Practical Techniques for Improving the Performance of Polymeric Membranes and Processes for Protein Separation and Purification

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ABSTRACT: *Protein separation and purification technologies play an essential role in various industries including but not limited to pharmaceuticals, dairy as well as the food sector. Accordingly, a wide variety of techniques such as chromatography and electrophoresis have been developed and utilized extensively over the years for this purpose. Despite their widespread acceptance, conventional techniques still suffer from major limitations and complexities such as short lifetime, low productivity, high-pressure drop and difficulty in scale-up among others. Membrane separation processes have received significant attention in recent years as a promising alternative that can potentially overcome the problems associated with the conventional technologies due to their spectacular features. The prime advantages offered by the membrane-based processes for protein separation and purification include tunable properties, cost-effectiveness, superb productivity, as well as energy efficiency. The present manuscript aims to highlight the significant aspects of the established protein separation and purification technologies by addressing the principal concepts and highlighting their characteristics. Special attentions are paid to the membrane-based processes by providing detailed features and specifications involved in each individual process, especially from the industrial perspective. Furthermore, the recent and ongoing progress on strategies and practical techniques towards improvement in performance of membranes for separation and purification of various proteins is introduced and discussed in details.*

KEYWORDS: *Protein separation and purification; Performance improvement; Polymeric membranes; Membrane modification.*

INTRODUCTION

Over the past decades, membrane technologies have progressed from a simple laboratory-scale research into

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a wide variety of industrial applications such as food, biotechnology, chemical and pharmaceutical for separation, fractionation and purifications of molecules and species [1, 2]. Development of membranes with improved characteristics and high yields has escalated the importance of diverse membrane-based processes [3-13]. Accordingly, numerous traditional separation techniques including distillation [2, 14], crystallization [15] and solvent extraction [16, 17] are now either totally substituted or improved by the membrane processes. This is due to the fact that in many cases, membrane separation processes are more reliable, more efficient or more cost-effective than the rivals [18-21].

The membrane can be described as an interphase between feed and product streams, allowing specific species to pass through, while retarding or rejecting others. In the process of protein separation by the membranes, selectivity is achieved based on either size, Molecular Weight Cut-Off (MWCO), electric charge or a combination of them. It should be noted that the MWCO is the molecular weight of the smallest soluble molecule being rejected more than 90% by a membrane and is considered as a standard parameter for performance evaluation in the experimentations [22].

A typical protein molecule is comprised of charged, bipolar and hydrophobic groups that can interact with the functionalities present at the membrane surface via different mechanisms such as electrostatic and hydrophobic interactions, hydrogen and dipolar bonding, dispersion forces or a combination of them. Thus, proteins can be adsorbed by almost all types of surfaces. The affinity between a protein and membrane surface is often increased by giving rise to the hydrophobicity of the intended surfaces. Additionally, the increase in entropy is one of the key driving forces in the adsorption of proteins onto the surface [23]. Difficulties associated with the separation of proteins are mainly due to the complexity of the molecular structure. Proteins possess complicated primary to tertiary structures and are often multipolar. Proteins may also possess several charges and the net charge depends on the pH of the medium. Since proteins have interactions with water molecules and ions present in the medium, their active size is greatly influenced by the ionic strength and salt concentration as well [24]. Due to these characteristics, separation of proteins and similar biological molecules has been facing a number of challenges and complexities. Accordingly development of effective approaches for separation and purification of proteins and biological molecules in large scale is of a great interest for researchers as well as industrialists.

SIGNIFICANCE OF PROTEIN SEPARATION AND PURIFICATION

Following the rapid growth of biotechnology, purification of new therapeutic proteins with emphasis on economy and throughput has gained attention for applications such as cancer treatment and gene therapy [24-26]. The purified and isolated proteins have many applications in the medical industry including but not limited to monoclonal antibodies such as trastuzumab for treating breast cancer [2] and Infliximab for treating rheumatoid [16]. Table 1 provides some of the important purified proteins, their applications, and properties. Often, therapeutic proteins are found in multi-component aqueous solutions in low concentrations. For example, Factor VIII, which is a clotting agent for blood with a concentration of 0.1 mg/L, exists along with about 750 additional proteins in blood plasma. Since therapeutic proteins are directly injected into the body, they have to be supplied in a specific purity and be free from any pathogens and other proteins causing side effects [24, 27]. Therefore, it is required to separate the target molecules out of the mixture with high sensitivity and specificity [28]. Accordingly, the complexity of biochemical and biological systems necessitates more sophisticated techniques with high efficiency and selectivity for separation and purification of individual components. It should be noted that the separation or isolation of desirable proteins from compounds is usually the most expensive part of downstream processes in protein manufacturing [29]. In a normal biological protein production process, purification and separation of proteins may constitute more than 70% of the total costs [24]. Selectivity and the rate of separation are the key factors which determine the level of efficiency and economics of the process [29].

Essentially, protein separation and purification can be categorized into three main categories:

(1) Removal of the solvent, most often water, from solutions containing macromolecules also known as concentration [30-32]. Particularly, highly concentrated solutions of the antibody are increasingly gaining value in the therapeutic industry [33].

Table 1: Some important purified proteins and their applications.

Name of purified protein	Applications	Properties
Albumin	Drug delivery, act as an important extracellular antioxidant	Biodegradable [14] Biocompatible [14] Less-immunogenic protein [14]
Lysozyme isolated from egg whites	Natural food preservative [15] Inhibition of HIV replication [16]	Anti-inflammatory properties [15] Antibacterial activity against food spoilage bacteria and pathogens [15] Antitumor activities [16]
lactoferrin	Limits the proliferation and adhesion of microbes [17] Functions as a natural iron scavenger and an activator [17] Preventing inflammatory pathway activation[17]	Antimicrobial activities [17] Interact with the molecular and cellular components of hosts and pathogens [17]

(2) The practice of eliminating suspended particles from a solution also known as clarification [32]. For instance, in downstream antibody purification, the broth needs to be clarified so that plugging elements are eliminated [34].

(3) Fractionation indicates the highly precise separation of solute species with close molecular weights [32]. For instance, whey proteins with Immunoglobulin G (IgG), lactoperoxidase (LPO) and lactoferrin which are the proteins found in whey must be fractionated due to their economic importance [35].

PROTEIN SEPARATION AND PURIFICATION TECHNOLOGIES

Protein separation and purification methods have been evolved over the past 200 years. Before the 20th century, only simple separation methods like precipitation and crystallization were practiced for protein separation. The traditional techniques such as distillation, absorption, and solvent extraction [36] were not very attractive for protein purification, since proteins are intrinsically unstable and prone to degradation by heat, solvents and even shear stresses [24]. Over times, promising approaches including electrophoresis and centrifugation were devised for protein separation and purification. In 1906, chromatography was introduced by *Botanist Mikhail Tswett*. During the 1950s to 1980s, chromatography systems were established according to different properties of proteins such as size, charge, hydrophobicity and isoelectric point. Researchers are still working on chromatography to make it more reliable and effective.

Besides chromatography, advances in materials engineering and science, coupled with using progressive facilities have contributed to the advancement of membrane technology. Nowadays, protein separation by membranes has predominantly found its position among the competing rivals [37-39]. The following sections provide further insights into the chromatography, electrophoresis, and membranes as the prominent techniques for protein separation and purification.

