Chapter 3

Transport Studies of Proteins and Amino Acids through Chitosan/ Polystyrene Sulfonate Multilayer Membrane

TRANSPORT STUDIES OF PROTEINS AND AMINO ACIDS THROUGH CHITOSAN/POLYSTYRENE SULFONATE MULTILAYER MEMBRANE

Abstract

This chapter presents the details of the fabrication of chitosan/polystyrene sulfonate (CHI)/(PSS) polyelectrolyte multilayer on polyether sulfone supports. Different multilayer systems were prepared by varying the parameters such as pH, ionic strength and molecular weight of polyelectrolyte solutions. The transport studies of bovine serum albumin (BSA), ovalbumin and lysozyme were conducted through these multilayer membranes by varying pH, ionic strength and concentration of the protein solutions. A dramatic change was noted in the transport behavior of the proteins through these multilayers depending on the number of deposited layers and the protein solution pH. The transport studies through the composite membranes shows 95% rejection of BSA by a 9 bilayer (bl) CHI/PSS membrane (permeate flux of 0.49 m^3/m^2 day) at 10 psi. Ovalbumin showed permeate enrichment and more than 100% of ovalbumin is permeated (permeate flux of 25.7 m^3/m^2 day) and 98% lysozyme is rejected (permeate flux of 11.7 m^3/m^2 day) at 10 psi by a 5 bilayer (bl) membrane at a protein solution pH 8.8. The individual transport studies of ovalbumin and lysozyme indicates that these egg white proteins could be separated using CHI/PSS multilayer system. The supporting membrane was permeable to all the three proteins. It is observed for the first time that a uniform coating of polyelectrolyte multilayer on a microfiltration membrane can change its sieving characteristics to the extent that it achieves the characteristics of an ultrafiltration membrane.

The influence of ionic strength of protein solution on protein transport through CHI/PSS multilayers have been studied by taking BSA, ovalbumin and lysozyme as model proteins. The percentage transmission and flux of BSA, ovalbumin and lysozyme were found to increase with increase in salt concentration in the protein. The percentage transmission of BSA through 9 bilayer membrane was found to increase from 5.3 to 115.6 when the salt concentration was varied from

0 to 1 M. It was observed that 0.1 M NaCl in BSA solution is capable of permeating all the BSA. When the salt concentration in BSA was further increased, a negative solute rejection (solute enrichment in permeate) was found to take place. With 9 bilayer membrane, the percentage transmission of ovalbumin was found to increase from 23.3 to 125.8 when the salt concentration in protein was increased from 0 to 0.05 M. The effect of protein concentration on protein transport is studied by taking BSA as a model protein. BSA was rejected by the multilayer membrane at all the studied concentrations (0.25, 0.5, 1 and 2 mg/ml). With increase in feed concentration, maximum rejection of protein occurred at higher number of CHI/PSS bilayers. BSA solution flux was found to decrease with an increase in BSA concentration. This study indicates that it is possible to fine tune the transport properties of proteins through multilayer membranes by varying the pH, concentration and ionic strength of protein solutions.

The transport studies of a number of selected amino acids were also carried out through CHI/PSS multilayer membrane under ultrafiltration condition. Multilayers were fabricated on polyether sulfone supporting membrane. The amino acids selected were glycine (neutral), histidine and lysine (both basic) and aspartic acid (acidic). The transport studies indicate that the percentage transmission of acidic and basic amino acids through the multilayer was strongly dependent upon the solution pH and the number of bilayers. A 7 bilayer CHI/PSS membrane was capable of rejecting 96.4% lysine and 93.6% aspartic acid at pH 5. The transport of glycine through the multilayer was not much affected by pH or number of bilayers. This study indicates that even nanofiltration properties can be imparted to microfiltration membranes by the deposition of a few CHI/PSS multilayers. The fact that small molecules like amino acids get rejected from the multilayer has significant implications especially in amino acid enrichment and separation with these multilayer systems. This study also suggests that in the transport of charged species through multilayer system, the charge factor outweighs the size factor.

Scientific background and motivation

Thin polymer films are of great scientific interest due to their potential industrial applications. Ultrathin separation layers of nanometer thickness can be fabricated on suitable substrates by layer-by-layer deposition of cationic and anionic polyelectrolytes from aqueous solutions by self assembly method.[1] Polyelectrolyte multilayers (PEM) can be made on a number of substrates such as glass, silicon wafers, alumina sheets etc. The polyelectrolyte multilayers find a large number of applications in ion permeation, pervaporation, integrated molecular optics, electronics, biosensors and water purification.[2-9] Large scale protein purification has become important in the field of biotechnology and ultrafiltration using membranes is one of the most commonly used processes for the fractionation of proteins and other macromolecules.[10-12] The driving force in ultrafiltration process is the pressure difference across the membrane. Ultrafiltration (UF) is quite often viewed as purely size-based separation with the larger components retained by the membrane while smaller species pass into the filtrate. Water and other small solutes are passed relatively unhindered through the membrane pores.

