Chapter

Membrane and Bioseparation

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Abstract

Although one of the strongest methods of purification is chromatography, the major problem of porous bed chromatography is that purification takes place using the diffusion. This will prolong the purification process and bring down the efficiency. In recent years, membrane methods have greatly overcome this limitation due to low membrane thickness, low pressure drop, and convective flow, and they are a great alternative to chromatography columns. Unfortunately, the membranes have a low surface area. For solving such problem, membrane modification with polymeric brushes and layer-by-layer adsorption in polyelectrolyte films can be attractive. Accordingly, in this chapter we introduce types of biomolecule purification methods, the best purification method, membrane modification techniques, and their limitations and assets. Also, we introduce the membrane as an attractive tool for selective purification and separation of biomolecules.

Keywords: membrane, polyelectrolyte multilayers, polymeric brushes, biomolecules, layer-by-layer adsorption

1. Introduction

The rapid development of biotechnology needs more reliable and effective methods for isolation and purification of bio-products (proteins, enzymes, peptides, or nucleic acids). Since the introduction of recombinant insulin as a therapeutic agent in 1982, the global protein therapeutic market is rapidly expanding with the continuous growth of biotechnology. However, due to the complexity of protein mixtures, the purification of proteins remains a problem in their production. Since purification and recycling are then about half the costs of producing cell-derived drugs, high fecal separation techniques and high recyclability are fundamental to produce the essential therapeutic proteins.

The therapeutic proteins currently constitute a very effective pharmaceutical industry, predicting that they would expect their sales to reach 165 billion dollars [1]. So far more than 100 proteins have been accepted as therapists, many are undergoing therapeutic testing. Recombinant therapeutic proteins, drug-antibody mixed, vaccines, enzymes, recombinant/normal cytokines, interferons, monoclonal antibodies, growth hormones, and coagulation factors are known as biochemical therapists. They have been proven effective in the treatment of many potentially fatal diseases, such as cancer, diabetes, and cardiac disorders [2]. Protein purification is essential for basic protein research and the production of therapist antibodies [3–5], and the expansion of the need for pure protein [6] is challenging the existing purification methods [7]. Separating a protein is especially important to reduce degradation, to remove impurities that can interfere with protein function, and to remove toxicity from proteins that are used in therapy [8].

Packed columns have been the primary tools for protein isolation and analysis for decades. However, it has a number of problems such as compressibility of the beads, plugging and fouling, and especially the slow flow speed through the column.

Membrane chromatography is able to overcome the mentioned problem of packing column and minimize it. Because it provides a higher flow rate, much lower pressure drops, and illustrates greater productivities per unit time. In comparison with the bead-packed column, flow through pores of the membrane (convective transport) quickly brings protein to binding sites. However, despite their potential, a major disadvantage of the membrane absorbers is low internal surface area that leads to a relatively low binding capacity. To overcome this problem, membrane modification, especially with two methods of coating and grafting polymerization, can be efficient, in such a way that membranes with multiple binding sites and specific functional groups for the capture of different biomolecules are achieved.

A wide range of polymeric and porous inorganic supports have been used in order to develop protein adsorbing membranes with high protein binding capacities and selectivity. Functional groups containing carboxylic acid, epoxide, $-SO_3H$, $-NH_2$, and $-CH_2OH$ are particularly interested for membrane modification. Based on the various interactions between the groups mentioned on the membrane and biomolecules, various types of ion exchange membranes, hydrophobic interactions, covalent bonding, affinity, etc., for the separation and purification of enzymes, proteins, and antibodies from various sources, have been developed. In this regard, our goal is to introduce the membrane as an excellent tool for the selective separation and purification of biomolecules with high binding capacities as well as the introduction of the best membrane modification methods to improve membrane performance in this area.

2. Types of macromolecular purification methods

Because an organ contains thousands of proteins and their amounts can change over a wide range, isolating a target protein is often challenging. To overcome this challenge, scientists often attach an affinity tag to recombinant proteins. **Figure 1** shows the overall schematics of the production and isolation of the recombinant proteins that the special binding of the marked protein (tagged) is the strongest level in the purification of the protein [9, 10]. When this technique is performed in a column, it is often called "affinity chromatography."

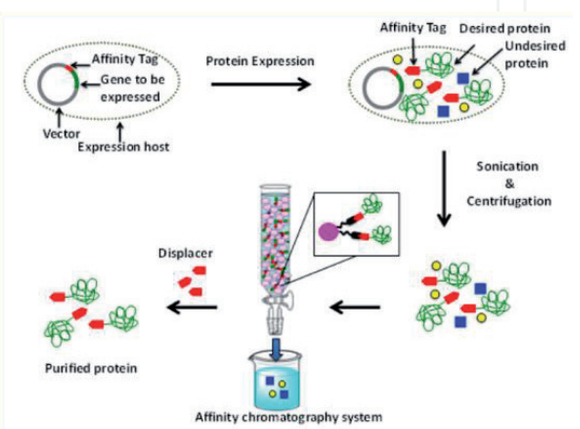

Figure 1.
Expression and purification of a recombinant protein [11].

Several methods for purifying the protein are available [3, 4, 6, 10, 12, 13], and the methods of chromatography are the most powerful and versatile methods. In these techniques, stationary functional groups such as ion exchange groups [14], hydrophobic molecules, or affinity ligands [15] capture the desired proteins.

Reversed-phase chromatography is relatively selective and separates proteins based on their relative hydrophobicity on a large scale. But this method requires an organic solvent mobile phase, which certainly denatures a number of proteins and eliminates the operation [12]. Ion exchange chromatography [12] separates proteins based on their charge density (**Figure 2a**), although gel filtration chromatography (size-exclusion chromatography) separates these molecules based on their size and is useful for the condensation of protein samples [16] (**Figure 2b**). In affinity purification, the scientist designs an affinity tag on recombinant proteins, and this special tag acts as a facilitator for the desired protein separation from the protein mixture (**Figure 2c**) [15, 17].

Affinity chromatography due to its high selectivity is the most robust method to isolate a single target protein from complex biological fluids (probably, affinity adsorption is a better name for this technique, which usually occurs in a batch mode). This isolation relies on the interaction between the functional groups (ligands bound to a solid surface) and the inserted tag in the protein. Some examples of affinity interactions include the interaction between antigens and antibodies, the binding of the histidine tags to the ion-metal complexes [18, 19], adsorption of maltose tags to carbohydrate matrices [20], the binding glutathione-S-transferase to glutathione [21], and the binding of streptavidin to biotin [22].

2.1 Immobilized metal affinity chromatography (IMAC) for His-tagged protein purification

IMAC is a very versatile and powerful way to purify the protein based on the tendency of specific amino acids to the variable metal ions attached to a solid support. Porath et al. introduced IMAC in the mid-1970s [23–26]. In this way, metal ions such as Ni^{2+}, Zn^{2+}, Co^{2+}, or Cu^{2+} are attached to ligands (e.g., iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA)) that are fixed on a support (**Figures 1**–**5**). A wide range of solid supports are available to immobilization of metal chelates, and polymer materials with hydroxyl groups are particularly common [25]. Usually in protein purification, the interaction of various metal ions with proteins, depending on the metal ion complex, is carried out through histidine, tryptophan, or cysteine residues [23, 25, 27]. For metal ion complexes that are especially attached

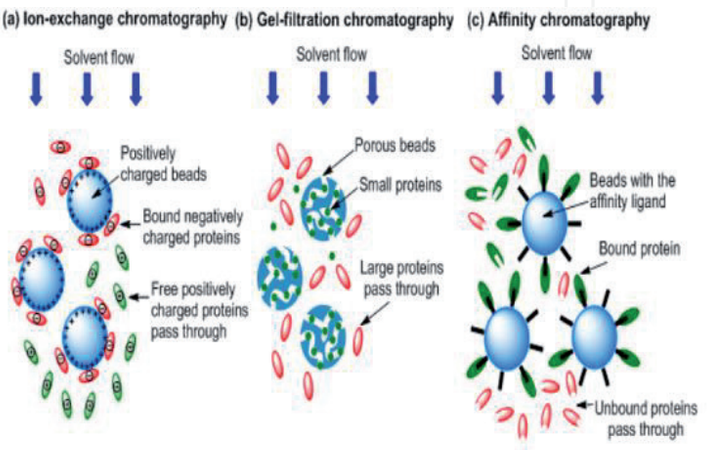

Figure 2.
Different types of chromatographic methods for protein purification [11].

to imidazole, the number and relative position of the available histidine residues determine the binding of protein. Therefore, in the expression of recombinant proteins in bacterial cells, to add a short sequence of histidine residues to each of the terminals C or N of the recombinant protein (typically 6), a short sequence of DNA binds to the desired gene. This histidine tag strongly binds to Ni^{2+}, Co^{2+}, or Cu^{2+} complexes (**Figure 3**) [26]. Because most proteins contain one or a relatively large number of histidine residues, the selected metal ion complex to capture the proteins labeled with histidine should not have very strong interactions with imidazole or many of the various proteins that will be attached to the support. For this reason, Ni^{2+} and Co^{2+} complexes are more commonly used to purify the proteins labeled with histidine than the Cu^{2+} complex [25, 28, 29].