Chromatography

Chromatography is a method to separate constituents of a mixture by passing a mobile phase over a stationary one. Molecules that tend to stay in stationary phase have lower speed while moving through the apparatus in comparison with those that have a tendency towards the mobile phase. Column chromatography is the most popular physical formation of chromatography that contains a packed column acting as a stationary phase and the mobile phase should be pumped into it. The sample enters the column at one terminal and different species are separately collected at the other end since the components transport at distinct rates [40]. Fig. 1 demonstrates a schematic of

Chromatography allows high-resolution separation and has the ability to use different absorbents to form various modes, such as gel filtration [41] (using a porous medium),

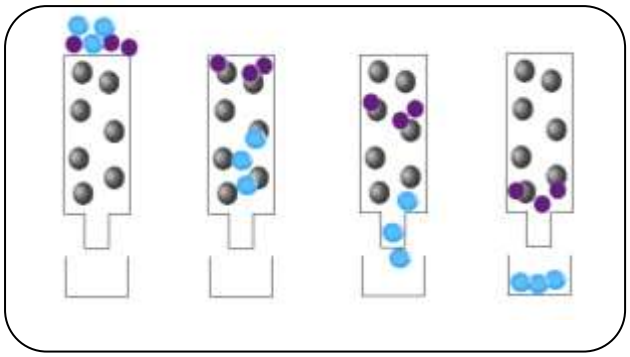

Fig. 1: Schematic representation of the chromatography process.

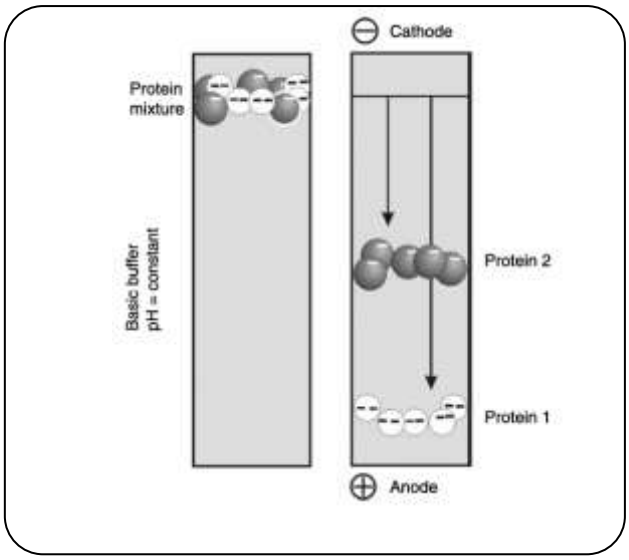

Fig. 2: Separation principle of the electrophoresis process. Proteins with different net charges and sizes migrate in an electric field in the presence of a buffer with different migration velocities. Different proteins form discrete zones [47].

ion-exchange [42] (using charged surface) and affinity chromatography [43] (using ligands). For example, Atasever et al. applied affinity chromatography for purification of LPO from bovine milk [16]. They synthesized an affinity matrix by using Sepharose 4B, L-tyrosine (acting as spacer arm to provide full access to ligand) and sulphanilamide (acting as a ligand). Results showed that LPO was successfully purified with the yield of 62.3%. Despite the attractive features, chromatography suffers from a few principal aspects. Difficulty in scale-up, high cost, long processing time, lengthy cleaning, and the necessity for a detailed understanding of the solution as well as the need for using multiple cycles are among these limitations [22, 24, 44-46].

Electrophoresis

Electrophoresis is the migration of particles and charged molecules in the presence of an applied electric field. The speed of migration is different based upon the charge and size of the different molecules. The concept of electrophoresis was discovered by Ame Tiselius and was named "moving boundary electrophoresis". Fig. 2 demonstrates a schematic and the basic principles involved in electrophoresis.

Electrophoresis allows the separation of human serum into four main components such as albumin, a-, b- and g-globulin. The easiest way for transportation of proteins is electrophoresis in the gel. Acrylamide gels are widely used for electrophoresis of proteins but in some cases the agarose gel is used. To reduce the heat generation, gels are kept cool during operation and the electric field strength is reduced. The result of separation is easily visible using the colors that bond to proteins. Thus, the electrophoresis of proteins in gels is an attractive separation method due to its advantages such as high throughput, simplicity, speed, and economy.

Based on the principles of electrophoresis, each protein has the special electrophoresis mobility (m) and its migration speed is determined under the electric field *E*. Mobility of electrophoresis depends on the net charge and the size of the molecule. Proteins with different electrophoresis mobility have different migration speeds. The electric field causes migration rather than current. Electric current depends on the composition and conductivity of buffer. The minimum concentration of buffer is required to maintain the constant charges of proteins. Mainly, basic buffers are used, but sometimes electrophoretic separations need to be carried out in acidic buffers where the proteins are positively charged and migrate towards the cathode [47].

Membrane separation

Over the decades, membranes have progressed in diverse areas for separation of numerous species and molecules from gas to solid and liquids [48-60]. Nowadays, membrane separation processes are applied as the first choice for purification and separation of numerous species as well as biological molecules such as proteins, peptides and amino acids [61, 62]. The transport of protein molecules across the membrane is governed by what occurs at the gateway of the pores. The molecules

are retained if bigger than the size of pores. In contrast, if the size of a pore is greater than a molecule, the passage does not necessarily take place. The convection force induced by the pressure difference between two sides of the membrane (transmembrane pressure,TMP) generates the driving force for transport of protein molecules. Resistance is largely caused by the electrostatic repulsion especially when both membrane surface and protein carry the same electrostatic charge. However, in the case of opposed charges between the membrane surface and the protein, a self-repulsive layer of protein on the surface forms due to the attraction of charged protein molecules by the surface. Therefore, protein molecules will only pass through the pore if the convection force prevails over the electrostatic repulsion; otherwise will be retained. It should be noted that proteins are neutral at their isoelectric point (*pI*) at which they possess no ionic layer and their actual volume would be minimum. The net charge and the effective volume of proteins are affected by the isoelectric point and pH of the medium [63]. Thus, pH of the solution and the ionic strength are the crucial parameters that determine whether proteins are transmitted or rejected [24, 64]. This effect could be used for selective separation of species. For example, the selectivity of about 2 has been recorded for separation of Bovine Serum Albumin (BSA) and IgG at pH=7.0 and 0.15 M salt concentration. However, once solution conditions changed to pH=4.8 and 0.0015 M salt concentration, selectivity reached values close to 50 for the same system due to the resulting electrostatic forces. Reverse selectivity, which allows passage of large molecules while retains small ones has also been observed. In this case, at pH=7.4 and 0.0015 M salt concentration, IgG which is larger than the BSA passed through a 300,000 MWCO membrane and BSA was totally retained. This was because at this pH, IgG is neutral while BSA carries the same charge (negative) as the membrane [24, 62].

Membrane separation at the ambient temperature provides a good approach for separating temperature-sensitive solutions without any risk for chemical changes or degradation and has many advantages in the food and pharmaceutical industries [65]. Also, separation by membranes includes advantages such as easy scale-up, continuous operation, low energy consumption, being environmentally friendly and no need for solvent extraction [64,66]. To reach the desired separation, the operational conditions should be adjusted well which is possible by having a good understanding of the system [24]. Membrane technologies still face some challenges for purification of proteins like fouling of the membrane, precipitation of solutes and irreversible absorption [67]. When solute molecules accumulate on the membrane surface because of the partial rejection of membrane, the selectivity of the membrane is negatively influenced. This phenomenon is known as Concentration Polarization (CP) [39].