There are many reports on the fabrication and characterization of polyelectrolyte multilayers and permeation of ions and small molecules through these multilayers.[13-15] Surface modification of reverse osmosis membrane with a single layer of polyelectrolyte is reported by Tsuru and co-workers.[16] PEMs on polymeric support polyether sulfone is also reported by Malaisamy and Bruening.[17] They prepared high flux, highly selective nanofiltration membranes by the surface modification of polyether sulfone ultrafiltration membrane by layer-by-layer deposition of anionic and

cationic polyelectrolytes. However, the transport of proteins through multilayers under different conditions of pH and ionic strength is not well documented. Miller and Bruening reported the permeation of BSA (MW 67,000; 0.25 mg/ml) and myoglobin (MW 17,000; 0.125 mg/ml) through hyaluronic acid-chitosan (HA/CHI) multilayer under nanofiltration conditions with a pure water flux of 5 m^3/m^2 day at 4.8 bar.[13] They observed 97% retention of BSA and 15% retention of myoglobin with 8.5 bilayer HA/CHI membrane, indicating that the MWCO of this membrane is between 17,000 and 67,000. They noted that the solution flux through HA/CHI membranes has been decreased by 80% for myoglobin and 90% for BSA.

The aim of this work is to investigate whether multilayer build up is possible on polyether sulfone microfiltration membrane and if so how it changes the sieving characteristics of the microfiltration membrane. By forming a uniform thin multilayer skin on the microfiltration membrane, the membrane may show ultra filtration properties (UF membranes usually consist of a skin layer over a more porous structure). Due to the presence of charged multilayers as skins, the microfiltration membrane may acquire somewhat the characteristics of a charged UF membrane.

Ultrafiltration finds applications particularly in protein separations. Polyelectrolytes with low charge density are capable of forming less cross-linked multilayers, which in turn result in swollen membranes that are capable of separating larger molecules.[13] As CHI/PSS pair is less cross-linked and comparatively swollen,[18,19] it is investigated whether CHI/PSS multilayer is suitable for protein separations. Before carrying out actual protein separations, it is necessary to study the transport

properties of individual proteins through PEMs under different pH, ionic strength and concentration conditions. This is due to the fact that although, ultrafiltration is generally considered as size-based separation technique, there are significant experimental and theoretical evidences that protein retention is also determined by solution pH, ionic strength and membrane charge.[20,21] The net charge carried by the protein depends upon the pH. A change in solution pH can alter the electrical charge on both the protein as well as the membrane, which can cause either attractive or repulsive interactions. The solution pH can also alter the conformation of proteins, which can affect the protein diffusion coefficient.[20,21] Three proteins (BSA, ovalbumin and lysozyme) with varying size and isoelectric point (pI) were selected and their transport properties through the PEM membranes at the pI of these proteins and at pHs above and below the pI were studied. Since the protein conformation and charge are pH dependent, there should be a change in the transport characteristics of proteins through the multilayers with pH.

The thickness, porosity and charge density of PEM depends on a number of factors like nature of polyelectrolyte, dipping conditions, pH and ionic strength of the polyelectrolyte solution. By varying these factors, it is possible to fine tune the sieving characteristics of PEM. Protein transmission can also be influenced by adjusting the salt concentration in protein solution. There are number of reports on the influence of ionic strength and pH on protein transmission through charged ultrafiltration membranes.[22-28] Balakrishnan et al studied the effect of ionic strength on the flux and transmission of lysozyme and ovalbumin through hydrophilic polyacrylonitrile ultrafiltration membrane

