Applications of Polymer Brushes in Protein Analysis and Purification

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Key Words

protein immobilization, phosphopeptide enrichment, protein microarrays, immobilized metal-affinity chromatography, ion exchange, size exclusion

Abstract

This review examines the application of polymer brush–modified flat surfaces, membranes, and beads for protein immobilization and isolation. Modification of porous substrates with brushes yields membranes that selectively bind tagged proteins to give 99% pure protein at capacities as high as 100 mg of protein per cubic centimeter of membrane. Moreover, enrichment of phosphopeptides on brush-modified matrix-assisted laser desorption/ionization (MALDI) plates allows detection and characterization of femtomole levels of phosphopeptides by MALDI mass spectrometry. Because swollen hydrophilic brushes can resist nonspecific protein adsorption while immobilizing a high density of proteins, they are attractive as substrates for protein microarrays. This review highlights the advantages of polymer brush–modified surfaces over self-assembled monolayers and identifies some research needs in this area.

1. INTRODUCTION

MALDI-MS: matrix-assisted laser desorption/ionization mass spectrometry

Polymer brushes are assemblies of polymeric molecules tethered to a substrate such that the graft density is high enough to force the polymer chains to extend away from the surface (1). The end of the polymer chain is held on the surface by physisorption or covalent bonding, whereas the bulk of the chain extends into the solution or air interface, as shown in **Figure 1** (2). In an appropriate solvent, brushes can be swollen and highly extended for rapid capture and purification of proteins or other analytes (3–5). Highly swollen, hydrophilic brushes are also useful for minimizing nonspecific adsorption of proteins (6–8), and the functional groups in such brushes can be readily tailored for specific separations.

This review focuses on the application of polymer brushes for protein immobilization, purification, and enrichment. We first describe different approaches for the synthesis of polymer brushes and then discuss applications of polymer brush–modified flat surfaces, membranes, and beads for protein immobilization and isolation. Modification of porous substrates with brushes yields membranes that selectively bind genetically modified (tagged) proteins to give 99% pure protein at capacities as high as 100 mg of protein per cubic centimeter of membrane. Moreover, enrichment of phosphopeptides on brush-modified matrix-assisted laser desorption/ionization (MALDI) plates allows detection of femtomole levels of phosphopeptides by MALDI mass spectrometry (MALDI-MS). The last section of the review discusses future research directions in this area.

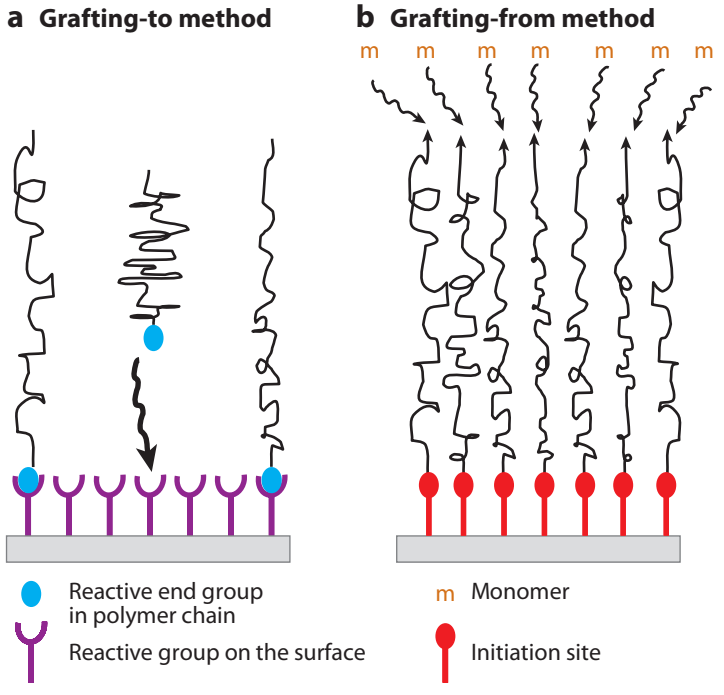

Figure 1

Schematic representation of different approaches to polymer brush synthesis. (*a*) Grafting-to method in which active groups on the surface and reactive end groups in the polymer chains react to form a covalent bond. (*b*) Grafting-from method in which the polymer chains grow from surface-tethered initiators.

2. SYNTHESIS OF POLYMER BRUSHES

ATRP: atom-transfer radical polymerization

Initially, polymer brushes were formed by physical adsorption of block copolymers (9, 10). In this case, one block has affinity for the surface, whereas the other block does not and extends into the solvent. However, because such systems are often unstable, recently developed synthetic techniques use covalent attachment of polymers to substrates to provide more robust brushes. Covalent grafting generally occurs through either grafting-to (11) or grafting-from (12–14) techniques. In the grafting-to method, end-functionalized polymer chains bind to a substrate via a chemical reaction between active groups on the surface and active end groups in the polymer chains (**Figure 1*a***) (11). This method results in relatively low grafting densities because steric hindrance prevents incoming polymer chains from diffusing through previously deposited chains to reactive surface sites. In contrast, in the grafting-from strategy, the polymer chains grow directly from surface-tethered initiators (**Figure 1*b***) (12–14). The grafting-from approach yields a high density of chains because small monomers can readily reach growing chains or initiators on the surface. Initiator immobilization on the surface is a vital step in the grafting-from method and affords some control over brush density (15, 16). Typical initiator-attachment strategies include reaction of surface hydroxyl/amino groups with acid chlorides or acid bromides (17, 18), modification of Au surfaces using thiols or disulfides (18, 19), reaction of alumina or silica with silanes (18, 20), and adsorption of polyelectrolyte macroinitiators (21, 22). A number of recent review articles provide an extensive discussion of the synthesis of polymer brushes (23–25).

The grafting-from approach has been used to modify various surfaces with almost all known polymerization techniques including cationic (26), anionic (27), radical (28), ring-opening metathesis (29, 30), photochemical (31, 32), and electrochemical (33) polymerization. Controlled radical polymerization techniques such as atom-transfer radical polymerization (ATRP) (34–36), reversible addition-fragmentation transfer (37, 38), and nitroxide-mediated polymerization (39) have emerged as some of the most powerful synthetic methods for brush formation because they afford controlled polymer growth and polymers with relatively low polydispersity. Additionally, surface-initiated polymerization with these techniques frequently results in minimal polymerization in solution. ATRP is especially attractive due to its mild reaction conditions (room temperature in many cases); use of readily available catalysts, initiators and monomers; and tolerance to impurities (40–42).

The use of controlled polymerization is particularly important for creating films in complex geometries such as the pores of membranes, where the brushes can serve as high-capacity, selective absorbents. In the synthesis of such materials, uncontrolled polymerization or formation of polymer in solution can rapidly plug pores and prevent flow. Surface-initiated ATRP in membrane pores minimizes polymerization in solution and allows for control over the polymer brush thickness through variation of polymerization time. Husson and coworkers (43) used ATRP to grow poly[poly(ethylene glycol)methacrylate] brushes inside regenerated-cellulose ultrafiltration membranes. At a constant pressure, the water flux through the membrane decreased monotonically with increasing polymerization time because growth of the brushes decreased the pore diameters. This study also showed that the molecular weight cutoff of the membrane decreases with increasing polymerization time, further confirming that ATRP provides control over the diameter of the pores in the membrane. Yusof & Ulbricht (44) studied the effects of photografting conditions and monomer concentration on polymer brush growth inside membrane pores. They found that the density of polymer chains in the membrane correlates with the surface density of the entrapped photoinitiator, benzophenone.