The most common metal ion ligands, IDA and NTA, occupy three or four of the metal ion coordination sites, respectively; in this case, at least two of the coordination sites remain free [30].Therefore, the proteins His-tagged coordinate to the metal ion complex during the purification process (**Figure 3**). However, most proteins contain one or more histidine residues, which can cause non-specific binding and reduce the purity of the protein. Selection of Ni^{2+} as a coordinating ion leads to relatively weak complexes with single histidine residues and low non-specific adsorption [11]. In contrast, the hexa-histidine tag forms very strong complexes with immobilized Ni^{2+} [31, 32] to effectively capture the tagged protein. Replacement agents (usually free imidazole) that bind to immobilized metal ions can specifically eluate the proteins His-tagged; other elution methods include pH changes and ionic strength [28].

IMAC has many advantages: Low cost, high specificity (selectivity), simplicity, and mild elution condition. In addition, the binding site can be rearranged several times without loss of performance, and selectivity can be controlled by selecting different metal ions and change physical properties such as pH, ionic strength, and temperature [30, 33]. This technique can quickly isolate polyhistidine-tagged proteins with 100-fold enrichment in a single purification step, and purity can increase by more than 95% [34]. However, the non-specific binding of proteins due to histidine or cysteine clusters creates an important challenge in purification; adding low concentrations of a competitive agent (such as imidazole) to the loading environment can help to overcome this challenge, but it often reduces protein binding capacity [32, 34]. Also, exact selection of the phase for IMAC is important to get high yield and low production cost.

Among the many methods available for the purification of biomolecules, salt deposition, dialysis, electrophoresis, etc., chromatographic methods are

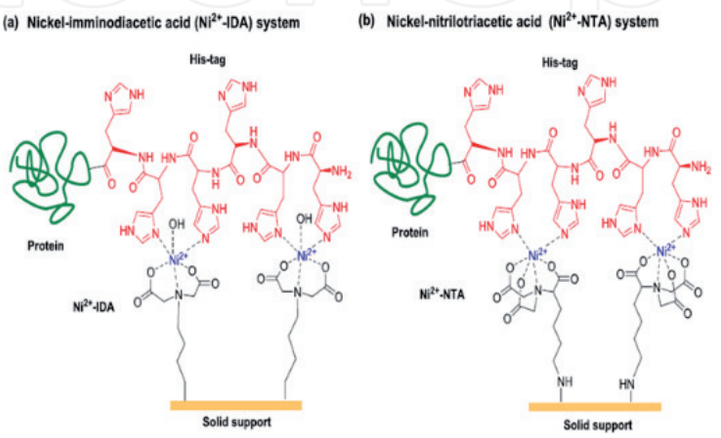

Figure 3.
Models of interaction between the polyhistidine affinity tags and two stationary metal ion-ligand complexes. (a) Ni^{2+}, imminodiacetate (Ni^{2+}-IDA), and (b) Ni^{2+}, nitrilotriacetate (Ni^{2+}-NTA) [27].

remarkable because of their selectivity and particular. Also, the mentioned methods cause impurities in the process of separation as well as more stages of separation. Membrane-based chromatography because of its superiority over conventional chromatography columns is a very good alternative to these columns. Membranes are economically more affordable than stacked columns. It can be remarked that the membrane's superior advantage over packed columns is passing the convection flow through the membrane pores, which speeds up the purification and separation process. In the next section, these two are closely compared.

3. Common phases for IMAC and their advantages and limitations

The most popular IMAC template uses packed-bead columns (**Figure 4a**). Packed-bead columns have been used for decades as the main means of purifying proteins for both analytical and preparation needs [35]. In a chromatographic separation based on column, the solution that contains the target molecule is loaded onto a chromatographic matrix, and moving phase separates the components, so the goal is apparent in a group elution of the column [36, 37]. In comparison, with a column based on affinity, the target selectively binds to the ligand, while other compounds along with the moving phase pass through the column [35]. The subsequent washings with the buffer will remove the remaining impurities, and in the final stage the target protein, as soon as the surface is replaced by a competitive factor, denatured or other mechanism in a pure form, is eluated of the column [26]. The main limitation of the most bead-packed columns is the transfer limited by the slow diffusion of proteins into the bed pores, which leads to a long separation and low productivity; this limitation also refers to large amounts of eluate and the need for analyte concentration after separation [38–41]. In addition, stacked bed phases create a high pressure drop across the stacked bed, and the same packing of large-scale columns is difficult [4, 38, 42, 43].

The development of homogeneous nonporous chromatography may overcome diffusion constraints, but these systems are relatively expensive, due to the low surface area having a low binding capacity, and also create high pressure drop [44, 45].

The porous membranes are forming as an attractive solid support for IMAC, and various studies discuss the progress of membrane adsorbents over packed columns for protein purification [3, 8, 46–48].

Compared to the bead-packed columns, the flow through the membrane pores (convective transfer) brings the proteins to the binding sites (**Figure 4b**). Convection transfer minimizes the constraints caused by the diffusional mass transfer resistance [10]. In addition, the membranes are thinner than the packed

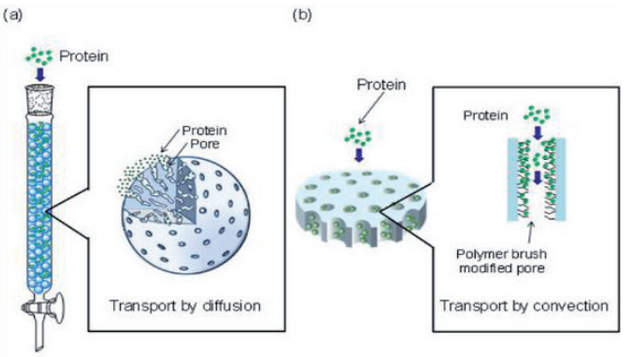

Figure 4.
Transfer of protein to binding sites. (a) Diffusion in nano-porous beads. (b) Convection flow in membrane pores [10].

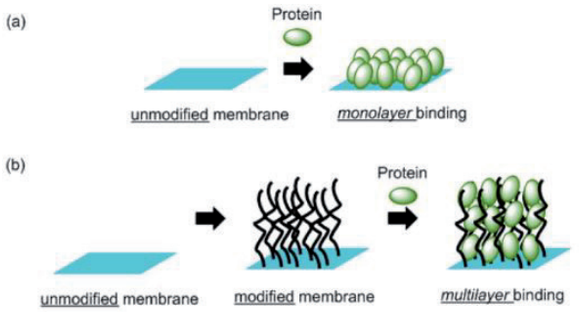

Figure 5.
Capture of (a) a single layer of protein on the unmodified membrane surface and (b) a multilayered protein on the surface of a membrane modified by a polymeric brush [51].

substrates, so the pressure drop across the membrane is significantly lower than that of a packed column. These advantages make membrane purification systems copacetic for very fast and large-scale protein purification. Although membrane adsorbents are very interesting for purification, due to the low surface area, they suffer from lower binding capacities [49, 50]. Membrane modification is a very effective method for providing the desired functional groups as well as increasing the surface area of the membrane. Usually, the unmodified membranes only have a surface area of $10m^2/g$ [51], and they mainly bind less than one layer of protein in their pores. Grafting of polymer chains in membrane pores is a common approach to increase biomolecule capture (especially proteins); more detailed explanations of this method are given in the next section.

In 1990 Müller et al. suggested the use of polymer brushes containing ion exchange sites to capture protein multilayers in membrane pores [52]. The membrane pores are modified with polymer chains binding several layers of protein (**Figure 5**) [51].