MEMBRANE PROCESSES FOR PROTEIN SEPARATION AND PURIFICATION

Various membrane processes could be employed for protein separation and purification, but pressure-driven operations like MicroFiltration (MF) [68], UltraFiltration (UF) [69] and NanoFiltration (NF) [70] have made the most contributions [22]. These membrane processes separate species based on the prevailing mechanisms under an exposed pressure. As the size of pores become smaller from MF to NF, the endurable pressure by the membrane would increase [71]. Such membranes can be used in various geometries including hollow fiber, flat sheet and spiral-wound modules [72]. Flat sheet membranes analogous to a filter press, are comprised of a sequence of cassettes. Tubular membranes are made in the form of tubes of circular cross-section with diameters in the range of 3-15 mm. This type of module is able to tolerate fluids that contain large particles or solids. Hollow fiber membranes, possessing diameters of 0.05-2 mm, are usually used in compact bundles. Spiral wound and hollow fiber modules also offer large area per unit volume and are considered appropriate for relatively clean feeds [24]. *Li et al.* [73] developed two-layer hollow fiber membrane made from polyethersulfone (PES) and sulfonated polyethersulfone (SPES) for separating BSA/hemoglobin protein mixtures. Due to the strong electrostatic interactions between sulfonic groups of SPES and hemoglobin, BSA diffused to permeate flow and hemoglobin was rejected by the membrane. Various types of membrane processes used for protein separation and purification are discussed in the following sections.

Microfiltration

MF is a process appropriate to separate particles in the size of 0.1-10 micrometer and has extensive applications

for separation, clarification, and purification of protein solutions [22]. MF is particularly used for eliminating bacteria and some viruses from pharmaceutical proteins [74], clarification of juices [75] and wine [22, 76], dairy industry applications including whey protein separation [77] and milk protein fractionation [78, 79]. Operation modes of MF membranes can be either dead-end or cross-flow (Fig. 3). When the membrane operates in a cross-flow mode, the fluid enters parallel to the surface of the membrane and can pass through membrane because of the pressure difference. In this case, the rate of cake formation on the skin layer is low [22] and is preferred in the industry as it allows more run time between cleaning cycles [80]. MF membranes, besides centrifugation, deep filtration and bed chromatography, can be utilized for the primary withdrawal of medical products from mammal cells, yeast cells, and bacterial cell cultures. In comparison to the centrifugation method, MF makes harvested solutions free from particles using membranes with a pore size of ~0.2 μm and there is no need for additional clarification before purification [81]. *Tremblay-Merchand et al.* investigated eliminating caseins from Serum Protein (SP) of skimmed milk with SP removal rate of 0.58 kg/h.m^3 using ceramic MF membranes [82]. They also investigated the influence of some key parameters like TMP and Volumetric Concentration Factor (VCF: the ratio of the SP concentration in foulant to that in feed) on membrane fouling and the efficiency of the process. They reported the optimal TMP of 152 kPa and VCF of 3X for high-efficiency separation. Jørgensen et al reported utilizing a cross-flow ceramic MF membrane for fractionation of skimmed milk proteins and scrutinized the impact of pore size and temperature on the process [83]. In this experiment, they compared the performance of MF membranes having pore sizes of 0.05μm, 0.1μm and 0.2μm with VCF of 2.5. Results demonstrated that the most suitable pore size of MF ceramic membrane for skim milk, protein fractionation was 0.1μm. The only problem associated with this membrane was that during filtration, an increase in temperature from 50˚C to 60˚C led to decrease in permeate flow due to the interactions between membrane surface and whey proteins.

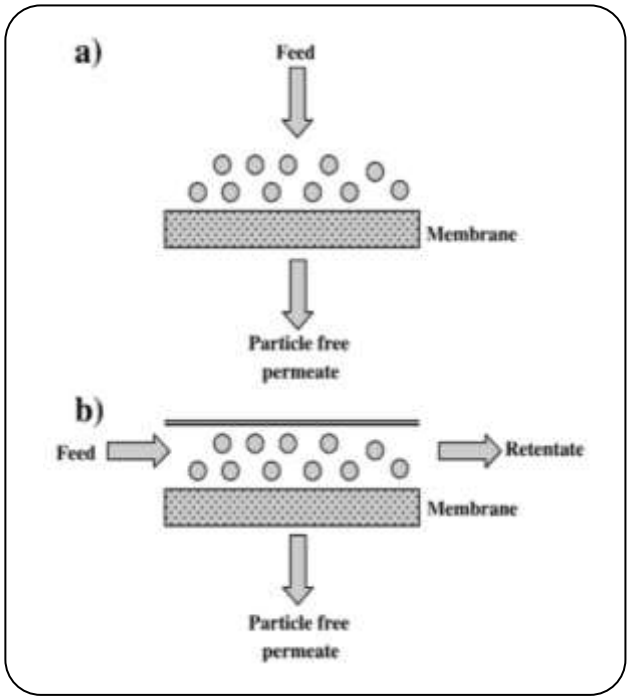

Fig. 3: The standard modes utilized in MF membrane process a) dead-end, b) cross-flow [22].

Ultrafiltration

UF is a pressure-driven process with the pores in the range of 10 to 100 nm and is suitable for retaining molecules with a molecular weight of 10 KD to 1 MD. The operating pressure in the UF is between 0.2 to 4 bars. UF membranes are ideally used for concentrating macromolecules such as proteins [24, 84] in the production of foods and beverages, dairy products [85], industrial enzymes and therapeutic proteins [24, 86]. Also, UF is applied in the food industry to improve the taste and durability of beverages [87]. UF membranes are usually made of polymers [88, 89], ceramic [90-92] and metallic [93, 94] materials. However, membranes based on metallic materials are not often used whereas ceramic membranes, especially those possessing hydrophilic surface, are widely applied in biotechnology and pharmaceutical industries [21].

The properties of polymers used for the fabrication of membrane play the main role in the performance of UF membranes. Various polymers can be incorporated for fabricating membranes, however, polysulfone (PSf) and polyethersulfone (PES) are mostly utilized in the commercial scales [24]. PSf and PES decrease wettability of the membrane due to their hydrophobic surface. For this reason, many research studies are devoted to increasing the hydrophilic properties of these polymers in order to diminish their fouling during protein separation [87]. For instance, Ulbricht et al [95] modified polyacrylonitrile

(PAN) ultrafiltration membrane by increasing the hydrophilicity of the surface by water plasma and 2-hydroxy-ethyl methacrylate (HEMA). After examining the modified membrane for BSA separation, they obtained a 150% increase in flux. Investigations regarding the influence of solution pH on membrane fouling indicated that intense fouling occurred when pH and *pI* were equal [96]. *Kumar et al.* prepared novel UF membranes based on chitosan and Poly Vinyl Alcohol (PVA) for separation of ovalbumin and lysozyme [97]. The organic-inorganic UF membrane was hydrophilic and porous with controllable charge density. Results revealed that the best separation performance occurred at pH=11 while BSA had extremely negative charge and Lysozyme was neutral. This hydrophilic negative charged membrane showed low fouling and high selectivity (15.4) for protein separation.