3. PROTEIN IMMOBILIZATION AND PURIFICATION WITH POLYMER BRUSHES ON FLAT SURFACES, BEADS, AND MONOLITHS

Poly(AA): poly(acrylic acid)

Polymer brushes are attractive for protein immobilization and purification because three-dimensional, water-swollen brushes have sufficient accessible volume to bind the equivalent of many monolayers of protein. Increases in binding capacity may enhance the efficiency and/or sensitivity of analytical devices such as membrane absorbers, protein microarrays, and modified MALDI plates used for protein capture. Several methods for immobilizing proteins in brushes have been reported, including covalent binding, electrostatic adsorption (ion exchange), and binding to metal-ion complexes (20, 45, 46). Brushes containing carboxylic acid and epoxide groups are particularly common because they can be readily derivatized (47, 48).

Poly(acrylic acid) [poly(AA)] brushes are especially attractive for protein immobilization because in aqueous solution these films swell to four times their initial thickness to facilitate binding of large biomolecules (45, 48, 49). Dai and coworkers (49) modified Au-coated Si with poly(AA) brushes and their derivatives and immobilized protein in these films via ion-exchange (**Figure 2*a***) and metal-ion affinity interactions (**Figure 2*b***). Both methods give high protein-binding capacities. Remarkably, using the first method, about 80 monolayers (16.2 μg cm^{-2}) of lysozyme absorb

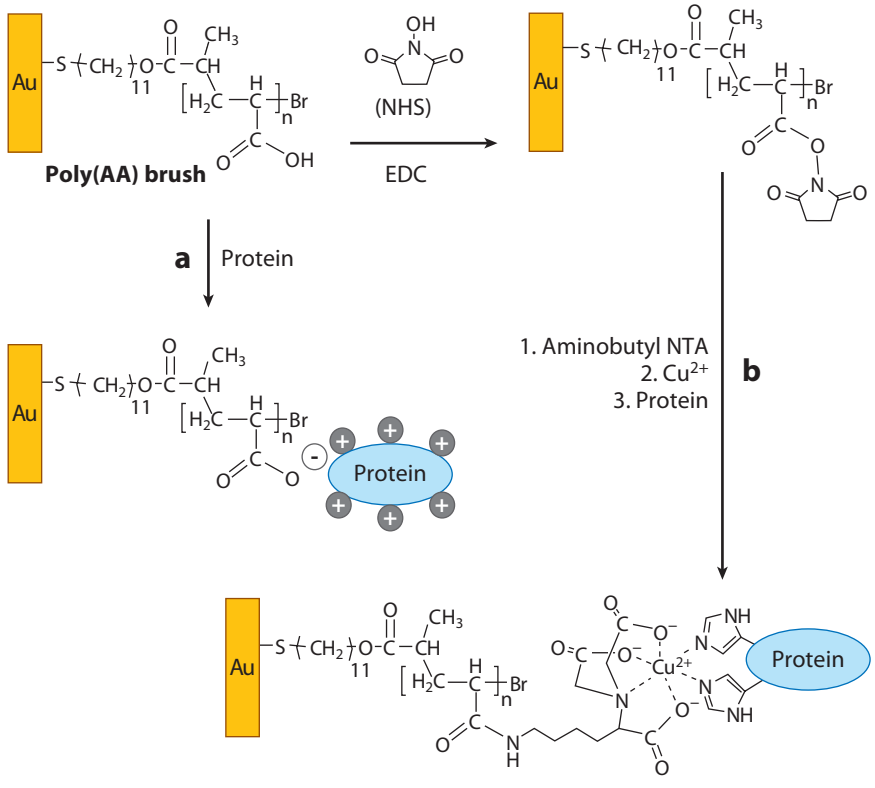

Figure 2

Protein immobilization on poly(acrylic acid) [poly(AA)] brush-modified films via (*a*) ion-exchange interactions and (*b*) metal-ion affinity interactions. For metal-ion affinity binding, polymer brushes were activated with *N*-hydroxysuccinimide (NHS) in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC). The NHS-activated poly(AA) brushes were derivatized with nitrilotriacetate (NTA)-Cu^{2+} complexes.

electrostatically on a 55-nm-thick poly(AA) film on Au. Functionalization of the poly(AA) brushes with nitrilotriacetate (NTA)-Cu^{2+} complexes yields films capable of absorbing large amounts of protein via metal-ion affinity interactions. A 55-nm-thick poly(AA) film modified with NTA-Cu^{2+} binds 5.8 μg cm^{-2} of bovine serum albumin (BSA), 7.7 μg cm^{-2} of myoglobin, and 9.6 μg cm^{-2} of anti-immunoglobulin G. This corresponds to approximately 20 monolayers of protein in these films. Recently, Cullen et al. (48) used poly(AA)-NTA-Cu^{2+} brushes to immobilize ribonuclease A at a capacity of 11 μg of ribonuclease A per square centimeter of film, which is ∼30 monolayers of the immobilized enzyme.

Groll and coworkers (50) captured polyhistidine-tagged (His-tagged) proteins from a crude cell lysate using glass surfaces modified with NTA-Ni^{2+}-terminated poly(ethylene glycol) (PEG). Through use of microcontact printing (51), amine-modified NTA was covalently patterned on films of isocyanate-terminated, star-shaped PEG, and His-tagged enhanced green fluorescent protein was selectively captured from crude cell lysate using these films. Such coatings are attractive as substrates in protein microarrays because they resist nonspecific adsorption and are highly selective.

Beads are attractive substrates for protein purification because of their high surface area, and several groups used beads modified with polymer brushes to purify proteins from egg white via ion exchange (52–54). As an example, Bayramoğlu and coworkers (53) used poly(methacrylic acid) [poly(MAA)]-grafted chitosan beads for purification of lysozyme from 50% diluted egg white at pH 6.0. The chitosan-*g*-poly(MAA) beads showed a lysozyme binding capacity of ∼66 mg of lysozyme per gram of beads. Single-step purification of lysozyme from egg white with these beads resulted in 94% pure lysozyme as determined by high-performance liquid chromatography. The high purity is achieved because lysozyme has a net positive charge at pH 6.0, whereas all other proteins in egg white are negatively charged at this pH.

Magnetic beads are attractive for use in drug delivery; immunoassays; protein and enzyme immobilization; and in the separation, isolation, and analysis of biomolecules because they can be easily collected or focused using a modest magnetic field. Recently, a number of groups demonstrated the immobilization of proteins on magnetic beads modified with polymer brushes (**Figure 3**) (55, 56). Huang and coworkers (55) immobilized BSA in poly(glycidyl methacrylate-*co*-glycerol monomethacrylate) brushes grafted to magnetic microspheres. This copolymer brush contains both hydrophobic and hydrophilic polymer blocks; the epoxide groups in poly(glycidyl methacrylate) [poly(GMA)] units can covalently bind proteins, whereas the hydrophilic poly(glycerol monomethacrylate) units enable the microspheres to disperse efficiently in aqueous solution. These materials have a binding capacity of ∼27 mg of BSA per gram of beads. The poly(GMA-*co*-glycerol monomethacrylate) brushes can also immobilize penicillin G acylase (PGA), and the activity of this enzyme depends on the ratio of the constituent monomers (57). The maximum PGA activity in the hydrolysis of penicillin G (753 μmol min^{-1} per gram of beads) occurs when the weight ratio of GMA to glycerol monomethacrylate used to form the brushes is 60 to 40. Enzyme activity decreases when more of the hydrophilic monomer, glycerol monomethacrylate, is present because the enzyme substrate, penicillin, must diffuse through the hydrophilic polymer to the enzyme. When >60% of the hydrophobic polymer GMA is present, the activity of the beads also decreases because the brushes collapse in water and bind little enzyme. Compared to its free form, microsphere-immobilized PGA is less sensitive to changes in temperature and pH. The activity of the immobilized enzyme decreased by 8.5% when the temperature was changed from 45° C to 55° C, whereas the activity for the free enzyme under the same conditions decreased by 80%. Approximately 64% of the enzyme activity was retained after ten cycles of repeated use (57).