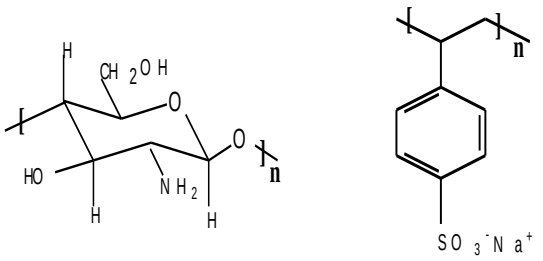
at pH 6.8 and they observed a dramatic change in protein transmission with ionic strength.[22] Increase in protein transmission with increase in ionic strength is observed on the surface modification of composite regenerated cellulose membranes by the attachment of quaternary amine functionality.[23] The sieving coefficient of BSA through a 100,000 MWCO polyether sulfone membrane is reported to be increased by nearly 2 orders of magnitude as the NaCl concentration was increased from 1.5 mM to 150 mM.[24] Similar results were obtained for the transmission of BSA and lysozyme through hydrophilic inorganic membranes.[25] In these cases, the increase in protein sieving coefficient with increasing ionic strength was consistent with the enhancement in electrostatic shielding of repulsive interaction at high salt concentration. The protein transmission through a hydrophilic membrane is governed by electrostatic phenomenon. Salt addition causes decrease of protein-membrane interaction, which may be quantitatively obtained from streaming potential data. It is reported that as the salt concentration in protein increases, streaming potential decreases to minimum value and no electrostatic repulsion or attraction is opposed to protein transfer across the membrane.[26]

The protein transmission can also be affected by concentration variations in protein solution. The effect of protein concentration on protein transport through charged ultrafiltration (UF) membranes have been discussed in literature.[22,29-32] Most of the works attribute the change in the percentage transmission of proteins with protein concentration due to concentration polarization effect.

Separation of amino acids is yet another area of interest. Amino acids are usually produced commercially by microbiological fermentation which generally yields a complex mixture of amino acids that need to be separated. There are some reports on the nanofiltration of amino acids.[33-38] However separation by UF is not yet reported. It is investigated whether amino acid separation is possible with CHI/PSS multilayer membrane under UF conditions which is rather a mild technique in the sense that the applied pressure is low compared to NF.

3.1 Film characterization

Chitosan-polystyrene sulfonate is selected as the polyelectrolyte pair. The structures of the polyelectrolytes are shown in fig.3.1. Chitosan is a weak polyelectrolyte and can serve as polycation in multilayer coating.[13] Polystyrene sulfonate is one of the most widely used polyanion in the preparation of multilayers.



Chitosan **Polystyrene sulfonate**

Figure 3.1: Polyelectrolytes used for coating

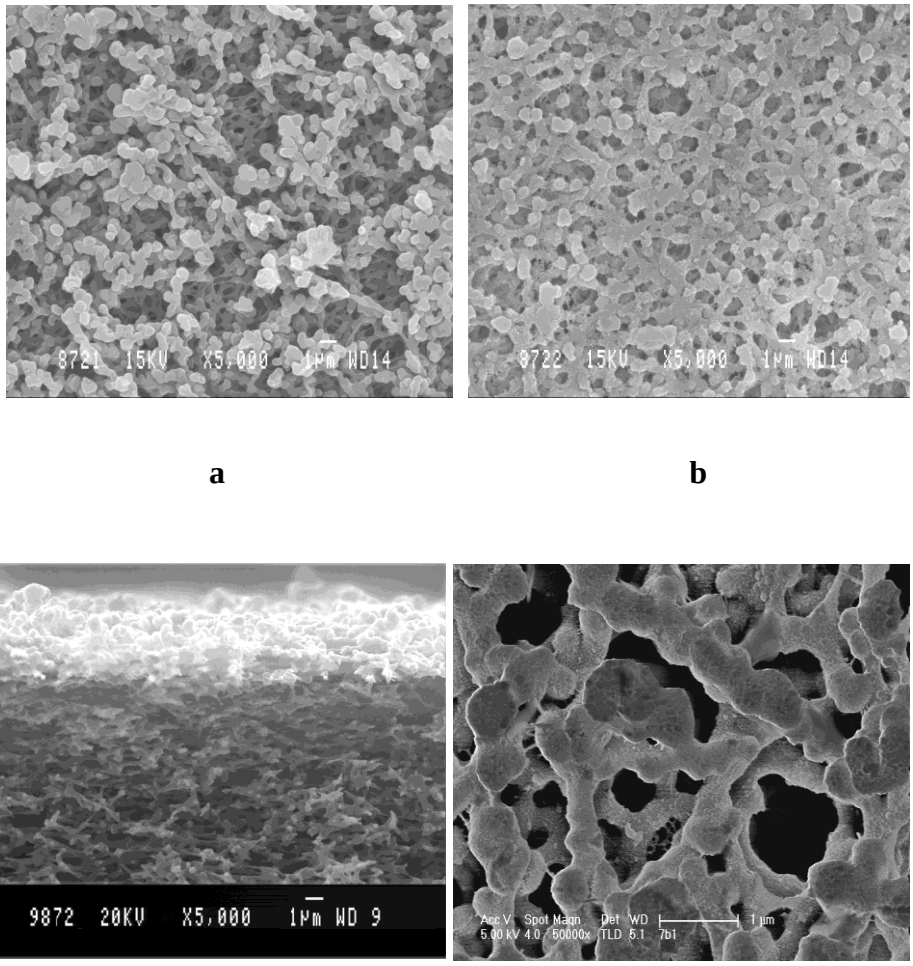


Figure 3.2: SEM images of supor and 7 bilayer CHI/PSS membranes **[a:** bare membrane, **b:** 7 bl CHI/PSS coated membrane, **c:** cross-section of 7 bl CHI/PSS membrane, **d:** 7 bl CHI/PSS (0.1 M NaCl) membrane**]**