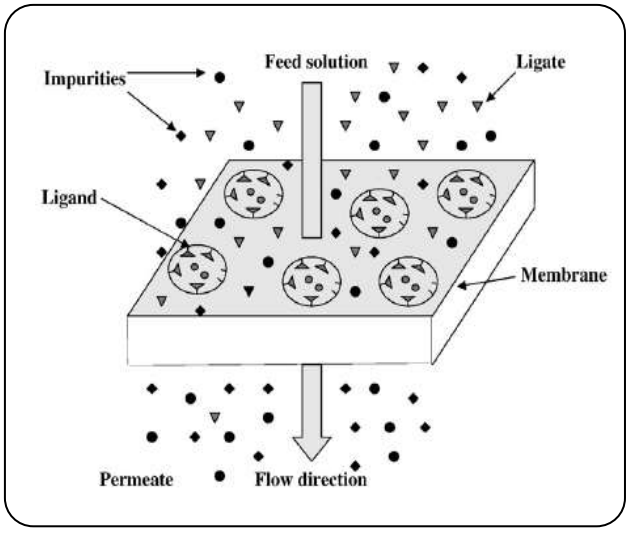

Fig. 4: Schematic representation of membrane chromatography process [22].

Nanofiltration

One of the most recent developments in the field of membrane technology is the emergence of NF membranes. The pore size of NF membranes is typically in the range of about 1–10 nm. Thus, this type of membrane is basically applied for separating peptides [98], monovalent salts and tiny or large bivalent ions [99-101]. NF is specifically used for separating peptides due to appropriate cut-off and also electrochemical impacts which play an effective role for charged molecules [22]. The main advantage of NF is that it is carried out at much lower operating pressure with higher flux than reverse osmosis [102]. Much of the efforts to improve the performance of NF membranes are focused on the enhancement of flux and pressure reduction through modifying membrane, tuning surface charge and reduction in the thickness of separation layer. The surface coating is a common approach to modifying NF membranes. For example, PVA can be incorporated in polyamide NF membrane for enhancing antifouling properties [103]. Grafting using UV [104] and plasma grafting are among methods used for applying a charge to the membrane [105]. Das et al. reported separating lactose and protein in whey through a two-step filtration using NF membranes [106]. They first separated lactose from proteins by a UF membrane in a filtration unit in order to enhance the yield of operation and then the permeate flow of ultrafiltration was fed to NF unit for concentrating lactose. Protein and lactose recovery of 80% and 90% was obtained due to less fouling of the membrane.

Membrane Chromatography

Membrane chromatography is an alternative method for a variety of resin chromatography column models like ion-exchange resin chromatography, reversed phase and affinity chromatography [107, 108]. A schematic of membrane chromatography is shown in Fig. 4. Membrane chromatography is applied for the separation of many different biological components such as DNA [109], viruses [110] and proteins [111]. This technology also can be used for separating single proteins like BSA, lysozyme [112, 113] and myoglobin, but it is specially applied for binary and multiple protein separation [114]. In most cases, membrane chromatography is applied for purification of subsidiary proteins in a mixture like antibodies that exist in much less amount than albumin in plasma [38, 115]. Particularly, manufacturing monoclonal antibody (MAB) as therapeutic biologics signifies a growing field of research where membrane chromatography can play a significant role [116].

In membrane chromatography, particular ligands are bound to the pores at the surface of the membrane in order to adsorb target proteins. Pores with large sizes facilitate access of the proteins to the binding sites. This results in a reduction of pressure drop and the processing time.

Reversible interactions between ligand and protein molecule lead to separation of the target protein in the mixture. In order to successfully implement purification via membrane chromatography, three requirements should be met:

(1) For separation or purification of the target molecule, a special bio-ligand should be available;

(2) Ligand should have chemically active groups to bind covalently to the chromatographic matrix;

(3) The membrane matrix should be available for it's pair covalent connection.

Also, ligand selection for membrane chromatography is affected by two factors:

(1) Ligand should have a specific and reversible affinity toward target material;

(2) Ligand could be able to be chemically modified in order to link to the matrix without damaging the bonds.

Yoo and Ghosh reported using hydrophobic interaction membrane chromatography for eliminating aggregated and filtered proteins from monoclonal antibody [117]. Also, membrane chromatography was used as an effective technique for BSA-Lactoferrin mixture separation [46]. In another study, membrane chromatography systems based on chitin and chitosan were studied by *Zeng* and *Ruckenstein* [14]. They applied chitin-based membranes for separation of lysozyme (1mg/mL) and ovalbumin (1mg/mL) mixture. The oligosaccharide of macroporous chitin membrane and lysozyme possessed van der Waals and hydrogen bonding interactions which led to a high selectivity. The purity of 99% was achieved for lysozyme with the yield of 61.5%. The findings also revealed that the maximum adsorption of lysozyme using chitin-based membrane chromatography (40 mg/mL) was much greater than chitin-based beads (5mg/mL).

Among the advantages of membrane chromatography, easier access to the junction in comparison to column chromatography is notable [44]. Furthermore, membrane chromatography benefits from easy scale up and low cost in comparison to column chromatography because of its less buffer usage and equipment cost [116, 118]. However, in this method, flow is not distributed uniformly through the membrane because of the great diameter to length ratio of the unit. This limitation reduces the process efficiency [22, 44]. Additionally, in this method, it is required to design specific absorbers and use cyclic stages because of the small absorption capacity of absorbers [44].

STRATEGIES FOR IMPROVING THE PERFORMANCE OF MEMBRANES FOR PROTEIN SEPARATION AND PURIFICATION

In spite of the widespread applications of membrane technology, there still are some limitations and disadvantages associated with it. Although using membranes under optimal physical, chemical and operational conditions may enhance the selectivity, the product may not necessarily possess desired purity [119]. For instance, fouling is a major problem that limits the practical application of membranes. This occurs by aggregation of suspended molecules or precipitated biomolecules at the membrane surface or within its pores. Membrane fouling affects permeate flux and protein rejection and causes an increase in pumping expenses and energy consumption [96, 120]. A decline in the membrane performance due to its fouling and CP are common problems in the filtration processes. When solute molecules are rejected by the membrane, solute concentration near the membrane surface increases and aggregation of layers of foulants occurs [87, 121]. In addition to the reduction in flux, membrane fouling also alters the selectivity and reduces membrane efficiency. Membrane fouling caused by protein aggregates can be categorized into three different scenarios [22]:

(1) Creation of a gel layer because of the concentration polarization;

(2) Adsorption of proteins in membrane pores and on its surface;

(3) Denaturation of proteins and formation of protein aggregates that block the membrane pores.

Cleaning membranes using chemicals cause extra cost and shortens membrane lifetime. Costs for cleaning membrane fouling may comprise about 10-20 % of total costs for using a membrane in the first year. Hence, applying more efficient methods to solve this problem is required [97, 122]. Some of the major strategies and techniques for reducing the membrane fouling and improving their performance for protein separation and purification are being discussed in the following sections.