In addition to modifications performed on beads and flat surfaces, polymer brush–modified silica monoliths have been used for purification of proteins using ion exchange (58, 59), size-exclusion

NTA: nitrilotriacetate

BSA: bovine serum albumin

PEG: poly(ethylene glycol)

Poly(GMA): poly(glycidyl methacrylate)

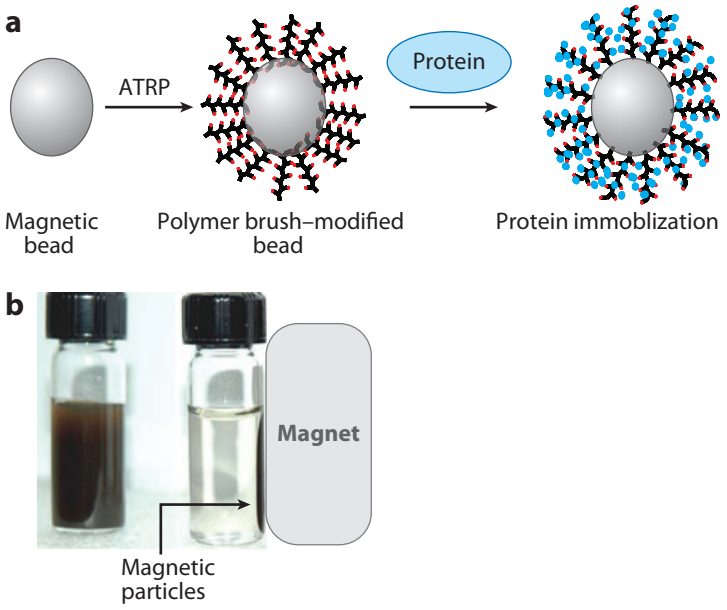

Figure 3

(*a*) Schematic representation of protein immobilization on polymer brush–modified magnetic beads. (*b*) Image of 100-nm-diameter silica-coated magnetic beads with (*right*) and without (*left*) collection by a magnet. Abbreviation: ATRP, atom-transfer radical polymerization.

(28, 60, 61), and hydrophobic-interaction chromatography (62–64). Kikuchi & Okano (63) reviewed the use of polymer brush–modified stationary phases for applications in different areas of chromatography. Importantly, surface-initiated radical polymerization enables fine control over the thickness of polymer brushes, so modification of porous stationary phases with brushes affords control over pore size (28, 60). Huang et al. (65) modified nanoporous silica gel with poly(acrylamide) brushes and used this gel for size-exclusion-based chromatographic separation of proteins. The thickness of the poly(acrylamide) brushes was 10 nm, which was much smaller than the average pore size of the silica gel (86 nm). Thus, the polymer film reduced the pore size of the silica gel but did not plug the pores. Using a column of the modified gel, the authors eluted thyroglobulin (molecular weight 66,430 Da), ovalbumin (44,000 Da), and ribonuclease (13,700 Da) in order of decreasing molecular weight.

In the case of smaller proteins, however, strong interactions between analytes and polymer chains limit the use of polymer brushes in size-exclusion chromatographic columns (65). Yoshikawa et al. (61) studied the interactions of proteins with poly(hydroxyethyl methacrylate) [poly(HEMA)]-modified silica as well as the effects of the poly(HEMA) on size-exclusion chromatography. Their findings suggest that the interactions of poly(HEMA) brushes with proteins are minimal on the outermost surface but more prominent inside the brushes. Thus, for large proteins that cannot penetrate the brushes, the separation is dominated by size exclusion. However, for smaller proteins that can enter the brushes, both size and absorption affect the separation. The ability to use both size-exclusion and absorption mechanisms may allow some separations that are not possible with size exclusion alone, but the combination of the two mechanisms can complicate interpretations of elution order.

Poly(HEMA): poly(hydroxyethyl methacrylate)

4. SELECTIVE PROTEIN PURIFICATION IN POLYMER BRUSH–MODIFIED MEMBRANES

Purification is often the bottleneck step in producing proteins for therapeutic or research purposes. Perhaps the most powerful method for protein purification is affinity adsorption, in which immobilized ligands interact specifically with an affinity tag that is genetically engineered into the protein of interest. Modified surfaces selectively bind those proteins containing the affinity tag, whereas other cellular proteins can be washed away. The most common affinity tags are polyhistidine (50, 66), streptavidin (67), glutathione-S-transferase (68), and maltose-binding protein (69). The use of polyhistidine tags allows purification by immobilized metal-affinity chromatography (IMAC), where selectivity is usually based on the interaction of the polyhistidine with a Ni^{2+} complex immobilized on silica beads or in a gel. However, drawbacks to column-based IMAC include slow diffusion of macromolecules into porous beads, difficulties in packing large columns, relatively high pressure drops, and long separation times (70, 71). These drawbacks become particularly important in large-scale separations.

Membrane absorbers (72) have the potential to provide more rapid affinity purification than column-based methods because convective flow, rather than diffusion, brings proteins to binding sites in membrane pores. Convective rinsing of pores may also help to remove nonspecifically adsorbed proteins (66). Additionally, the development of disposable membranes for one-time use would avoid cross-contamination between samples (43).

Unfortunately, typical protein-absorbing membranes suffer from low binding capacities relative to porous beads. We and other groups are working to increase the capacity of membrane absorbers by modifying the pores of membranes with polymer brushes (20, 66, 73–75). The brushes contain multiple protein-binding sites that give rise to high protein-binding capacities. Moreover, brushes can be easily modified with ligands that exhibit specific biological recognition and provide high selectivity. **Figure 4** schematically shows His-tagged protein binding to a NTA-Ni^{2+}-derivatized poly(HEMA) brush inside a membrane pore. A wide range of porous polymeric and inorganic materials such as alumina (20, 66), silica (76), polyvinylidene difluoride (PVDF) (75), nylon (77), polyethersulfone (22), regenerated cellulose (78), and polyethylene (79) have been modified with polymer brushes to develop protein-absorbing membranes with high protein-binding capacity as well as selectivity towards the protein of interest.

The ability of polymer brushes to enhance protein binding to membranes depends greatly on both membrane geometry and the selection of the polymer brush. Kawakita and coworkers (76) modified porous glass membranes with poly(GMA) brushes to obtain a BSA-binding capacity of 12 mg of protein per gram of membrane. Kumar et al. (80) grafted poly(vinylbenzyltrimethylammonium chloride) brushes onto cotton cellulose and achieved an equilibrium binding capacity of 40 mg of BSA per gram of membrane. We showed that modification of microporous alumina membranes with poly(HEMA) and subsequent derivatization of the poly(HEMA) brushes with NTA-Cu^{2+} give membranes with a BSA-binding capacity as high as ~95 mg of BSA per gram of membrane, which is several times higher than the capacities of other protein-absorbing membranes (18, 20, 76). This high capacity stems in part from both the relatively small pore diameter (0.2 μm) in the membrane and the thickness of the polymer brushes. Binding capacity should increase as pore size decreases, but unfortunately resistance to flow also increases.