4. Surface modification techniques to increase bonding capacity

The surface modification should be such that, in addition to the availability of desirable and appropriate functional groups, there is no conflict with the purpose of the membrane process and separation, but in line with it and contributing to this goal [53–64]. Among the membrane modification methods, two methods involving grafting polymerization through appropriate initiator and coatings are more significant. In this section two methods include layer-to-layer adsorption of polyelectrolyte films and the growth of polymer brushes (**Figure 6**), the first is one of the coating methods and the other is one of the polymerization methods, for membrane modification and the provision of multiple binding sites in membrane pores have been discussed.

In most membrane-based processes, hydrophilicity is one of the most important factors. Also, in the separation of biomolecules using membranes, this factor is important to prevent non-specific surface adsorption. The diameter of the membrane is also one of the important factors in the separation process, and, based on the purpose of separation, a suitable diameter membrane can be prepared. To improve the membrane's hydrophilicity, even a hydrophobic polymer as a base membrane (such as polyether sulfone) can be grafted with a hydrophilic moiety. Therefore, in membrane preparation for separation processes, the base membrane should be prepared in such a way that, in addition to having a sufficient density of suitable functional groups, be hydrophilic, and its pores diameter be appropriate for maintaining the flow velocity. In this regard, it can be concluded that, in general,

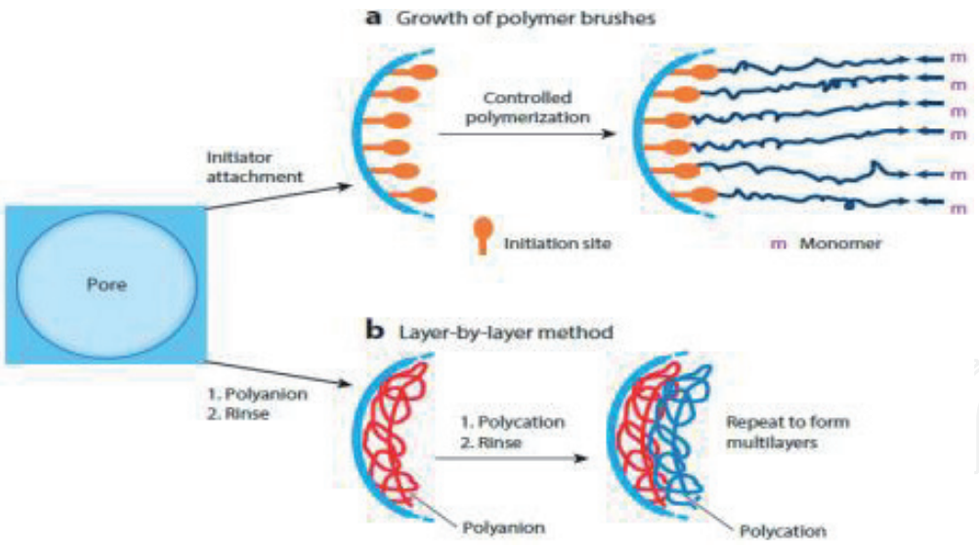

Figure 6.
Schematic diagram of (a) growth of polymer brushes and (b) layer-by-layer adsorption to form films that may capture proteins in membrane pores [65].

the diameter of the membrane pores, the hydrophilicity, and thickness are the main factors governing the process of separation; here the factors are also considered.

4.1 Modification of surfaces with polymer brushes

Polymer brushes are assemblies of polymer chains with one end attached to the surface and one end extended of the surface (**Figure 8**) [66]. These brushes are very moving and attractive to binding several layers of protein in many substrates such as membrane pores. Such brushes, when appropriately derived from the ligand, can capture several layers of protein through metal ion complexes (**Figure 7**).

4.1.1 Methods of growth of polymer brushes on surfaces

There are two main methods for the growth of polymer brushes on solid surfaces, physical absorption [68, 69] (**Figure 8a**) and covalent bonding [70] (**Figure 8**).

In the physical adsorption, one end of a block copolymer strongly adsorbed to the surface. A covalent bonding can be made through either "grafting-to" [71, 72] or "grafting-from" methods [73]. In the "grafting-to" method, polymers with end-functional groups to form polymer brushes react with a suitable functional group on the substrate. Alternatively, by "grafting-from" method the polymer chains grow directly through initiators that are covalently attached to the surface. These two covalent techniques provide different densities of polymer brushes [11]. In the

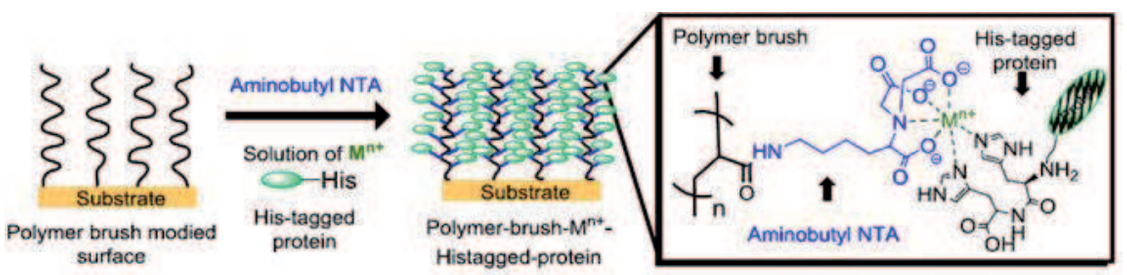

Figure 7.
Multilayer binding of the His-tagged protein to an acrylic acid brush derived with aminobutyl NTA [67].

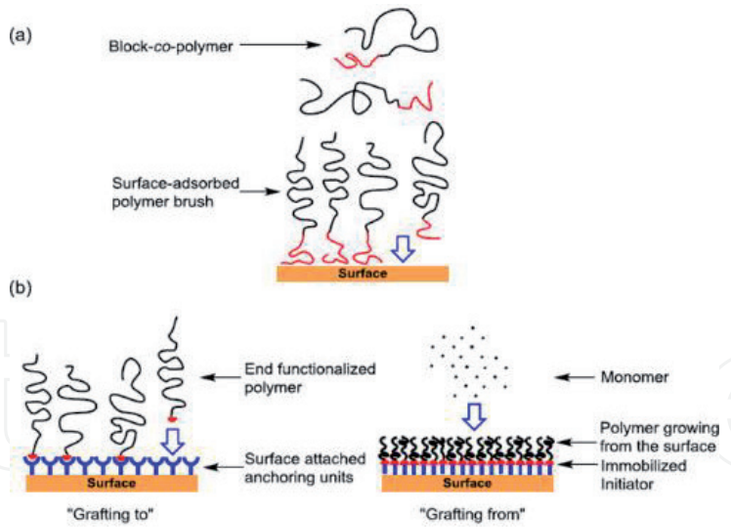

Figure 8.
Formation of a polymeric brush through (a) the physical adsorption of a block copolymer and (b) covalent bonding through "grafting-to" and "grafting-from" methods [11].

"grafting-to" method, the limitations of surface access for the incoming polymeric chains are referred to relatively low thickness and bond densities; in contrast, the "grafting-from" method uses small monomers, which, to provide relatively high bond densities, easily reach the surface growing reactive [11]. Controlled polymerization through the surfaces can create polymeric chains with adjustable lengths.

Polymerization methods used to synthesize polymer brushes include cationic polymerization [70], anionic [74], atomic transfer radical polymerization (ATRP) [75], ring-opening polymerization, and TEMPO-mediated radical [76].

4.1.2 Immobilization of biomolecules on polymer brushes

Several groups successfully made polymer brushes for biomolecule immobilization [12, 77, 78]. However, most designs require a separate derivative process to introduce a special functional group, for applications such as protein staining (**Figure 7**). Polymer brushes with hydroxyl groups, carboxylic acid, and epoxide are the most commonly used choices for simple derivation, among these, poly(acrylic acid) brushes are also more attractive, because these brushes in water multiply their initial thickness swells.