The formation of multilayers on the surface of bare membrane has been examined using SEM. Fig.3.2a and b shows SEM images of supor and a 7 bilayer CHI/PSS (pH 1.7) on supor membrane, respectively. The pores are visible even after coating of 7 bilayers. The cross section (fig.3.2c) clearly shows the presence of ultrathin layer of polyelectrolytes

on the surface. The deposition of salt containing polyelectrolytes results in uniform coating as seen in fig.3.2d. The presence of polyelectrolyte multilayer is clearly seen in the image. The development of multilayer films on the supporting membrane is also clear from FT-IR. Fig.3.3 presents the characteristic infrared frequency region for the sulfonate group (1033.7 cm^{-1}) deposited at a pH of 1.7. The supor membrane has no peak in this region. The sulfonate peak height increases with the number of deposited layers as can be observed from the figure. Modification on the absorbance values was also observed in the regions 1400 to 1700 cm^{-1} which are characteristic of symmetric and antisymmetric-$NH3^{+}$ deformations.

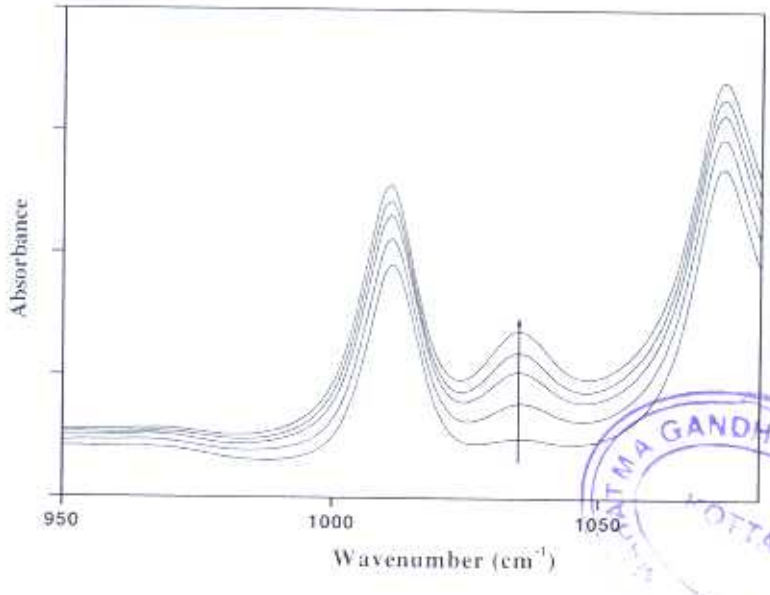


Figure 3.3: FT-IR of supor membrane and CHI/PSS 0,3,5,6,7 bilayers at pH 1.7 (sulfonate region, 1033cm^{-1})

The percentage transmission was calculated using the following relationship

% rejection = $[(C_f - C_p)/ C_f] \times 100$

Where C_f = concentration of feed

C_p = concentration of permeate

% transmission = 100 - % rejection

The protein flux was determined by measuring the time necessary to collect a known volume of permeate. The solution flux is given as the volume of the permeate/unit membrane area/unit time. The flux values were calculated in $m^3/(m^2$ day). The surface area of the membrane was 13.4×10^{-4} m^2.

3.2 Effect of coating pH

CHI/PSS multilayers on polyether sulfone support were prepared from chitosan and PSS solutions having pH 1.7 and 2.1. The effects of pH of the coating polyelectrolyte solution on the transport of proteins through CHI/PSS multilayers were investigated. The results obtained for the transport of BSA are summarized in Table 3.1. Transport of BSA across the membranes reduces with the increase in the number of bilayers. At the make up pH of 1.7, a 9 bilayer membrane rejects 94.7% BSA with a flux of 0.49 (m^3/m^2 day). But when the pH of the make up solution was 2.1, a 9 bilayer membrane rejected only 72.9% BSA. An 11 bilayer membrane was required for the rejection of 86% BSA. A better sieving efficiency is

clearly observed by the multilayers prepared at pH 1.7 even though flux is slightly lower (fig.3.4 and 3.5). A wide variation can be seen in the percentage transmission of BSA for multilayers prepared under the two pH conditions (fig.3.4). Rejections are higher for multilayers prepared at pH 1.7 for the number of bilayers selected indicating that multilayers are thicker at this pH. The transport and flux values at pH 2.12 gives a clear indication that multilayers formed are thinner and less selective. This indication is very well supported by IR data (fig.3.6). The area under the plot of the sulfonate peak at 1033 cm^{-1} is larger for the one at pH 1.7.