To create membranes for selective purification of His-tagged proteins, we modified poly(HEMA) brushes with NTA-Ni^{2+}, rather than NTA-Cu^{2+}, because copper ions can interact with any protein containing accessible histidine residues, whereas nickel ions are selective for His-tags. Alumina membranes modified with poly(HEMA)-NTA-Ni^{2+} can bind 100–150 mg of

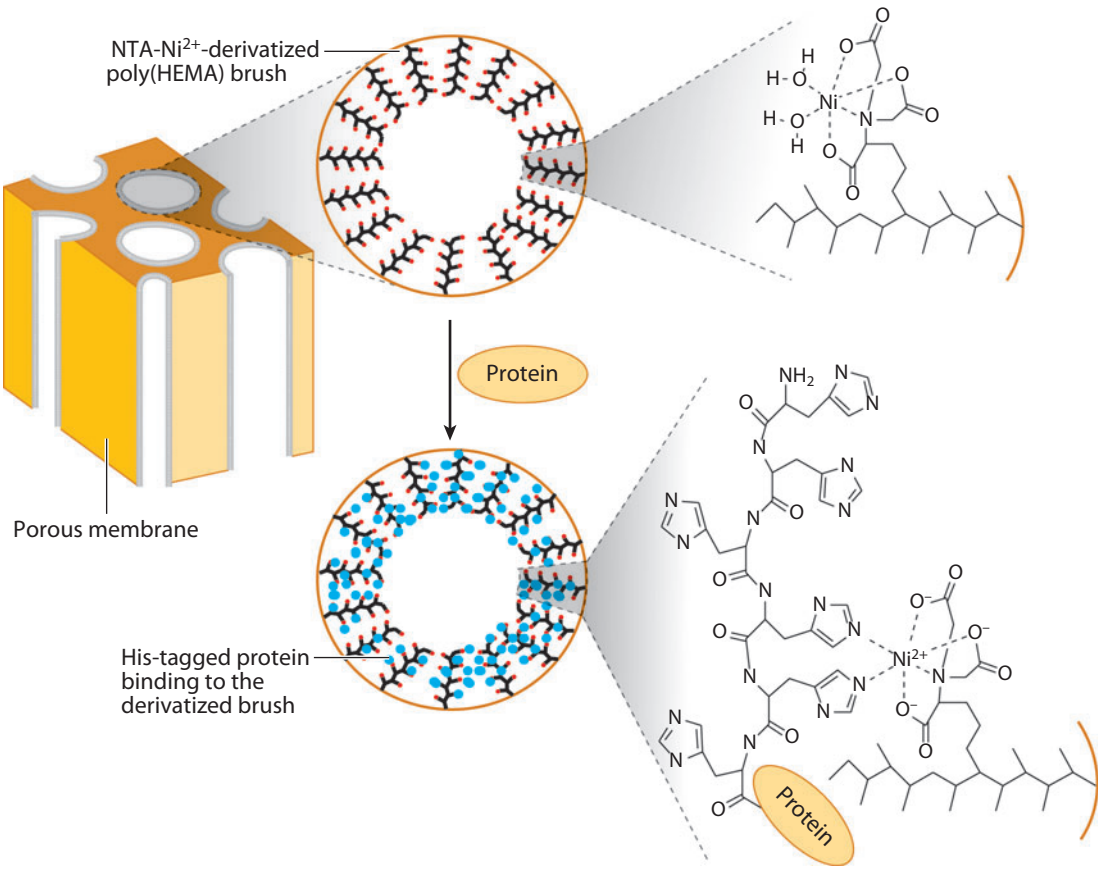

Figure 4

Schematic illustration of the binding reaction between a His-tagged protein and a nitrilotriacetate (NTA)-Ni^{2+}-derivatized poly(hydroxyethyl methacrylate) [poly(HEMA)] brush inside membrane pores. Adapted with permission from Reference 66.

His-tagged ubiquitin (HisU) per cubic centimeter of membrane (66). Perhaps more importantly, these membranes are highly selective. To demonstrate the selectivity of membranes modified with poly(HEMA)-NTA-Ni^{2+}, we isolated HisU from a solution containing 10% bovine serum (66). **Figure 5** shows the gel electropherograms for both the initial bovine serum solution spiked with HisU and the eluent from a membrane loaded with this solution. The uniquely strong band for HisU in the eluent suggests a purity of approximately 99%. In recent work, we showed that polymeric membranes modified with polymer brushes are capable of purifying His-tagged proteins directly from a cell lysate.

Membrane absorbers have also been used for protein immobilization via ion-exchange interactions. Ulbricht and coworkers (31, 44) formed cation-exchange microfiltration membranes by photografting copolymers of poly(AA) and a cross-linking monomer, methylene bisacrylamide, inside the pores of polypropylene membranes. The incorporation of cross-links within the grafted polymer layers led to higher dynamic protein-binding capacities than did grafting of a linear polymer, even when the number of functional groups was the same in both cases (44). Overall, the poly(AA-*co*-methylene bisacrylamide)–modified membranes exhibited a lysozyme-binding

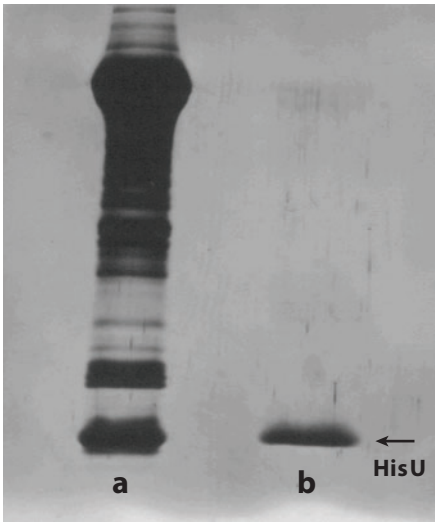

Figure 5

Electrophoretic analysis of (*a*) 10% bovine serum spiked with 0.3 mg ml^{-1} His-tagged ubiquitin (HisU) and (*b*) the imidazole eluent from a poly(HEMA)-NTA-Ni^{2+}-modified alumina membrane loaded with this solution. Silver staining was used to develop the gel. Abbreviations: NTA, nitrilotriacetate; poly(HEMA), poly(hydroxyethyl methacrylate). Adapted with permission from Reference 66.

capacity of 60 mg cm^{-3}, which is 30 times higher than the binding capacity of an unmodified membrane. Husson and coworkers (78) reported that regenerated cellulose membranes modified with poly(AA) brushes via 1 h of ATRP show static lysozyme-binding capacities of 99 mg ml^{-1}. This capacity is two to three times higher than the capacity of commercial Sartobind C membranes. However, the Sartobind C membranes are 40 times more permeable because of their larger pore size.

Iwanade and coworkers (81) modified porous hollow-fiber membranes with poly(GMA) brushes and reacted the epoxide groups of the polymer with ampholite molecules containing amino and anionic groups such as carboxylic acids (**Figure 6**). The resulting membranes showed multilayer protein binding for lactoferrin, cytochrome *c*, and lysozyme. In a specific example, membranes containing (2-aminoethyl)phosphonic acid as an ampholite had equilibrium absorption capacities of 130 mg g^{-1}, 150 mg g^{-1}, and 190 mg g^{-1} for lysozyme (feed concentration of 0.2 g $liter^{-1}$), cytochrome *c* (feed concentration of 0.5 g $liter^{-1}$), and lactoferrin (feed concentration of 1.0 g $liter^{-1}$), respectively. Moreover, the elution efficiency was >99%. However, mutual repulsion between the anionic groups caused extension of the polymer brush and a threefold decrease in the flux relative to unmodified membranes. In fact, decreased membrane permeability due to extended polymer brushes is a major challenge in grafting charged polymer brushes into porous membranes. To overcome this drawback, Iwanade and coworkers (81, 82) suggested ionic cross-linking of the negatively charged polymer brushes with bivalent cations to reduce swelling. However, at some point, decreased swelling also limits access to binding sites. Thus, controlled polymerization techniques are likely to be vital in controlling brush thicknesses to optimize the balance between protein-binding capacity and membrane permeability.

