALDI-TOF or ESI instruments that can detect peptides with larger m/z values. If these larger fragments are present, membrane-based digestion may prove useful for middle-down proteomics.
4.5 References


Chapter 5 Conclusions and future work

5.1 Conclusions and future work

This dissertation describes the development of two kinds of materials for protein separation and analysis: polymer brush-modified magnetic nanoparticles and trypsin-modified membranes. Chapter 2 demonstrates that the magnetic nanoparticles selectively capture His-tagged proteins. Chapter 3 explores the trypsin-modified membranes facilitate the digestion of proteins prior to their analysis by mass spectrometry. Chapter 4 expands the work on membrane-based proteolysis and presents a preliminary investigation of how the number and size of peptide fragments varies with the protein residence time in the membrane.

Growth of Ni\(^{2+}\)-NTA-SA-PHEMA brushes on silica-coated Fe\(_3\)O\(_4\) yields stable MNPs that bind an order of magnitude more protein than typical commercial magnetic microparticles. These particles can rapidly isolate 8 monolayers of His-tagged protein directly from a cell extract with high purity, and the \(\sim 50\) nm thickness of the polymer brushes reduces diffusion limitations to allow purification in as little as 5 min. After protein elution, the beads can be reused if desired.

We can expand this work by using modified-MNPs for phosphopeptides enrichment. Protein phosphorylation is an important cellular regulatory mechanism, so identifying phosphorylation sites on regulatory proteins is a key for understanding diseases such as cancer and finding new pharmaceutical targets.\(^1,2\) Fe\(^{3+}\)-NTA complex and titania are two commonly used materials for enriching phosphopeptides.\(^2\) Fe\(^{3+}\)-NTA is more specific for multiply phosphorylated peptides than monophosphopeptides, whereas titania prefers to bind monophosphopeptides.\(^2,3\) We have successfully modified MNPs with NTA-SA-PHEMA brushes, which readily binds Fe\(^{3+}\) ion, and

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titania (or oxo-titanium) can be immobilized to beads by reacting SA-PHEMA with titanium(IV) isopropoxide (Ti(i-PrO)$_4$) (Figure 5.1). The mixture of the two kinds of beads will present unbiased phosphopeptide enrichment.

Figure 5.1 Schematic drawing of the formation of the Polymer-oxoTi hybrid structure, and the binding and elution of phosphopeptides in this material.

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 $\alpha$-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 nearly twice as many MS-detectable peptides for BSA. Miniaturized membranes consume only a few $\mu$L of sample, and processing of a 10 $\mu$L solution takes about 6 min, which 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. Due to the above advantages, as well as their long-term stability and simple fabrication, the trypsin-modified membranes can potentially simplify protein analyses. However,
all digestion by nylon/PSS/trypsin membranes is currently syringe-based and analyzed using MALDI-MS. It is promising if we can couple our membranes with online LC separation systems, which allows high through-put analysis of complexed samples (such as immunoprecipitates) (Figure 5.2).5

Figure 5.2 Experimental setup for integration of the enzyme reactor into the nanoLC−ESI-MS system. Figure reprinted with permission from Krenkova, J.; Lacher, N. A.; Svec, F. Anal. Chem. 2009, 81, 2004-2012.

Another direction to expand the work on nylon/PSS/trypsin membranes is to generate long peptides (3-20 kDa) by varying residence times for applications on middle-down proteomics. We successfully digested α-casein in different residence times in a trypsin-containing membrane and labeled the digests with H5-acetyl and D5-acetyl groups to compare peptide intensities. Quantitative examination of the peptides patterns in MALDI-mass spectra of the digests reveals selective cleavage at highly accessible sites on the protein. Large protein fragments may also be
present in the digest, but their analysis will require further studies with MALDI-TOF or ESI instruments that can detect peptides with larger m/z values. If these larger fragments are present, membrane-based digestion may prove useful for middle-down proteomics.
5.2 References