The membrane modification can be done through the growth of the brush by initiators located in the membrane pores (**Figure 9a**). Membrane modification with brushes usually employs polymerization from surfaces to achieve high polymer-chain areal densities [17, 18, 79]. Thus, brush synthesis typically includes initiator attachment to the membrane and polymer growth from these immobilized initiators [18, 79]. Among many techniques for brush growth, surface-initiated atom transfer radical polymerization (SI-ATRP) is particularly useful because ATRP offers controlled polymerization of a wide range of monomers under mild conditions and uses readily available catalysts and initiators [19–22]. Several groups modified a variety of membranes using ATRP from immobilized initiators, and binding capacities of such membranes often exceed 100 mg of protein per milliliter of membrane [42, 80–83]. The amount of protein binding in polymer brushes varies with polymer-chain areal density; low-chain densities yield few binding sites and minimal protein capture, whereas high densities may result in steric hindrance to protein entry into the brush [65]. Hence, an intermediate chain areal density will likely lead to the most protein binding [65]. Chain density depends in part on the density of initiation sites anchored to membrane surfaces, and anchoring typically occurs through surface functionalities such as hydroxyl groups [84] and carboxylic acids [85]. However, some membranes have low densities of such surface functional group.

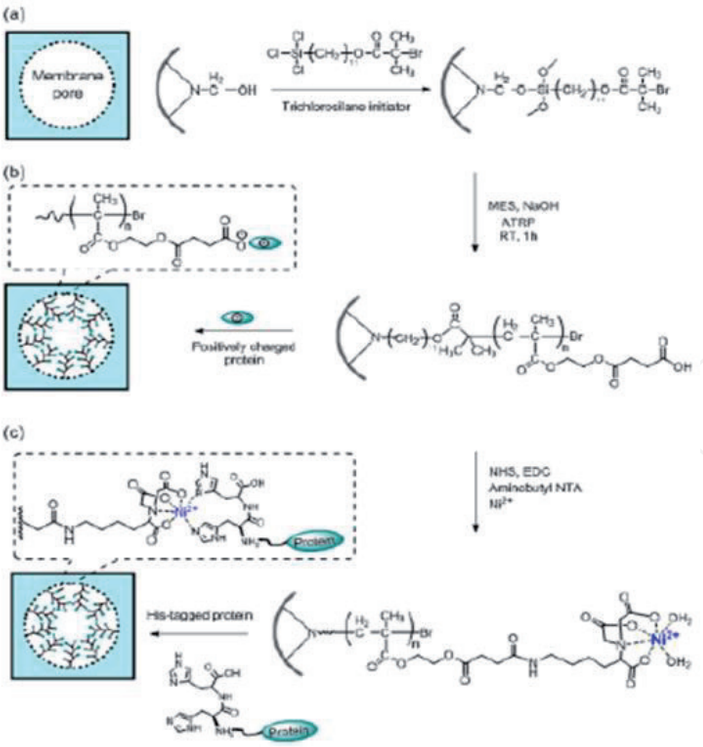

Figure 9.
Functionalization of membrane pores with poly(HEMA) brushes, activation (PHEMA) for forming poly(MES), and binding of His-tagged protein to a PMES-NTA-Ni2+ brush in a membrane pore. (a) Membrane modification though brush growth from initiators immoilized in membrane pores. (b) Protein capture in brush_modified membrane pores via ion_exchange. (c) Further functionalization of brushes for more selective purification of tegged protein [73].

In one study [80], to solve this problem, layer-by-layer adsorption of a macroinitiator was performed on a polyethersulfone membrane, and then the membrane was successfully modified using the ATRP from this macroinitiator. In protein capture through ion exchange, the brush-modified membranes show a significant protein binding capacity of 80–130 mg per $cm^3$of membrane (**Figure 9b**) [42, 48, 76, 86].

Further functionalization (**Figure 9c**) enables brushes to selectively purify the protein tagged. Alumina membranes with PHEMA-NTA-Ni^{2+} bind 120 mg His-tagged ubiquitin (His U) per cubic centimeter of membranes [87]. Also, nylon membranes with PMES-NTA-Ni^{2+} are functionalized. These membranes had larger pores than alumina membranes but still absorb 85 mg His U per cubic centimeter of membrane [88]. In addition, these membranes selectively bind His-tagged retinaldehyde binding protein from a cellular extract in less than 10 minutes. In general, the ability of polymer brushes to increase the binding capacity of the protein in the membrane depends on the type of polymer brush and the geometric shape of the membrane.

Even though the MES polymerization is carried out in water, attachment of the trichlorosilane initiator to the membranes is done in tetrahydrofuran (THF), which is sometimes incompatible with polymer membranes. To overcome this problem, Anuraj et al. utilized aqueous immobilization of a macroinitiator that was absorbed to the membrane through the hydrophobic interactions [89]. The subsequent polymerization of MES requires less than 5 minutes, and after functionalization with NTA - Ni^{2+}, these membranes provide the protein binding capacity as high as those after 1 hour of polymerization through modified membranes using the trichlorosilane initiator.

The main problem of the membrane modification with polymer brushes is the complexity and inefficiency of brush synthesis and derivation. Usually, the growth of brushes involves at least two steps: initiator attachment and polymerization under anaerobic conditions [67, 82]. In addition, often the monomer growth in

brush does not end, and controlling the density of the initiator and the polymerization conditions for optimization of the binding is challenging [90]. Derivation is also inefficient. To develop more simple ways to modify the membranes, the Bruening group began a layer-by-layer adsorption study, which is described in the next section in detail about this method.

4.2 Modification of surfaces with polyelectrolyte multilayers (PEMs)

Polyelectrolytes are formed through alternating (layer-by-layer) adsorption of polyanion and polycation. These films can bind proteins and multilayers through electrostatic interactions or, when they contain appropriate ligands, may capture special proteins (**Figure 10**). Such films are versatile materials for binding several layers of protein on surfaces, including membrane pores.

4.2.1 Growth mechanisms and structure of polyelectrolyte films

In 1990, Hong and Decher [91, 92] demonstrated the basic principles of layer-by-layer (LbL) polyelectrolyte adsorption by exposure to a charged substrate with alternating solutions of polyanions and polycations (**Figure 11**). After adsorption of each polyelectrolyte, the surface (location) takes reverse charge, and one quasi-equilibrium adsorption requires only a few minutes.

Although the polyelectrolyte spray provides a quick way to form them [93], the absorption from the solution is the most common method for making these films [91]. Also, among many methods for forming thin films such as dip and spin coating or single-layer adsorption, layer-by-layer deposition of the complementary polymers has emerged as a technique, especially for controlling the thickness and performance of the film (**Figure 11**). **Figure 11** shows the most common layer-by-layer method indicates the alternating (continuous) absorption of polyanions and polycations. Currently, this method, by simply immersing a substrate selected in polyanion and polycation solutions, is performed with rinsing to remove excess polymer after each deposition step.

Polyanions used to deposit these films include poly(acrylic acid) [94], poly(styrene sulfonate) [95], poly(vinyl sulfonic acid) [96], hyaluronic acid (HA), and so on. However most polycations contain ammonium groups of type IV [97, 98] or protonated amines [99, 100]. **Figure 12** shows a number of these polyelectrolytes. The layer-by-layer method can also employ a wide range of charged components including proteins [101, 102], viruses [16], nanoparticles [103–105], and flaky minerals [106, 107]. A number of layer-by-layer methods employ interactions such as hydrogen bond [16, 108–111] or covalent bond [112–115].

The PE adsorption depends on the charge density and polymer structure. Polyelectrolytes with constant positive charge, such as poly(sodium styrene sulfonate) (PSS) and poly(dialyldimethyl ammonium chloride) (PDADMAC),

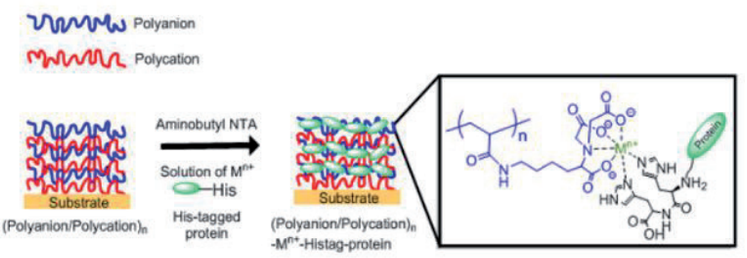

Figure 10.
Multilayer protein binding in a PEM derived with NTA-Mn^{+} complexes [11].