Growth of polymer brushes in membranes can also help to decrease the dispersity of pore sizes. A relatively narrow pore-size distribution is important for obtaining sharp breakthrough curves. (Breakthrough curves are plots of protein concentration in the permeate versus permeate volume.)

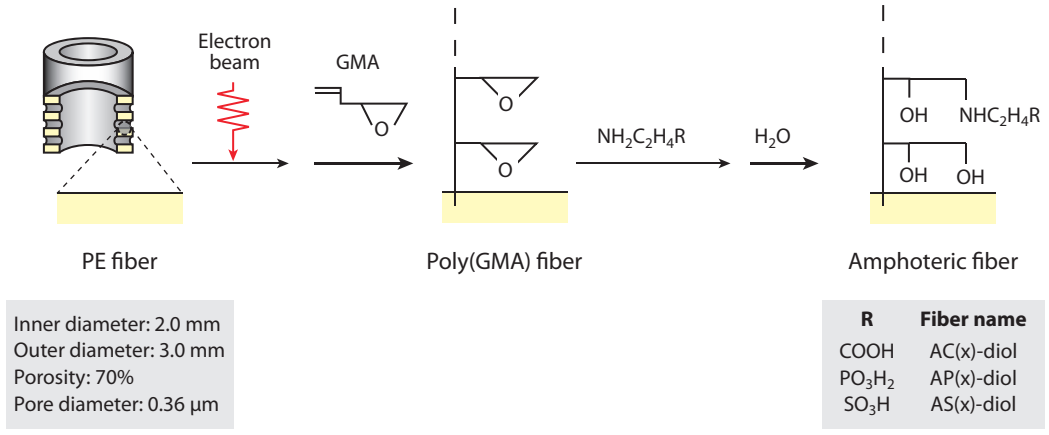

Figure 6

Modification of a porous high-density polyethylene (PE) hollow-fiber membrane with poly(glycidyl methacrylate) [poly(GMA)] brushes and subsequent reaction of the brushes with 3-aminopropionic acid (AC), (2-aminoethyl)phosphonic acid (AP), and 2-aminoethane-1-sulfonic acid (AS). Adapted with permission from Reference 81.

Singh and coworkers (75) tuned the ion-exchange capacity and the pore size of commercially available microporous PVDF membranes using controlled polymerization of poly(2-vinylpyridine) inside membrane pores. Growth of the brushes decreased the width of the pore-size distribution and prevented premature breakthrough of proteins in the largest pores. Controlling the polymerization time also provided control over the average pore size of the membranes and the ion-exchange capacity.

5. ANTIBODY AND ENZYME MICROARRAYS

A protein microarray is a physical matrix in which specific protein molecules are immobilized on a solid surface at defined locations. Within the past decade, microarrays of antibodies and enzymes have emerged as important tools for rapid parallel analyses of a range of analytes (45, 83). In the immobilization of proteins in arrays, however, unwanted nonspecific adsorption often lowers the signal-to-background ratio and also generates false-positive identifications (84). Thus, the array should demonstrate specificity toward the desired protein in appropriate areas and should at the same time show minimal nonspecific interactions.

Recent studies suggest that surfaces modified with polymer brushes are more efficient substrates for microarrays than nonpolymeric self-assembled monolayers (SAMs) containing the same binding functional groups (85, 86). Brushes are advantageous over SAMs for several reasons. First, as shown in Section 3 above, brushes have a high protein-binding capacity that can enhance the sensitivity of protein arrays. Second, the three-dimensional structure of swollen brushes should allow ready access to binding sites. In the case of nonpolymeric SAMs, immobilized antibodies/enzymes lie flat on the surface and are not highly accessible to the antigen/substrate molecules. Si wafers modified with copolymer brushes containing poly(2-methacryloyloxyethyl phosphorylcholine) [poly(MPC)] and poly(GMA) can immobilize four times more antibody F_{ab}' fragments than a SAM containing epoxy groups (85). Moreover, the antibody fragments immobilized on the polymer brushes show approximately six times higher activity than antibodies attached to epoxysilane films. This increased activity suggests that the antibody fragments immobilized in polymer brushes are more accessible to antigens than the antibodies immobilized on the monolayers.

Poly(MPC): poly(2-methacryloyloxyethyl phosphorylcholine)

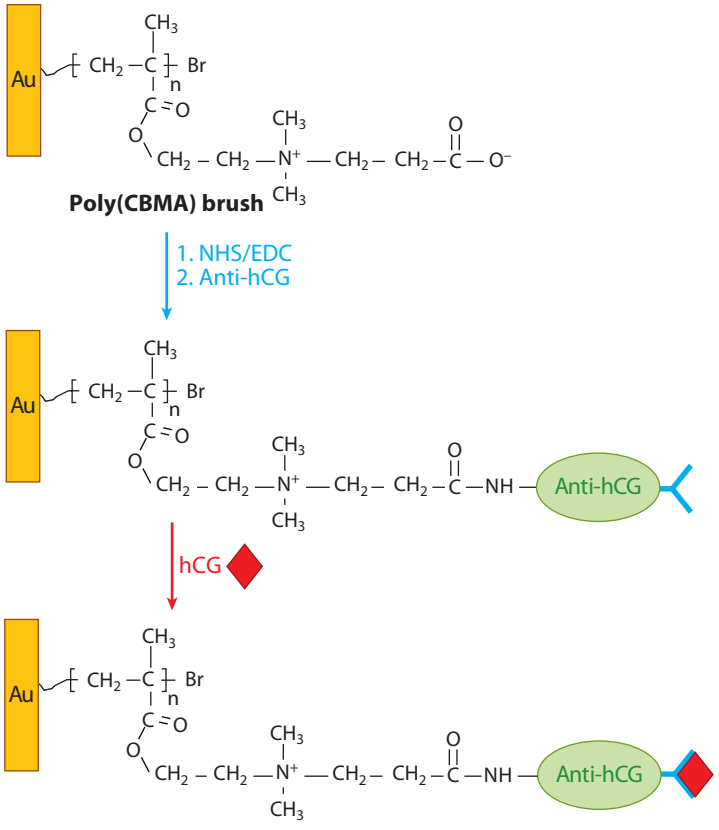

Figure 7

A schematic illustration of anti–human chorionic gonadotropin (anti-hCG) immobilization on *N*-hydroxysuccinimide (NHS)-modified poly(carboxybetaine methacrylate) [poly(CBMA)] brushes. Immobilization of anti-hCG results in specific binding of hCG protein. Abbreviation: EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

Hydrophilic polymer brushes are especially attractive substrates for protein arrays because water-swollen films typically show low nonspecific adsorption. PEG (6, 7) brushes are recognized as biocompatible materials that resist protein adsorption, but poly(AA) (49), poly(HEMA) (61, 87), and poly(carboxybetaine methacrylate) [poly(CBMA)] (8) brushes also exhibit low nonspecific interactions and can be functionalized to allow binding of proteins. Zhang and coworkers (8) modified gold films with poly(CBMA) brushes using surface-initiated ATRP and showed that these brushes prevent the nonspecific adsorption of fibrinogen, lysozyme, and human chorionic gonadotropin (hCG). Immobilization of anti-hCG on *N*-hydroxysuccinimide (NHS)-modified poly(CBMA) brushes (**Figure 7**) results in specific binding of hCG while maintaining resistance to nonspecific protein binding. Similarly, Tugulu et al. (87) utilized glass slides modified with poly(HEMA), poly[oligo(ethylene glycol)methacrylate], or poly(MPC), for synthesizing nonfouling films for protein microarrays. The presence of the polymer brushes prevents nonspecific protein binding, and at the same time, immobilization of O^6-benzylguanine onto the brushes results in chemoselective immobilization of O^6-alkylguanine-DNA-alkyltransferase–fusion proteins.