Back-flushing/back-pulsing

In back-flushing, the transmembrane pressure or filtrate flow is periodically reversed. Reversing the

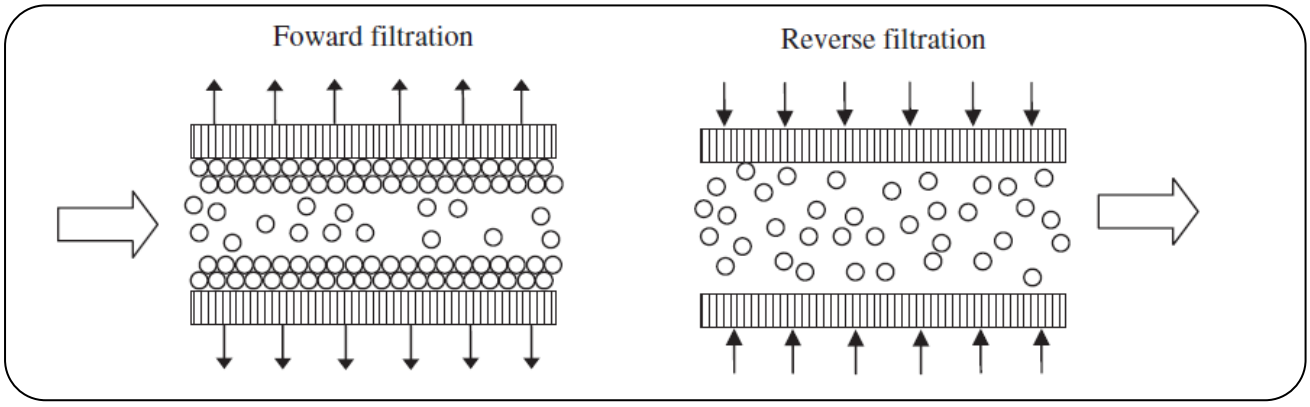

Fig. 5: Schematic representation of forward and reveres filtrations [123].

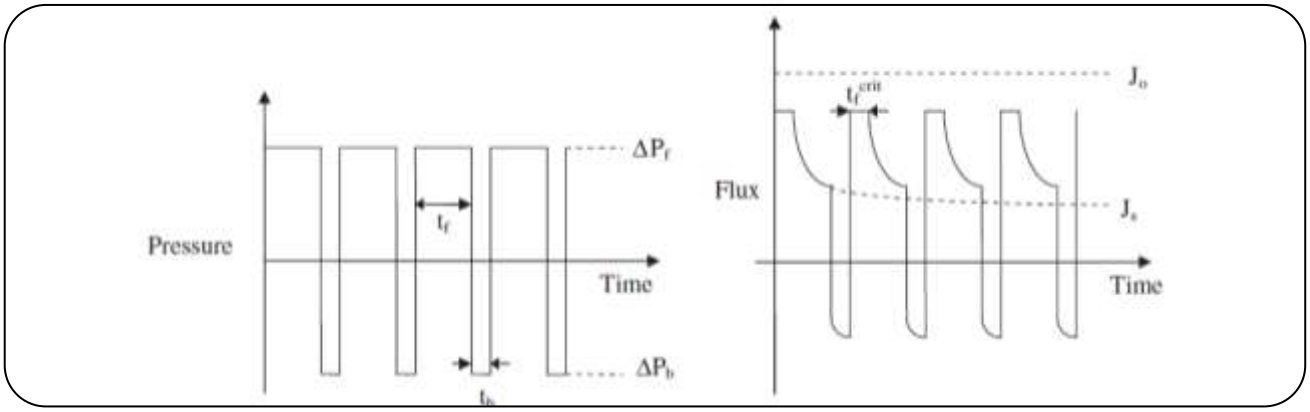

Fig. 6: The trends of pressure and flux within back-pulsing [123].

filtration direction leads to detachment of accumulated precipitates on the membrane surface (Fig. 5). Back-pulsing is an alternative technique that has been studied in recent years. The main concept of the two methods is similar. In back-pulsing, the duration of reverse flow is usually less than one second. Also, the back-pulsing method can prevent the membrane from fouling or at least release the foulants immediately after deposition [123] (Fig. 6). It is also claimed that back-pulsing cleans the membrane surface and causes flux reinforcement and reduction in membrane fouling [22].

Rodgers and *Sparks* in their study on binary protein mixtures found that flux of solute was significantly improved by using back-pulsing method for cleaning UF membrane pores [124]. The pulse frequencies of 0Hz, 0.5Hz, 2Hz and 5Hz were applied to membrane and results indicated the decrease in TMP and increase in permeate flux. *Wenten* investigated using back-flushing for 0.1 s at 5 s intervals for MF membranes [125]. Results demonstrated that during membrane performance, fluxes maintained high and protein transmission improved from 68% to 100%. Also, it has been reported that during MF of skim milk, the membrane began to foul after 2.4 hours at a cross-flow velocity of 5.5 m/s [126]. *Arkell* and *Jönsson* [17] investigated the performance of milk MF under back-pulsing. They found that both frequency and the duration of pulses contribute to effective utilization of back-pulsing. Skim milk MF was further examined with different back-pulse frequencies of 6 min^{-1}, 1 min^{-1} and 0.2 min^{-1} with durations of 0.2 s and 2 s. Permeate flux was constant at 350 L/m^2h in all the experiments. For this aim, TMP was increased to make up for the flux loss as fouling advanced. The operation time until TMP was increased to 0.8 bar was used to compare the performance of the processes. Results showed that the intermediate frequency (1 min^{-1}) along with the shorter duration (0.2 s) provided the longest durability. Probably, the lowest frequency was not strong enough to eliminate fouling, while the highest frequency pushed the foulants to the membrane due to the higher flux. Finally, it was observed

that under the optimum condition (1 min^{-1} and 0.2 s duration) runs for 9.7 hours at a cross flow velocity of 4.5 m/s. However, the virgin MF process lasted for 7.3 hours at a cross flow velocity of 5.8 m/s. This indicates that using back-pulsing, it is possible to decrease the required cross flow velocity and thereby save energy consumption [115]. However, once a higher flux of 500 Lm-2/h was tested, back-pulsing showed no positive impact on fouling condition. It was concluded that due to exponential increases of fouling rate after 550 Lm-2/h and the high value of flux itself, back-pulsing is inefficient for higher flux milk MF [116]

Gas Sparging

Gas sparging is one of the promising methods for increasing permeate flux by injecting gas in a liquid and forming a turbulence flow [127]. Gas sparging has proven to promote the transport of retained molecules to the bulk and reduce the CP layer by enhancing cross-flow hydrodynamics near the surface of the membrane [62]. This easy and inexpensive method has been widely used in industry for protein separation and fractionation [24]