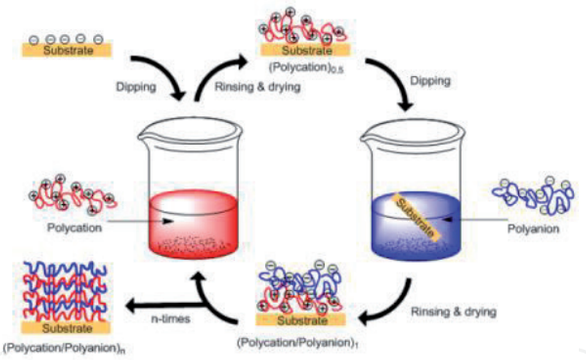

Figure 11.
Layer-to-layer absorption of polyelectrolyte multilayer [11].

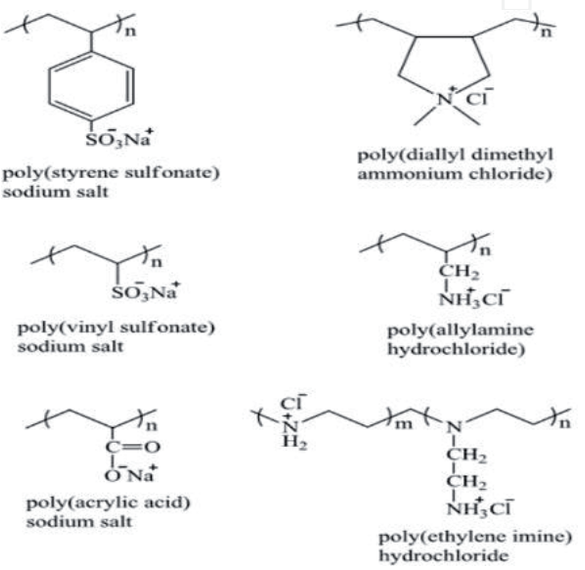

Figure 12.
The structure of conventional polyelectrolytes used in the manufacture of multilayers [116].

are called strong polyelectrolytes [117]. In comparison, for weak polyelectrolytes such as poly(vinyl amine) (PVA), poly(L-lysine) (PLL), poly(acrylic acid) (PAA), poly(allylamine hydrochloride) (PAH), and linear poly(ethylene imine) (LPEI), the charge depends on the pH and ionic strength [116]. Since both the density of charge and the PE conformation change with pH and ionic strength, these deposition parameters can dramatically alter the thickness and film conformation [116]. Typically, the thickness of PEM increases with increasing ionic strength of the sedimentation solution, because of the separation of the charge and the formation of loops and trains [118]. For weak polyelectrolytes, usually the thickest films are formed at pH values where polyelectrolyte has a low density of charge [119].

The binding and release of a protein, or other macromolecules, in a PEM greatly depends on the porosity and size of the mesh pores in the film (**Figure 13**) [120, 121]. In addition, film properties such as hydrophilicity-hydrophobicity balance and network charge complicate the binding and release of protein.

LBL films are often similar to a network structure (**Figure 13**), which includes cross-links caused by electrostatic interactions of polyanions and polycations.

The main factor governing the porosity of the network is the density of electrostatic complexation sites. A low-density cross-link refers to more open films and wider protein binding, but such films may be unstable. The change in polyelectrolytes, ionic strength, pH, or temperature can change the cross-link intensity and the protein binding as well as the film's stability and thickness.

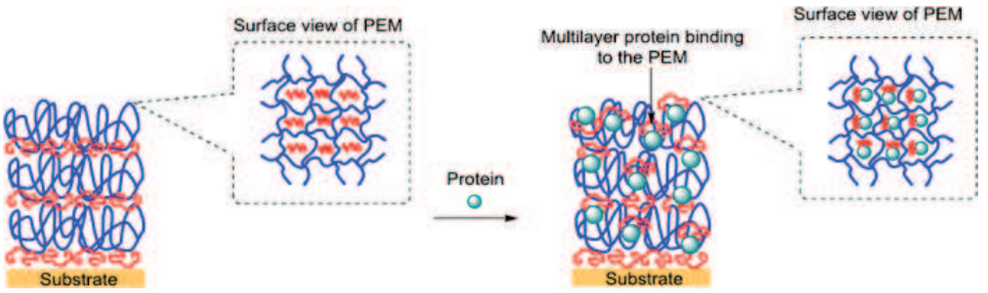

Figure 13.
Schematic representation of polyelectrolyte matrices designed for widespread protein binding [11].

4.2.2 Factors that change the growth of the film during layer-to-layer polyelectrolyte adsorption

In the addition of the selected polyelectrolyte for deposition, a series of adsorption parameters such as concentration and composition of support electrolytes [122–133], the molecular weight of polyelectrolytes [134–142], pH of polyelectrolyte solutions [143–150], adsorption time [122, 151–156], and temperature [157–159] affect the amount of polyelectrolyte deposited in layer-by-layer methods. Understanding the mechanisms of polyelectrolyte multilayer formation and the role of process parameters on determining the thicknesses and interfacial properties of multilayer films is essential for future film applications. Below we discuss the effects of a number of these variables on the growth of the film layer by layer.

4.2.2.1 The effect of electrolyte support

A number of studies investigated the importance of electrolyte support on the growth of polyelectrolyte films [122–126]. In the absence of salt added, polyelectrolytes, to maximize the intervals between charged repeating units (monomers) of polymers, are very broad [116]. Under these conditions, the adsorbed layers are thin, and the charge compensation of the surface is done only slightly (**Figure 14**) [160].

Excess salt may separate charges on polymeric chains and allows them to spiral and form thicker layers (**Figure 14**). Additionally, charge separation may require more polyelectrolyte adsorption to compensate for the opposite charge in a previously adsorbed film [116]. However, very high salt concentrations may lead to film lamination [161].

In addition to the electrolyte support concentration, the support electrolyte identity also changes the thickness of the film. Less hydrated cations [162] offer an increase in thicker polyelectrolyte films.

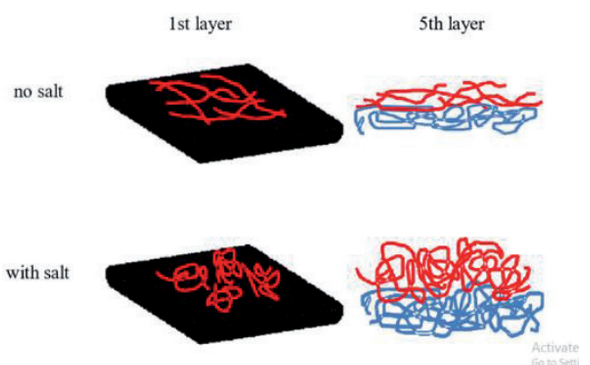

Figure 14.
The design of adsorbed polyelectrolyte layers in the presence and absence of salt. The lack of salt leads to thin layers of widespread polyelectrolytes, although in high ionic strength (salt presence), the coiled polymers form thicker layers [116].

Several other studies have tested that salt support in polyelectrolyte solutions changes the growth of films [163–165]. In these studies, the support electrolytes are all sodium salts, but the type of anion changes along the Hofmeister series from the cosmotropic anions to the chaotropic (F^-, $HCOO^-$, BrO_3^-, Cl^-, ClO_3^-, Br^-, NO_3^-, ClO_4^-) [165,]. Cosmotropic anions are strongly bound to water molecules and induce a number of structures in solution which leads to the deposition of molecules such as proteins; such anions increase the power (ability) of a hydrogen bond between water molecules to reduce the freedom of movement [116]. Chaotropic anions, due to their low electronegativity, high polarizability, and their weak electrostatic fields, destabilize the hydrogen bond between ions and solvent molecules to increase the solubility of a number of molecules [166]. Chaotropic anions strongly bind to polycations, thus reducing the density of the charge on the polyelectrolyte [116]. This refers to the formation of a coil structure, which increases the thickness of the layer.

4.2.2.2 Polyelectrolyte effect on multilayer adsorption

A number of polyelectrolyte properties, including chemical structure, molecular weight, concentration, and degree of ionization, affect the growth layer by layer of the polyelectrolyte layers. In this section, we will briefly explain the effects of these properties.

Studies showed that polyelectrolytes with different molecular weights have different effects on film thickness [91, 97]. Based on all these studies, it can be concluded that it is hard to predict how the thickness of the film will change with molecular weight.

The effect of concentration of polyelectrolyte solution is greater for strong polyelectrolytes. In high polyelectrolyte concentrations, many polyelectrolyte chains interact with the interface at the same time, and each one can only absorb on a small number of binding sites, which leads to relatively thick films. Inversely, in lower polyelectrolyte concentrations, polyelectrolytes interact with many binding sites at the surface to produce thinner films [167, 168].