Wang et al. (88) developed a chitosan-*g*-methyl-PEG-coated polydimethylsiloxane (PDMS) microchip to minimize nonspecific protein adsorption. The methyl-PEG units provide hydrophilic

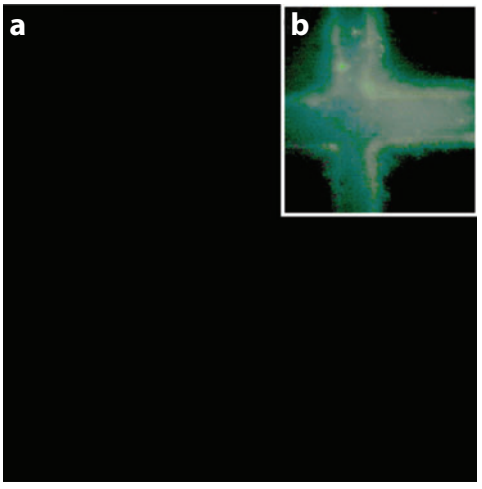

Figure 8

Fluorescence images of two microchannels. (*a*) A chitosan-*g*-methyl poly(ethylene glycol)–coated polydimethylsiloxane (PDMS) surface exposed to fluorescein isothiocyanate–labeled bovine serum albumin (FITC-BSA) for 24 h. (*b*) An unmodified PDMS surface exposed to FITC-BSA for 24 h. Adapted with permission from Reference 88.

domains and minimize nonspecific adsorption of biomolecules. **Figure 8*a*** shows a fluorescence image of the polymer-coated microfluidic channels after exposure to fluorescein isothiocyanate (FITC)-labeled BSA for a period of 24 h. The figure shows no detectable fluorescence, indicating effective suppression of BSA adsorption on this microchip. On the other hand, a PDMS microchip without polymer modification shows bright fluorescence in channels after exposure to FITC-BSA for 24 h (**Figure 8*b***).

In addition to minimizing nonspecific interactions, substrates for protein microarrays should also prevent denaturation of protein molecules. In one method of achieving this, Ober and coworkers developed a lithographic method (**Figure 9**) to produce protein patterns with minimal denaturation and a low level of nonspecific interactions (89). A patterned surface was back-filled with ATRP initiators, and poly(AA) brushes were grown from the initiator-containing regions. FITC-labeled BSA was covalently immobilized on poly(AA) brushes that were activated with NHS/EDC (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride). **Figure 10** contains the fluorescence image of these films, which contain well-defined, bright BSA patterns. The darker PEG-modified regions show minimal attachment of BSA and demonstrate that this method can yield protein microarrays with low nonspecific binding.

Proper orientation of an enzyme or antibody is required to maintain biological activity and should be taken into account when developing a substrate for immobilizing biomolecules. Randomly oriented proteins frequently show decreases in activity due to the inaccessibility of the active site. Control over protein orientation is generally achieved by (*a*) changing the surface charge (90) or (*b*) using site-specific immobilization through biotin-streptavidin interactions or thiol-disulfide interchange reactions (85, 87, 91, 92). Iwata and coworkers (85, 91) utilized well-defined block copolymer brushes consisting of poly(MPC) and poly(GMA) on silicon wafers to immobilize antibody F_{ab}' fragments in a defined orientation. The orientation of the antibody fragments was defined by derivatizing the GMA units with pyridyl disulfide and immobilizing the antibodies via thiol-disulfide interchange reactions (**Figure 11**). Increases in the length of poly(GMA) units resulted in increased loading of the antibody fragments due to the availability of more binding sites.

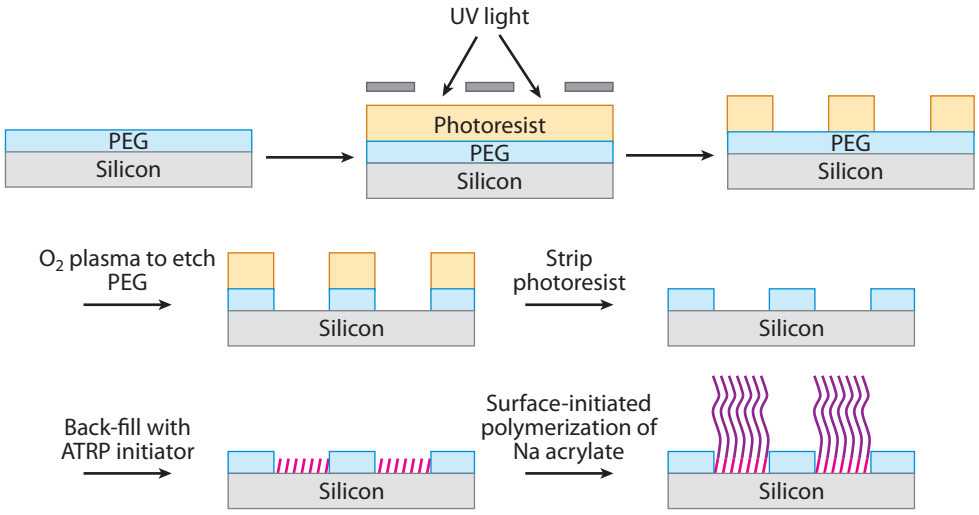

Figure 9

Patterning of poly(ethylene glycol) (PEG) and poly(acrylic acid) brushes on a silicon surface. Abbreviation: ATRP, atom-transfer radical polymerization. Adapted with permission from Reference 89.

The fluorescence intensity after reaction of immobilized antibody fragments with FITC-labeled antigen also increased with an increasing length of poly(GMA) units because of the increased loading of the antibody fragments. Polymer brushes without pyridyl disulfide moieties were also used to immobilize antibody F_{ab}' fragments via reaction with the epoxy groups on poly(GMA) units. The fluorescence intensity arising from binding of labeled antigen to antibodies linked to unmodified poly(GMA) blocks was 20 times lower than that obtained using antibodies attached to pyridyl-disulfide-modified brushes. Presumably, the random orientation of antibodies attached to unmodified poly(GMA) results in a lower activity, but lower antibody loading can also occur.

Enzymatic reactions in nonaqueous media are also important in industrial applications (93, 94), but enzymes frequently do not show sufficient activity in nonaqueous solvents. To overcome this

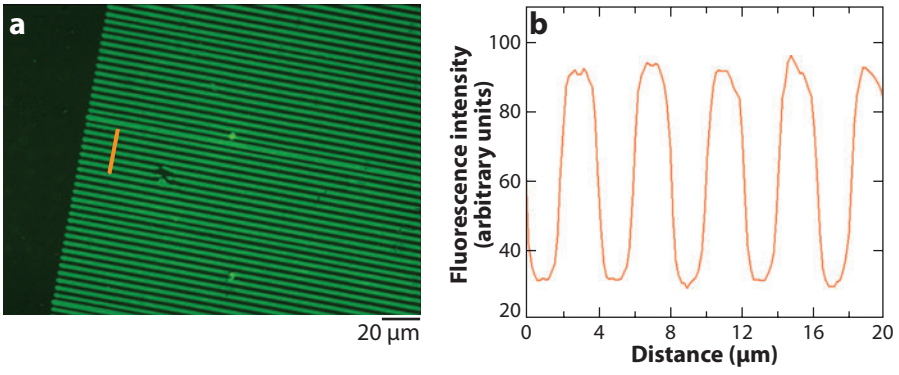

Figure 10

(*a*) Fluorescence image of fluorescein isothiocyanate–labeled bovine serum albumin immobilized on 2-μm-wide poly(acrylic acid) brush patterns in a background of poly(ethylene glycol) self-assembled monolayers. (*b*) Fluorescence intensity profile along the line section shown in panel *a*. Adapted with permission from Reference 89.