Charoenphun and *Youravong* reported using gas sparging in a UF membrane for peptide separation and by using this method, the permeate flow enhanced up to 42% [128]. *Hashemi Shahraki et al.* investigated the effects of direct gas injecting on the filtration of skim milk using UF and found that permeate flow increased up to 40% and 72% while using CO_2 and N_2 as bubbling gas, respectively [129]. This technique has also been applied for hollow fiber, tubular and spiral wound modules for reducing CP [123]. Also, Fouladitajar et al [28] investigated the influence of liquid flow rate (1.5 and 2.4 L/min), gas flow rate (0.5, 0.8 and 1.0 L/min) and TMP (1 and 2 bar) on the efficiency of air sparging during MF of whey using flat sheet membranes. It was observed that at the higher liquid flow rate and TMP of 1 bar, the flow was turbulent. Therefore, gas sparging failed to induce any significant increase in the wall shear stress, which would, in turn, decrease the fouling. However, at TMP of 2 bar and the same liquid flow rate, gas sparging was more successful than the previous case and flux enhancement of 14% -17% was achieved by increasing gas flow rate from 0.5 to 1.0 $L.min^{-1}$. This was explained by the more severe deposition of cake layer due to the higher TMP. On the other hand, at the lower liquid flow rate, gas sparging produced much more promising results. Permeate flux improved by 20% and 26% for TMP of 1.0 and 2.0 bar, respectively, while the gas flow rate was held at 1.0 L/min. From their observations, they concluded that at higher gas flow rates (inducing slug flow regime rather than bubble flow), gas sparging would perform better at disrupting the fouling layer, thereby increasing the flux. However, in an investigation by *Lv et al.* [38] on UF of soybean whey coupled with air sparging, it was noticed that there was a limit to the intensity of gas sparging. They also found that filtration duration was maximal at 5.0 m^3/h (1200 min), but further increase in the intensity of air sparging deteriorated the filtration duration. Perhaps, following the increase in gas flow rate beyond the optimum point, the excess air penetrated through the membrane and intensified the flux decline. This easy and inexpensive method has also been utilized for enhancing protein separation and fractionation [17, 24, 130]. For instance, gas sparging helped to achieve more selective separation of binary protein mixtures, including HSA and IgG (three times increment), and BSA and lysozyme (six times increment) [62]. In fact, screening factors, in this case, were independent of filtration flux and cake porosity. Thorough information on the hydrodynamics of the phenomenon in membrane processes is discussed elsewhere [127].

Electrophoretic Membrane Contactor

Electrophoretic Membrane Contactor (EMC) is based on a microporous membrane which provides mass transfer between two fluid flows and the separation is achieved due to the difference in mass flow rates of different components [131]. There are two electrodes situated in each side of the stack which provide the electrical potential difference. When a potential difference is applied, charged constituents in the solution are transported from the feed to the other part through the membrane. Each component has special velocity due to its charge and this leads to obtaining two exit flows containing distinct composition [132]. This process is presented schematically in Fig. 7.

There are two different modes for electrodialysis: in the first mode, a single feed solution possessing target molecules that should be separated is fed into two sides of the porous membrane (separation mode). In the other mode which is used for negatively charged molecules,

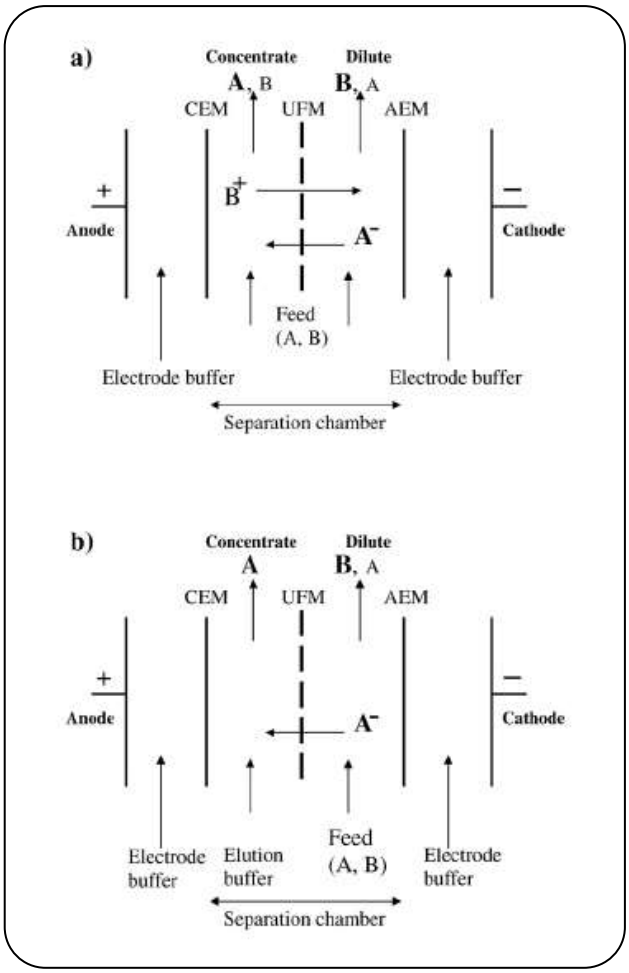

Fig. 7: a) Electrophoretic membrane contactor: separation mode; UFM: ultrafiltration membrane; AEM: anion-exchange membrane; CEM: cation exchange membrane. b) electrophoretic membrane contactor: elution mode [22].

the feed solution is directed to just one section and a buffer enters the other section (Elution mode). Elution mode is employed when high purification is required [22]. This separation method has been applied for the extraction of proteins from constituents like plasma, egg white and whey [133]. For instance, *Ogle et al. Ogle*, *Sheehan*, *Rumbel*, *Gibson* and *Rylatt* [134] used twelve channel electrophoretic membrane units including an anode, cathode and twelve thin (0.15 mm) PAA membranes and background electrolyte solution containing separated proteins recirculated through separation units. The applied electric field was perpendicular to flow paths of electrolyte solution in order to move sample constituents across the membranes. By using this effective apparatus, they successfully separated proteins of chicken egg white (ovalbumin, lysozyme, ovomucoid, and ovotransferrin) by their size.

Cascade Systems

Number of research studies have focused on developing novel membrane systems which are able to separate biomolecules with similar sizes that possess different physiochemical characteristics. One alternative is using several membranes linked optimally called membrane cascade systems [119]. In a membrane cascade system, permeate or retentate flow of one stage enters as a feed to the next stage. In addition, in this system, some streams should be recycled in order to increase the efficiency [135]. An internally staged UF cascade system was developed in which several flat membranes were compacted and placed in a device without using any artificial spacer or gasket in between [44]. Permeate flow exiting from the first membrane entered the second one, and similarly permeate from the second membrane fed into the third one and so on. Hence, protein rejection increased by adding each membrane and finally the species could be completely rejected. According to the results and Fig. 8, when three membranes were used, BSA was entirely rejected from the mixture and just hemoglobin remained in the permeate.

Patil et al. successfully used three-stage UF cascade operation for isolating whey proteins and examined different configurations and obtained a trade-off between purity and recovery [135]. They used three configurations as shown in Fig. 9. It was reported that configuration A (ideal-like cascade) reached high purity when operated at optimum pressure. Configuration B (adapted cascade) could be used when both high yield and recovery are required. Lastly, configuration C (counter-current cascade) suffered from low recovery due to the small flow rate came from previous stages. *Mayani et al.* incorporated cascade UF system for separation of lysozyme from egg white and single-stage, three-stage and four-stage cascade systems were examined [15]. The four-stage UF system consisted of two 50 kDa PS, one 3 kDa PS and one 3 kDa PES membrane obtained the highest lysozyme purity (97.5%) and highest recovery (71.5%), and fouling was negligible. The first membrane separated lysozyme from the bulk proteins and the second one used for lysozyme concentration and obtaining a lysozyme free permeate for the third membrane. The third

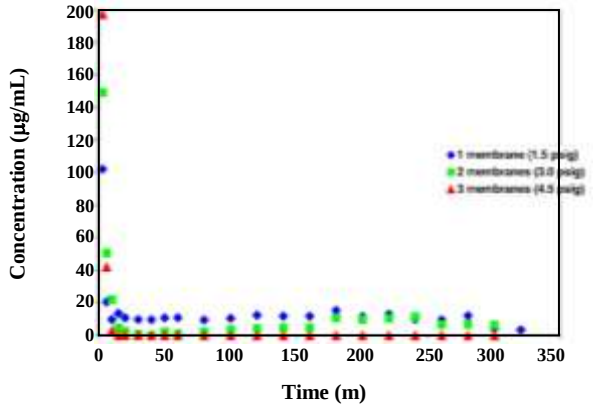

Fig. 8: Optimized ultrafiltration for separating BSA and bovine serum albumin at different pressures: by utilizing three stacked membranes the total rejection of serum albumin is obtained [44].