The results of a number of studies show that increasing the concentration of polyelectrolyte to a certain extent can increase the thickness of the film. On this basis, it can be concluded that there is a saturation limit to increase the thickness and adsorption of the film, preferably similar to an adsorption isotherm [116].

4.2.2.3 The effect of deposition pH (or polyelectrolyte ionization degree) on the growth of polyelectrolyte multilayers containing weak polyelectrolytes

The effect of this parameter on weak polyelectrolytes is observed. The pH of the weak polyelectrolyte deposition solutions greatly affects the thickness of the film, as well as its permeability and morphology [143–147]. In weak polyelectrolytes, the ionization of groups such as amines and carboxylic acids, and therefore the density of the polymer charge, is a strong function of pH [116]. Increasing the density of the charge over the polyelectrolyte will result in the formation of thinner films and a decrease in thickness; however, increasing the density of charge on previously adsorbed polyelectrolytes will help to form thicker films [116]. However, it should be noted that extreme pH values can be completely prevented by film growth with the aid of desorption [149]. Changes in the charge density due to differences in pH are specifically dependent on the polyelectrolyte system; in lower charge densities, due to less electrostatic repulsion between repetitive units (monomers), weak polyelectrolytes will form more coil conformations [116]. In addition, a weaker electrostatic repulsion between adsorption polyelectrolyte molecules should help to form thicker films [150].

4.2.2.4 The effect of temperature on the growth of polyelectrolyte multilayers

The effect of this parameter appears more in strong polyelectrolytes. Increasing the deposition temperatures significantly increases the thickness of the polyelectrolyte films. Polyelectrolytes tend to precipitate at higher temperatures, which leads to the formation of thick and rough layers [159]. Secondary interactions such as hydrogen bonding and hydrophobic and van der Waal's forces, which depend on temperature, also change the thickness of polyelectrolyte multilayer films [169].

It seems that the time parameter effect is less than the other parameters mentioned. Available studies on polyelectrolyte multilayers show a wide range of time estimates (seconds to hours) needed to form a layer [122, 151–156]. This widespread range of times may be due to differences in structure, molecular weights, and deposition pH amounts of polyelectrolytes used to form multilayered films [116].

5. Membrane modification and its application in biotechnology

5.1 Protein purification

Membrane-based processes are beginning to play crucial roles in the separation and purification of biotechnological products. Polyelectrolyte films and polymer brushes in porous support can be used as new membranes for biomolecule isolation and purification. Many studies investigated the interaction of proteins with LBL films [170, 171], In some cases, films can be used as protein storage with high binding capacity of proteins [172]. However, no theory has foreseen the insertion or loading of biomolecules in films, this is often due to the lack of experimental tools for accurately analyzing the molecular distribution and mobility [11].

LBL adsorption of polymer films and subsequent derivation were used to construct PEM-modified membranes, which easily capture the His-tag protein [173]. PEI/PAA multilayers selectively attach a protein from a mixture of concanavalin A and lysozyme. At pH 7/3, $(PEI/PAA)_3$ preferably adsorbed positive-charged lysozyme, and $(PEI/PAA)_2$ PEI adsorbed negative-charged concanavalin A [174]. Polyelectrolyte multilayer films formed in membrane pores that are terminated to a polyanion have cation exchange sites, as shown in (**Figure 15**).

Adsorption of proteins depends on the surface charge, protein charge, and the thickness of the polyelectrolyte film [175]. Generally, protein binding in LBL films depends on the size of the membrane pore, hydrophobicity, and surface charge [65].

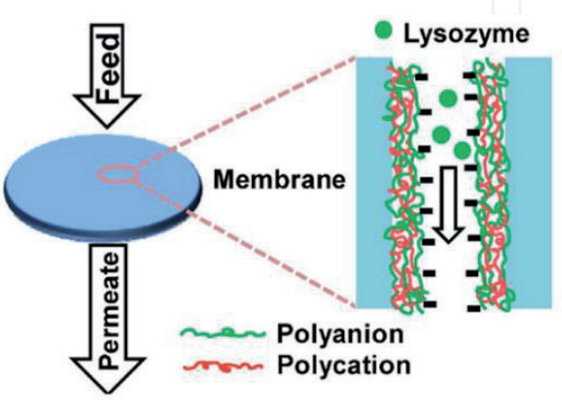

Figure 15.
Showing a schematic diagram of positive-charged lysozyme binding to a polyelectrolyte film terminated to the polyanion (the charges have been marked only for the end layer) [116].

Membranes containing film PAA/BPEI/PAA bind 100 mg/ml lysozyme through ion exchange [11], which is about twice the capacity binding of the commercial ion exchange membranes. So that, the Mustang S exchange membranes represent the binding capacity of lysozyme only 45-50 mg/cm^3 of membrane [133]. Also, after further modification of these layers with metal ion- NTA complexes (**Figure 16**), the membranes bind 70 mg/ml concanavalin A (ConA) (a 25-kDa protein) and 97 mg/ml of His-U (a 10-kDa protein). Interestingly, these membranes are selective, so that, optionaly,capture His-tagged COP9 (His-tagged COP9 signalsome complex sub unit 8) from a cell lysate with a purity of >95% [11]. More interestingly, and most importantly, the entire purification process takes less than 30 minutes from the beginning to the end of the process.

When the protein is captured from the cell extract, the size of the pores greater than 1 μm prevents the blocking of pores, and to maintain the flow speed is important [65].

Despite the successes mentioned in the membrane modification using polyelectrolyte multilayer films, because the derivation of these films is done using NTA ligand, which is an expensive ligand, the derivation of these films is costly, and also, only a small fraction of aminobutyl NTA is bound to the membrane; in addition, only one small portion of aminobutyl NTA attaches to the membrane. To overcome this problem, direct adsorption of metal ion binding polymers was performed without the need for further derivation with the NTA ligand [11].

Most purification processes employ a tool and method that, in addition to being inexpensive and productive in terms of time, are also consuming. Membrane-based purification processes are fast due to the fact that flow through membrane pores rapidly brings biomolecules to the binding sites. But despite this advantage, the biggest defect in the membrane is the lower surface area than the beds containing nanoparticles, which ultimately leads to lower binding capacity. In this regard, attempts to increase the membrane binding capacity and membrane modification methods should be advanced in a way that all these benefits are provided together. The advantage of polymeric film and brush-based modification techniques is that these polymers in water can swell several times their initial thickness and make the entry of biomolecules to the binding sites more rather easier and ultimately provide high

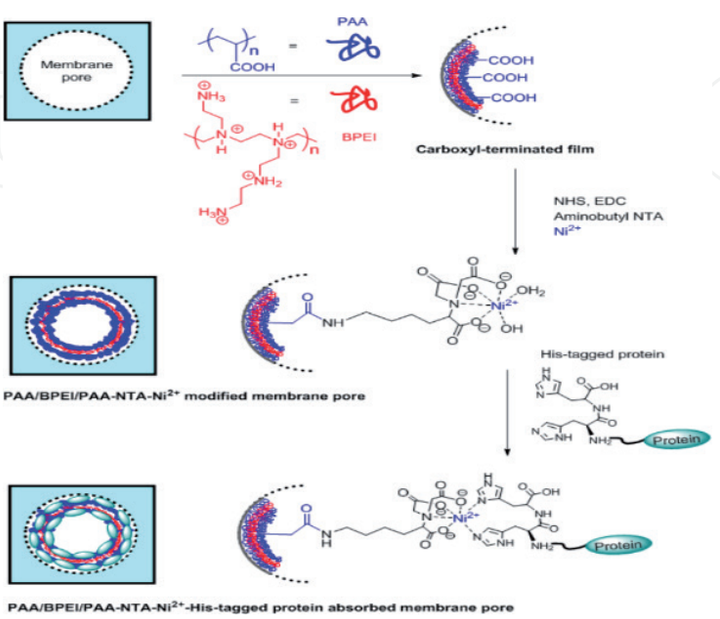

Figure 16.
The display of adsorption schematic $(PAH/PAA)_n$ in a membrane pore, functionalization with NTA-Ni^{2+}, and multilayer His-tagged protein binding [11].

binding capacity. Polymer films should have enough thickness and swelling to achieve high binding capacity and, on the other hand, do not block the membrane pore. In this regard, in most studies, three or lower layers are adsorbed in the membrane pores.