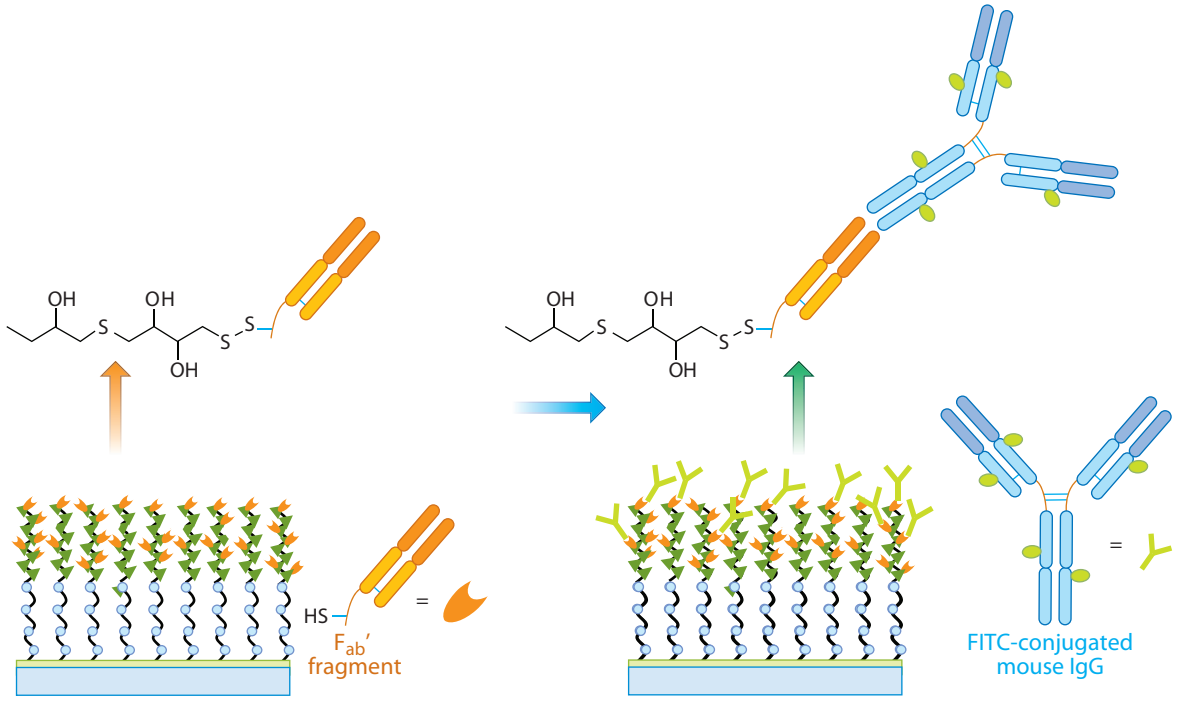

Figure 11

Reaction of fluorescein isothiocyanate (FITC)-labeled mouse immunoglobin G (IgG) with F_{ab}' fragments immobilized on poly(MPC)-b-poly(GMA) brushes. The F_{ab}' fragments were immobilized onto the polymer brushes via thiol-disulfide interchange reactions. Adapted with permission from Reference 85.

problem, enzymes have been coated with surfactants (95) and immobilized on microspheres (96) and in polymer brush–modified membranes (**Figure 12**) (97–99). A number of studies show that enzymes immobilized in polymer brush–modified membranes have higher enzymatic activity than enzymes coated with surfactants or immobilized on microspheres (97–99) because convective flow brings the substrates to the immobilized enzymes and minimizes mass-transport limitations. Goto and coworkers (98) immobilized lipases via ion exchange inside a porous polyethylene hollow fiber (**Figure 12**). The anion-exchange sites were created by radiation-induced grafting of poly(GMA) brushes (in a method similar to that shown in **Figure 6**), followed by reaction of the GMA units with diethylamine (98). The immobilized lipase showed 23-fold-higher activity than did the native lipase (suspended in substrate solution) in the esterification reaction between lauric acid and benzyl alcohol. The grafted poly(GMA) acted as a hydrophobic surfactant to stabilize the enzyme and to enhance activity. Moreover, reuse of the immobilized lipase three times in a batch reactor over a period of 24 h resulted in no loss in activity, whereas there was a 75% decrease in the activity of native lipase under similar conditions (99).

6. POLYMER BRUSH-BASED CAPTURE OF PROTEINS FOR ANALYSIS BY MASS SPECTROMETRY

The identification and analysis of the proteins associated with specific diseases are important goals in analytical chemistry. Identification of phosphorylated proteins, in particular, is important because changes in phosphorylation states can cause various diseases including cancer. MS is often

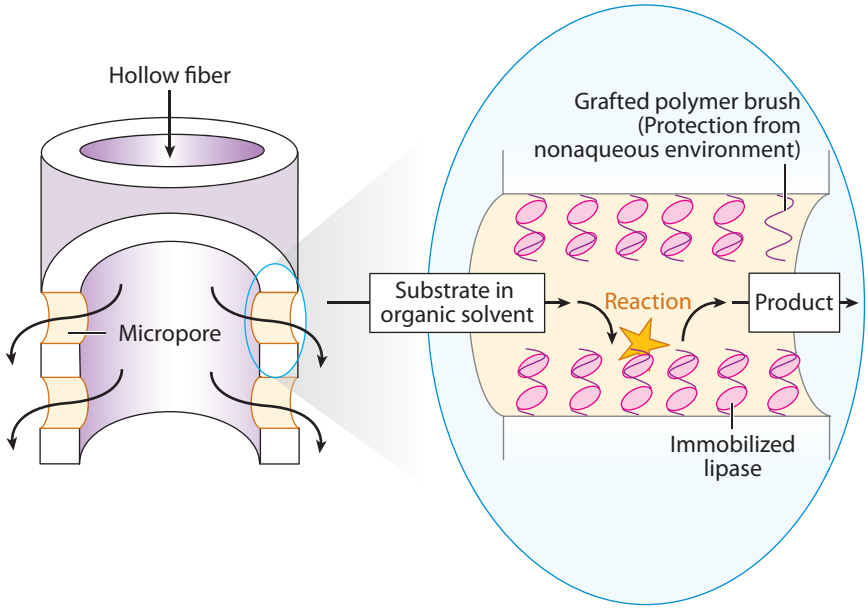

Figure 12

Schematic representation of lipase immobilized in a porous hollow-fiber polyethylene membrane. The pores of the membrane were modified with poly(glycidyl methacrylate) [poly(GMA)] brushes, followed by reaction with diethylamine. Lipase immobilization occurred via ion-exchange interactions, and the lipase-containing membranes were used to study the esterification reaction between lauric acid and benzyl alcohol. Adapted with permission from Reference 99.

employed for the identification and analysis of phosphorylated proteins, but the low abundance of the phosphorylated proteins, the number of closely related phosphorylated forms, the loss of the phosphoryl group in the course of fragmentation, and the suppression of signals by nonphosphorylated proteins make such analyses challenging. One way to overcome this problem is to develop methods for selective capture of the proteins of interest from a pool of unwanted proteins. A recent review describes a number of techniques for phosphopeptide enrichment such as IMAC, reversible covalent binding, and metal-oxide affinity chromatography (100). Capture of peptides directly in polymer brushes on a MALDI plate is attractive for high-throughput analysis of moderately complex samples.