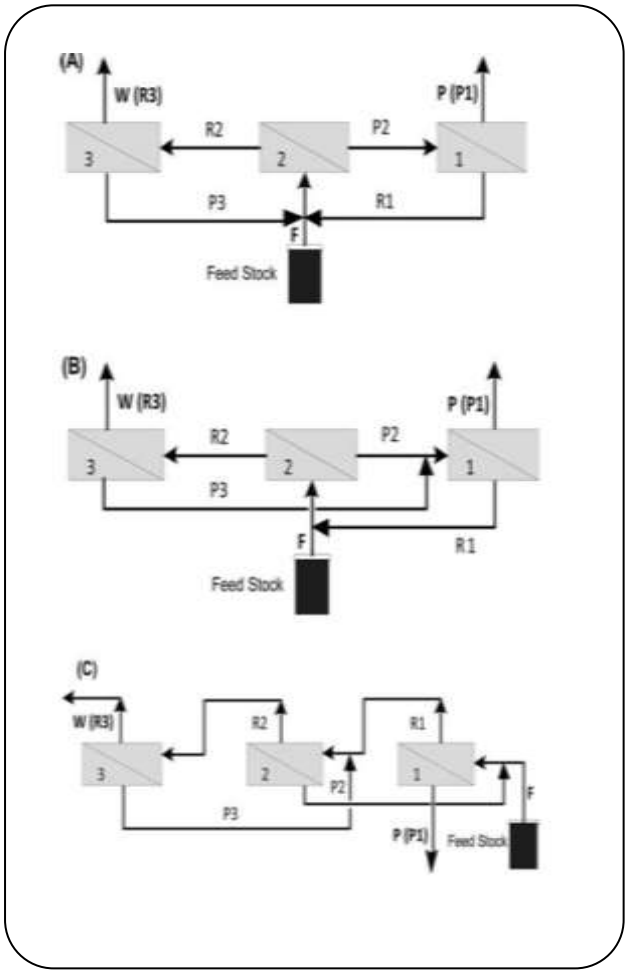

Fig. 9: Different configurations used for cascade ultrafiltration. A) ideal like configuration, B) adapted configuration and C) counter-current configuration [135].

membrane improved the lysozyme recovery. By adding the fourth membrane, more concentrated lysozyme stream was obtained.

Membrane Surface Modification

The surface properties of membranes are important factors that influence protein adsorption through hydrophobic interactions between protein molecules and membrane [136, 137]. The protein adsorption can be greatly reduced by membrane surface treatment using hydrophilic molecules. In addition, by using this method, cleaning of the membrane surface would be easier [138].

The most used methods for membrane surface modifications are attaching zwitterionic molecules [139, 140] and polyethylene glycol (PEG) [141]. Zwitterions which possess high resistance to protein adsorption are neutral molecules having the same number of positive and negative charged functional groups. Since these molecules possess high levels of hydration, they are impervious to bimolecular fouling [142, 143]. Zwitterions including phosphobetaine, sulfobetaine [144] and carboxybetaine [145] are popular for their antifouling properties [146]. Also, zwitterionic polymers containing sulfobetaine [147, 148], carboxybetaine [2] and acrylonitrile [149] have shown high fouling resistance [144]. Use of the PAN-based zwitterionic surfaces was first explored in order to decrease membrane fouling.*Sun et al.* utilized membranes made of sulfobetaine polymer and Poly(acrylonitrile) for ultrafiltration of BSA [149]. *Liu et al.* successfully applied a hybrid UF membrane consisting of poly(vinyl alcohol) and zwitterionic chitosan (ZICS) for separation of BSA and lysozyme [150]. It was reported that this zwitterionic UF membrane exhibited higher flux, fouling resistant properties and superb selectivity in comparison to the unmodified membrane. *Li et al.* reported fabricating a smart membrane which was capable of adjusting selectivity of the membrane via modification of its surface by PSB [64]. For this reason, PVP and (poly 3-(methacrylolylaino) propyl-dimethyl-(3-sulfopropyl) ammonium hydroxide) (PMPDSAH) (a type of PSB) were implanted inside and onto the hollow fiber MF membrane. Accordingly, a dense layer of PMPDSAH was formed on the membrane surface and subsequently the membrane was placed in electrolyte solutions to swell the PMPDSAH layer. It was observed that when

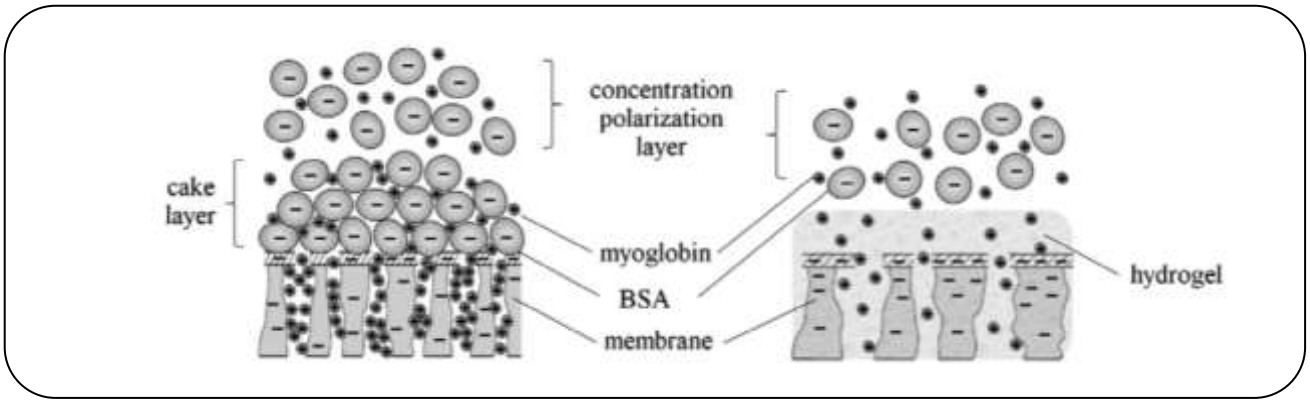

Fig. 10: Comparison between modified and unmodified membrane for separating BSA and myoglobin at pH=6. At this pH, myoglobin is positively charged and BSA and membrane have negative charge [87].

electrolyte concentration increased, PSB structure expanded and size of the pores reduced.