5.2 Antibody purification

Membrane purification processes are also used to purify and isolate antibodies. Common antibody purification processes using columns that contain immobilized protein A and G are costing.

Microporous membranes containing PAA/PEI films [176] were modified with small peptides and antibodies and then used to purify antibodies and proteins. Also, membranes containing small peptide, K19, selectively capture Herceptin from human plasma (**Figure 17**). And, the membrane modified with antibodies were successfully used to capture protein from cell lysate (Anti- (hemagglutinin A) (HA) antibodies captured HA-tagged regulator G-protein signaling2 (HA-RGS2) from cell lysate) (**Figure 20**).

Small peptides were immobilized to the membrane pores using the activation of the last PAA by NHS/EDC (**Figure 18**), and then the antibody was purified.

Although antibodies are important biotechnological therapists, their purification is highly costly; on the other hand, purification techniques that based on the column are long [176]. Therefore, trying to find the appropriate purification procedure for these therapists is essential. Membrane-based methods are promising candidates for this goal.

In this case, to purify the protein by immobilization of antibodies in membrane pores, there is the fact that the immobilization of antibodies by electrostatic is unstable, but provides high binding capacity (**Figure 19**). (A) In contrast, covalent immobilization provides stable binding to membrane pores but provides lower binding capacity (**Figure 19**). (B) In contrast, a two-step immobilization method [176], comprising electrostatic immobilization followed by a covalent linking (**Figure 19**) (C), maintains both the high capacity of electrostatic immobilization and the stability of covalent binding.

Tagged-protein selectively was captured using modified membranes with immobilized antibodies in membrane pores, which were immobilization with two-step immobilization method (**Figure 20**).

5.3 Phosphopeptide enrichment using TiO_2 nanoparticles containing membranes

Due to the relatively low abundance of phosphorylated proteins, detection and identification phosphorylation sites are challenging even with recent advances in

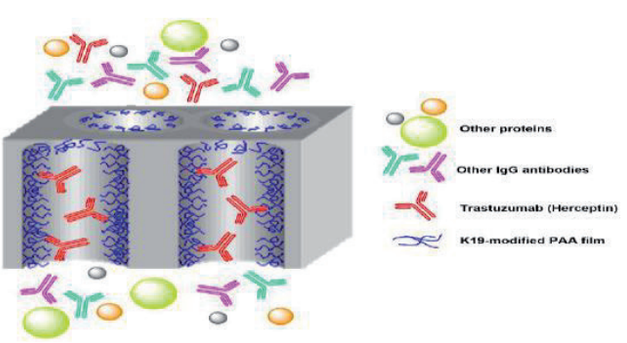

Figure 17.
Illustration of selective Herceptin capture in membranes modified with K19 peptide. K19 selectively binds Herceptin in the presence of other IgG antibodies [176].

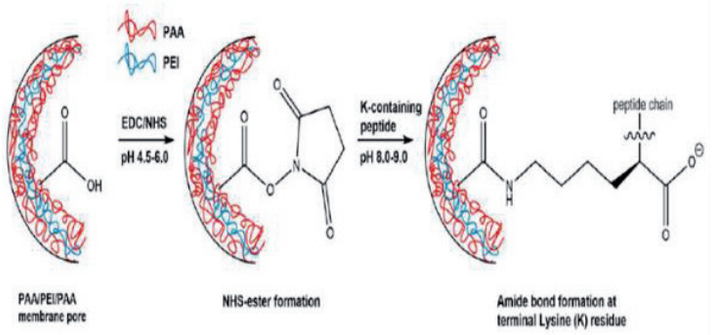

Figure 18.
Peptide/protein immobilization via EDC/NHS mediated coupling. For peptide in this research, a terminal lysine couples to PAA carboxyl groups. Proteins present surface amines for the coupling reaction [176].

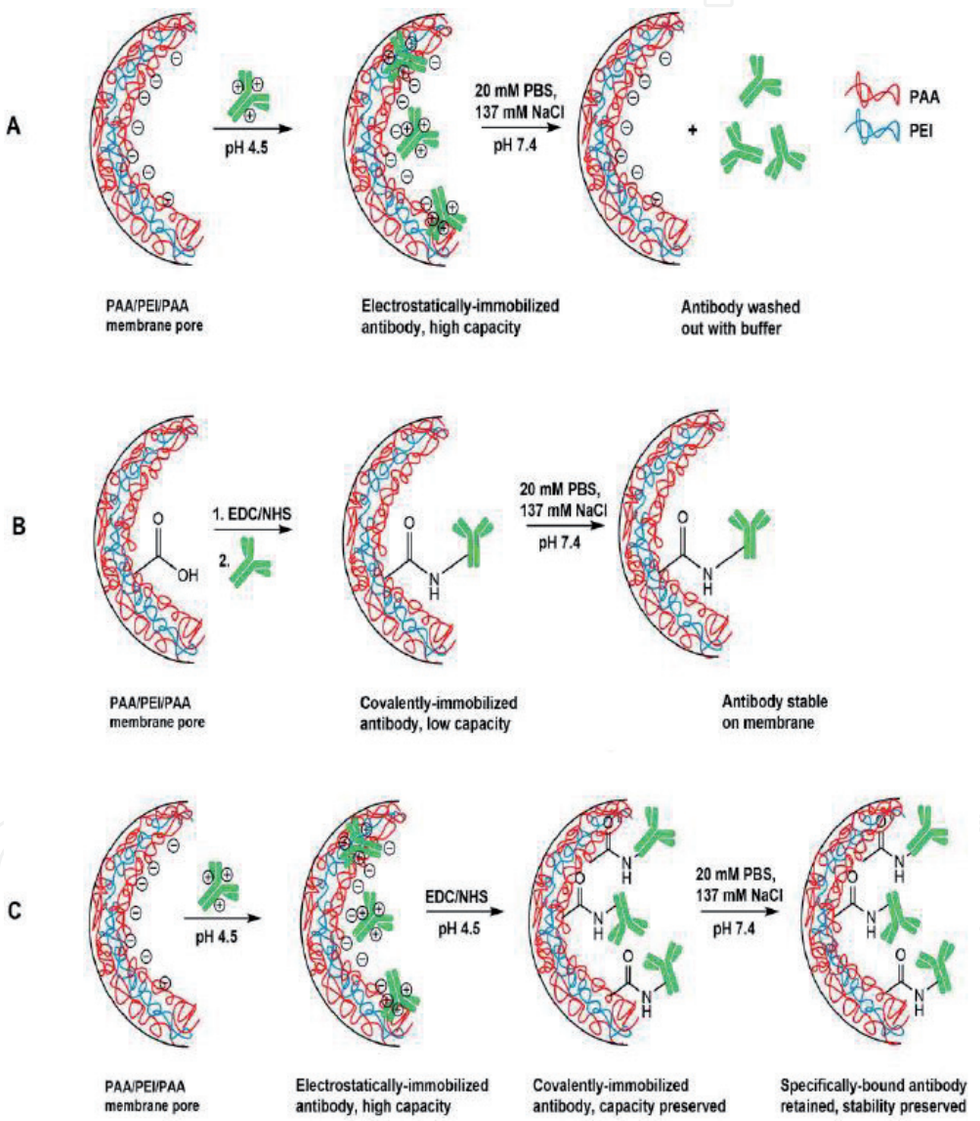

Figure 19.
(A) Electrostatic immobilization of antibodies yields high capacity, but the antibody elutes from the membrane in salt solutions. (B) Direct covalent immobilization does not yield the high capture capacity of electrostatic immobilization, but it does increase the stability of antibody on the membrane. (C) The two-step antibody immobilization of antibody first uses electrostatic capture to attain a high capacity and then covalently links the antibody to the membrane to increase stability [176].