Dunn and coworkers (101) used poly(HEMA) brushes in on-plate enrichment of phosphopeptides for analysis by MALDI-MS (**Figure 13**). Au-coated Si wafers were modified with poly(HEMA)-NTA-Fe(III) brushes, and small volumes (~1 μL) of digest containing phosphopeptide, nonphosphopeptides, and salts were spotted on these films. After incubation, the films were washed and dried, and a matrix was added for MALDI-MS analysis. In a specific example, mass spectra of a β-casein digest were obtained with and without enrichment on poly(HEMA)-NTA-Fe(III) brushes. Enrichment on the brushes yielded a 30-fold increase in the intensities of several phosphorylated peptides relative to the conventional MALDI-MS analysis. Additionally, the enrichment procedure resulted in signals from several phosphopeptides that were undetectable in conventional MALDI-MS as well as decreased signals for nonphosphopeptides (**Figure 14**). Finally, with ~30 fmol of a β-casein digest, enrichment resulted in an eightfold increase in signal for the peptide with a mass-to-charge ratio of 2062 compared to conventional MALDI-MS. The effectiveness of polymer brushes in the enrichment of phosphopeptides is likely due to the

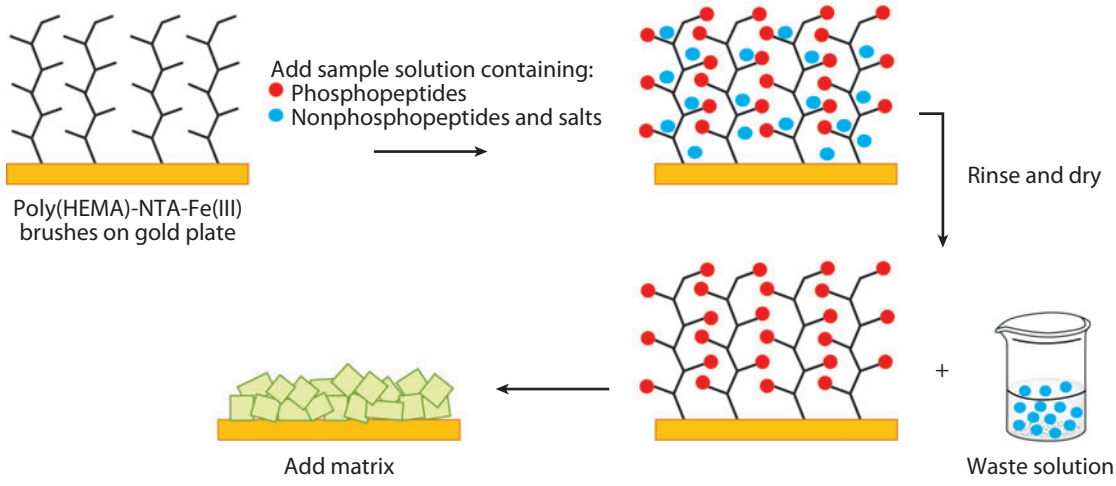

Figure 13

Protocol for phosphopeptide enrichment using poly(HEMA)-NTA-Fe(III) films on Au matrix-assisted laser desorption/ionization plates. Abbreviations: NTA, nitrilotriacetate; poly(HEMA), poly(hydroxyethyl methacrylate).

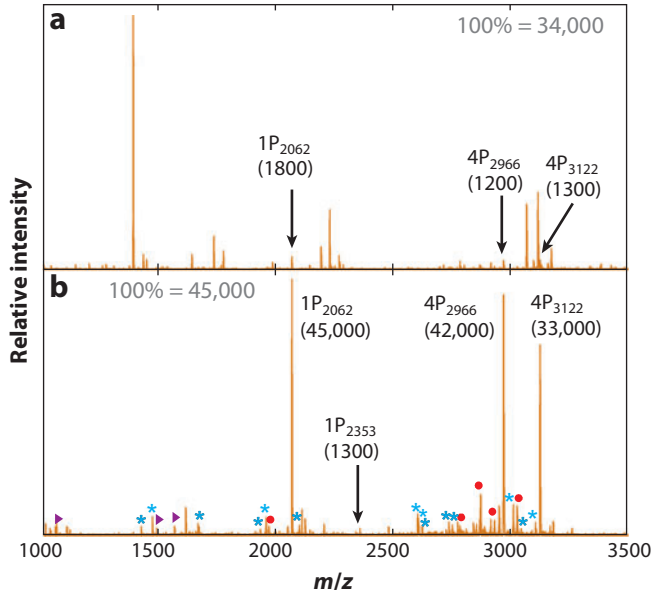

Figure 14

Positive-ion matrix-assisted laser desorption/ionization mass spectrometry analysis of a 7-pmol β-casein digest performed via (*a*) conventional analysis and (*b*) enrichment with a poly(HEMA)-NTA-Fe(III)-modified film. Peaks labeled with triangles represent doubly charged $1P_{2062}$, $4P_{2966}$, and $4P_{3122}$ phosphopeptides. Peaks labeled with circles are due to fragmentation of phosphopeptides. Peaks labeled with stars stem from α-S1-casein- and α-S2-casein-phosphorylated peptides. Numbers in parentheses are peak intensities. Abbreviations: NTA, nitrilotriacetate; poly(HEMA), poly(hydroxyethyl methacrylate). Adapted with permission from Reference 101.

high density of peptide-binding sites, as 60-nm-thick poly(HEMA)-NTA-Fe(III) brushes have a binding capacity of ~0.6 μg cm^{-2} for phosphoangiotensin. Remarkably, these brushes showed ~70% recovery of a synthetic monophosphopeptide and 100% recovery of a diphosphopeptide. In contrast, MALDI plates modified with a monolayer of NTA-Fe(III) gave a monophosphopeptide recovery of only ~9%.

SUMMARY POINTS

1. Surface-initiated polymerization allows growth of polymer brushes on nearly any chemically appropriate surface and gives control over thickness and composition. Moreover, the abundant functional groups on the brushes can be readily derivatized to tailor film properties for specific applications. Polymer brushes are promising candidates for protein purification, enrichment, and immobilization.
2. Because of their three-dimensional structure and high density of functional groups, polymer brushes have much higher capacities for protein immobilization than SAMs. At the same time, highly swollen brushes can resist nonspecific interactions. Thus, polymer brush–modified surfaces can form microarrays with high specificity toward desired proteins. Moreover, for nonaqueous reactions, enzyme activity is frequently higher with enzymes immobilized in polymer brushes than with free enzymes or enzymes coated with surfactants.
3. Affinity and ion-exchange membranes have several potential advantages over adsorption columns including reasonable scale-up, rapid flow–induced mass transport, and faster purification of large biomolecules. Modification of the pores of membranes with functionalized polymer brushes can help overcome the main disadvantage of membrane absorbers: low capacity.
4. Polymer brushes allow rapid enrichment of phosphopeptides on MALDI plates. This simple enrichment method should allow selective, high-throughput analyses of moderately complex samples.

FUTURE ISSUES

1. Polymer brushes are promising materials for protein purification and immobilization in microarrays. However, there are a number of challenges that must be addressed if these films are to find practical application.
2. In the use of brushes as substrates for protein microarrays, denaturation and loss of activity of immobilized enzymes and antibodies are still problematic. Although enzymes immobilized in polymer brushes often show higher activity in nonaqueous media than free enzymes, activity losses upon immobilization are observed in many reactions that take place in aqueous solution. Proper orientation of enzymes helps but does not solve this problem.
3. In the development of affinity membranes, modification of polymer substrates with large pore sizes (1–10 μm) is needed to achieve high permeability. However, it is not clear whether high capacities can be achieved with such large pores. Longer brushes may provide enhanced capacity, but low accessibility of binding sites in long brushes may be a problem.

4. In terms of brush synthesis, direct polymerization of functional monomers would avoid the need for brush derivatization and would simplify the membrane-modification process.
5. Studies of protein diffusion in polymer brushes will help to delineate the performance of these materials both as substrates for microarrays and as protein binders for purification.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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