Modification of membrane surface by PEG is another alternative that has been widely studied [151, 152]. PEG chains are soluble in water and are mostly used for increasing hydrophilicity and biocompatibility of membrane surface for fouling resistance [153]. *Peeva et al.* grafted poly (ethylene glycol) methacrylate (PEGMA) on PES membrane and a composite membrane with a thin layer of hydrogel on top was obtained [87]. Fig. 10 shows the general rule of fouling reduction and interactions between solutes and membrane surface during filtration of a protein mixture by the modified and unmodified membranes. In the unmodified membrane, hydrophobic interactions cause the deposition of foulants on the membrane surface. Due to the low concentration of water near the hydrophobic surfaces, water molecules are replaced by solute particles. This causes solute-solute interactions and gradually a cake layer is formed on the membrane surface resulting in declined permeate flux. In the modified membrane, the hydrogel layer can act like water molecules and make the interactions between membrane surface and solute molecules weaker. It should be noted that if the mesh size of the hydrogel is equal to that of the solute particles, the solute particles could be locked in the hydrogel network and negatively affect flux and selectivity of the membrane. For this reason, the hydrogel network should be compatible with the solute to be used in UF.

Membrane separations coupled with an electrical field

Applying an electric field across the membrane is another method to minimize the CP and fouling of proteins. The electric field pushes the colloidal particles away from the membrane surface and leads to a decrease in CP. In this approach, two electrodes which are located in each side of membrane and parallel to it provide an electric field. This method is particularly favorable for the separation of proteins because, by changing pH, the charge at the protein surface is controllable. Also, t he existence of an electric field as the auxiliary driving force eliminates the need for high shear stress. Therefore, during separation, the flux caused by shear stress increases without destructing sensitive proteins. Improved permeate flux from 23.4 to 36.7 L/m^2h was detected during UF of a combination of BSA and Lysozyme in an aqueous solution in the presence of an appropriate electric field of 1000 V/m [22, 123, 154]. For example, by incorporating electro-UF for filtration of amylase solution, the permeate flux was improved by 3 to 7 times. An example of improved flux is shown in Fig. 11. In this diagram, the flux in terms of electric field strength is observable for different concentrations [123]. *Chen et al.* investigated a new approach for fractionation of lysozyme and BSA proteins [155]. They combined ElectroDialysis (ED) and Electro-UltraFiltration (EUF) in order to gain electric filed with higher strength and results indicated that this combined system has better performance than EUF by increasing the permeate flux by 20%.

High-Performance Tangential Flow Filtration (HPTFE)

High-Performance Tangential Flow Filtration (HPTFF) is a relatively new concept in the purification of proteins and peptides and takes into account both difference in size and charge of species to perform separation process and

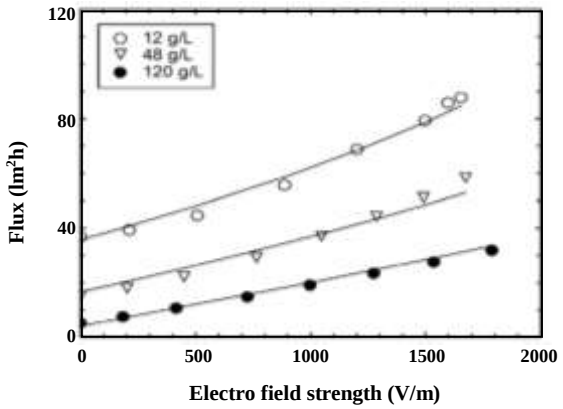

Fig. 11: Flux enhancement by applying an electric field for three different concentrations of solution. The solid lines represent experimental model (TMP=1.5 bar , v=0.07 m/s) [123].

is considered more beneficial in comparison to conventional membrane separations such as ultrafiltration. Several strategies are employed by HPTFF systems to achieve separation with high resolution including [123]:

(1) Making hydrodynamic volume of the target molecule or impurities maximum by selecting suitable pH and ionic strength

(2) Utilizing charged membranes to promote retention of proteins with identical charge

(3) Enhancing separation by using pressure dependent operations

(4) Applying diafiltration (a dilution filtration method) in order to remove impurities

In this method, by operating at the isoelectric point of one protein, that protein passes the membrane while other proteins are retained. Therefore, this technique is appropriate for separating proteins possessing the same molecular weight [22]. Many researchers have put effort to examine the probability of separation two proteins with similar sizes through HPTFF and have obtained successful results. Also, HPTFF has been effectively exploited for separating monomers from oligomers, monoclonal antibodies of mammalian cell proteins [156] as well as for separation of singly pegylated protein from highly pegylated molecules [157]. For example, HPTFF was used for final stage purification of mAbs (pI of 9.3) from Chinese Hamster Ovary proteins (additional proteins produced within fermentation by the host cell). By operation at pH of 5.3 and using positively charged membranes with large MWCO of 100-300 kDa, ultimately, sieving coefficients of 0.001 and 0.1 were observed for mbA and Chinese Hamster Ovary proteins, respectively. Therefore, positively charged mbAs were retained and Chinese Hamster Ovary proteins passed through the membrane [41]. *Rao et al.* [158] reported an alternative technique for controlling protein charge without manipulating solution pH. They used specifically binding ligands to manipulate charge of the desired protein. In their previous experiment, it was shown that binding dye Cibacron Blue to BSA molecule resulted in a transform in the net charge of the protein from +1 to -12 at fixed pH of 5 [159]. In consequence, BSA transmission was reduced 100 times once a negatively charged membrane was used. Inspired by this advancement, they investigated HPTFF of BSA and ovalbumin by addition of Cibacron Blue to the system. In the mixture solution, Cibacron Blue binds insignificantly to ovalbumin due to smaller equilibrium binding constant in comparison to BSA (110×10^3 for BSA and 1.3×10^3 for ovalbumin) [158].

CONCLUSIONS

Purified proteins are of great importance for various industrial applications. Despite huge advancements, conventional technologies for protein separation and purification still suffer from various drawbacks and limitations. The emergence of membrane processes has provided new promises for overcoming these limitations and improving efficiency, throughputs, and economics. However, despite the successful trials on the application of membranes for separation and purification of diverse proteins, complex structures and characteristics of proteins and interactions among themselves and with membrane surface have retarded the rapid progress of membrane developments and scale up for industrial applications. In this manuscript, several membranes processes including MF, UF, NF, and membrane chromatography were introduced and discussed in details in terms of potential applications for protein separation and purifications. Besides, several effective and practical techniques with their influences on the separation performance were reviewed. These methods have proven successful in laboratory experiments for decreasing membrane fouling, enhancing selectivity and often increasing product purity.

In overall, concerning membrane fouling, back-pulsing and back-flushing are industrial methods to decrease fouling in long terms and make cleaning

intervals longer. Gas sparging minimizes or removes CP layer via applying shear stress to membrane surface using gas slugs or bubbles. In surface modifications, antifouling properties of membrane surface are enhanced. However, providing robust bonds between the membrane and surface could be a challenge. EMF, prevents fouling by applying an external electric field to the membrane. Voltage and frequency are factors that affect lessening fouling and gaining effective flux. For improving selectivity, EMC can be used which incorporates both electrophoresis and membrane filtration. For high production rate and selectivity, process parameters such as electric field strength and MWCO of the membrane should be optimized. HPTFF provides us with high purity and selectivity by separating proteins based on size and charge and for the best results the flux regime should be pressure dependent. Cascade systems also offer high protein recovery and purity but they are used rarely. Since utilizing several stages with high-pressure reservoirs and pumps make these units hard to control. The mentioned methods operate under low protein concentration and low permeate flow, which is not compatible with the increasing demands in the industry. Thus, to satisfy industry needs, it is anticipated that future endeavors be dedicated to further enhancing these methods by coupling other units of operation with the membrane processes. In addition, probably integration of different methods of enhancing membranes into one system would result in enhanced membranes suitable for protein separation in the industrial scale.

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