MS [177]. The adsorption of nanoparticles in membrane pores is another way to provide selective binding sites. Phosphopeptide capture can be done through selective adsorption on ZrO_2 or TiO_2 columns or on matrix-assisted laser desorption/

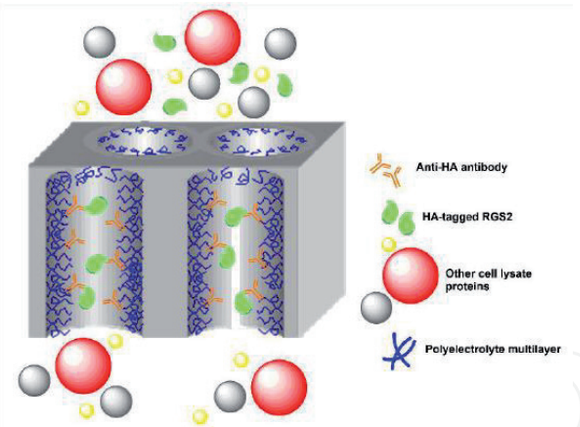

Figure 20.
Illustration of membrane-based selective capture of HA-tagged RGS2 from cell lysate. The capture employs immobilized antibodies [176].

ionization (MALDI) plates containing TiO_2 nanoparticles [178, 179]. Membranes are modified with sequential adsorption of poly(sodium styrene sulfonate) (PSS) and TiO_2 nanoparticles in membrane pores [180] (**Figure 21**). The membranes are attractive for the immobilization of TiO_2 nanoparticles [181–183], which are very small for column formats. These nanoparticles have a high surface area and can exhibit different and more binding capacities than larger particles. The binding capacities obtained in this way are less than the binding capacities of the brush-modified membranes, because the nanoparticle adsorption cannot provide films with high thickness and swelling.

5.4 Protease-containing membranes for controlled protein digestion before mass spectrometry analysis

By using existing methods for immobilization of protein in membrane, the Bruening group began employing enzyme-modified membranes as controlled reactors for protein digestion prior to analysis MS. MS is the most common and powerful technique for detecting proteins and their posttranslational modifications [184]. Although peptides, in comparison to proteins, are more capable of MS and liquid chromatography MS analysis [65]. Therefore, digestion is usually a critical initial step for analyzing MS proteins; digestion usually occurs after a protease such as trypsin is mixed with substrate proteins in solution [65]. Although this method requires low enzyme concentrations to restrict self-digestion of protease, digestion times are generally 1 hour or more [185]. To overcome this problem and

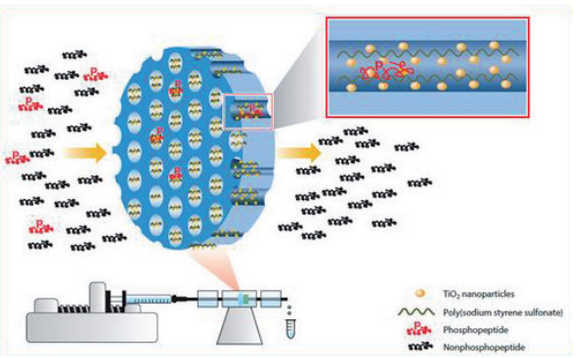

Figure 21.
Schematic of selective phosphopeptide capture in a membrane containing TiO_2 nano particles. A small holder attached to a syringe pump enables phosphopeptide elution in as little as 10 microliters of solution [65].

make it easier to analyze MS online, several research groups developed reactors with proteases immobilized on solid supports including monoliths [186, 187], membranes [188, 189], polymeric microfluidic channels [188, 190], and resins [191, 192]. With a thickness of only 10–200 micrometers, membranes provide excellent surface for controlling protein digestion [65]. Perhaps the biggest advantage of membrane digestion is controlling of peptide size afforded by varying residence times down to the millisecond level [65]. Little residence times should yield big peptides as a result of missed cleavage site, as a result of greater sequence coverage; larger peptides should enhance recognition of posttranslational modifications [65]. The purpose of current studies is to use large peptides to activate antibody sequences [65]. Limited digestion can also help reveal the presence of flexible regions in proteins because proteolytic sites are more accessible in these areas [193, 194]. (**Figure 22**) shows schematically preferred digestion in a protein flexible, accessible region, recognition of such regions is important for selecting shorted protein sequences to express for crystallization [65].

5.5 Isolation of enantiomer in racemic mixtures by membrane

Most of the drugs used today are racemic. An enantiomer may have the same effect as another enantiomer or even a harmful and different effect. Therefore, there is a need for tools and methods to detect and isolate enantiomers. The membrane's advantages over other separation methods in the previous sections are discussed in detail. As a new result of the use of membranes in the separation of racemic mixtures, modifying the regenerated cellulose membrane with chiral L-proline-copper complexes [195] through an intermediate epoxy-silane surface functionalization reaction for various times is a good example (**Figure 23**).

This chiral copper complex has various powers of coordination interactions with different enantiomers based on their space chemistry (stereochemistry) [195]. In this work, the ligand exchange chemistry is used to create membranes capable of separating the mixture of amino acids and potentially other drug substances that have functional groups capable to ligating with the metal complex. Such technique is simple, inexpensive, and scalable; also the method applied for membrane modification is very simple. The resulting membranes were evaluated in single component diffusion experiments with D- or L-phenylalanine (Phe), which showed much higher permeability for D-Phe than L-Phe. The high amount of Peclet number obtained (~400) [195] during the filtration process, combined with the complete fractionation of the enantiomer, shows that such system is very attractive and excellent as a competitor for chiral chromatography.

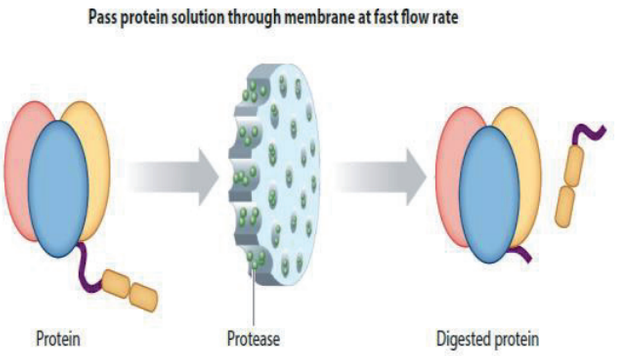

Figure 22.
Limited digestion at the most flexible and accessible site of a protein during rapid passage through a protease containing membrane (protein not drawn to scale, as it is much smaller than the membrane pores) [65].

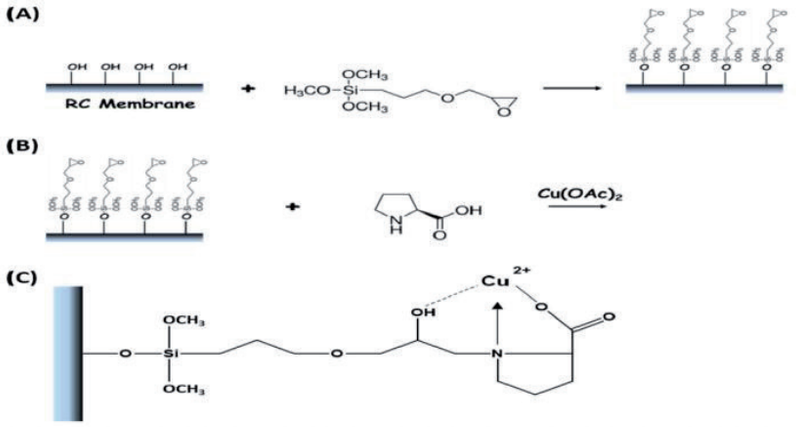

Figure 23.
Chemistry of ligand exchange. (A) Functionalization of RC membrane surface with the epoxy-silane. (B) Grafting of L-proline to membrane surface followed by immersion in aqueous copper acetate solution. (C) Resultant complex of grafted L-proline with copper [195].

6. Conclusion

Given that biomolecules now cover many areas of human life (most importantly the therapeutic area), identifying purification methods and isolating these materials and finding the right and most appropriate method are essential. Due to the constraints of column-based isolation and purification methods, the membranes provide the possibility of purification and rapid separation of biomolecules and, as a result, are excellent and unmatched substitutes and rivals for compacted bed-based chromatography systems. Membrane modification with polymer brushes provides three-dimensional and swelling structures for separation and purification of biomolecules with high-capacity binding. In terms of hard conditions, anaerobic conditions, initiator density control and their synthesis hard conditions, layer-by-layer adsorption of polyelectrolytes is a good alternative instead for brush synthesis, which is also explained in the brush section. In this way, with this simple modification method, membranes with binding capacities higher than conventional commercial membranes and as much as polymer brushes can be obtained. By controlling the thickness of the polymer films and also controlling the density of the polymer brushes,In addition to obtaining a high binding capacity, can maintain the flow rate through the membrane pores. The use of membranes in various fields of biotechnology indicates membrane's success in this area. In this regard, it can be said that the membranes will find great positions in the future of life.

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