Table 3.1: Transport of BSA (0.5 mg/ml), pH 8.8, through CHI/PSS (0.1M NaCl) multilayers at pH 1.7 and pH 2.12

No. of bilayers	CHI/PSS multilayer at pH 1.7		CHI/PSS multilayer at pH 2.12	
	% transmission of BSA	Flux (m^3/m^2day)	% transmission of BSA	Flux (m^3/ m^2 day)
0	96.5	42.8	96.5	42.8
7	28.3	0.73	65.4	1.34
8	21.2	0.56	36.8	0.82
9	5.3	0.49	27.1	0.56
10	12.4	0.27	20.4	0.51
11	-	-	14	0.43

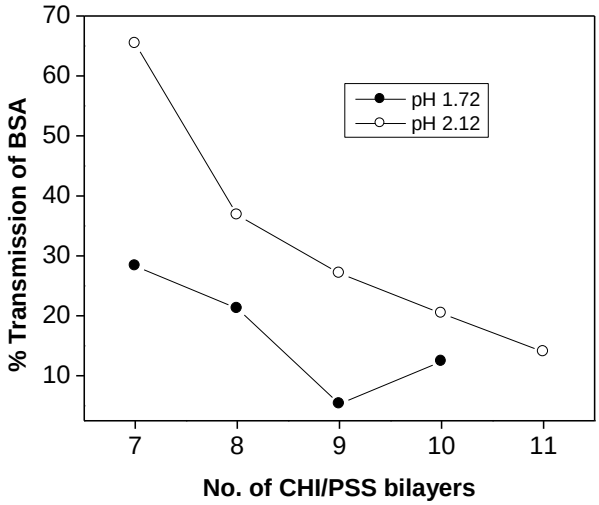


Figure 3.4: Percentage transmission of BSA as a function of number of CHI/PSS bilayers when the multilayers are fabricated at two different pHs 1.72 and 2.12.

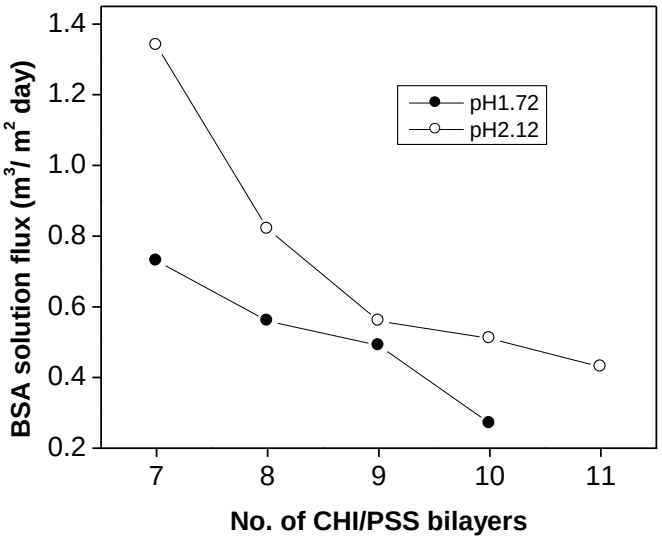


Figure 3.5: BSA solution flux as a function of number of CHI/PSS bilayers when the multilayers are fabricated at two different pHs 1.72 and 2.12.

As the thickness of the film is directly proportional to the amount of polyelectrolyte adsorbed, we can assume that the PEM formed at pH 1.7 may be slightly thicker compared to the PEM formed at pH 2.1. Tieke et al[14,15] observed similar influence of pH on the flux values. The authors noticed that in the case of PAH/PSS pair fabricated at the two selected pH, *viz* 2.1 and 2.4, the flux value was increased from 822 g m^{-2} h^{-1} to 3.88 kg m^{-2} h^{-1} while the permeation was dropped by 13%. The thickness and porosity of polyelectrolyte multilayers depends on a number of factors such as pH and charge density of the polyelectrolyte solution.[39-42] By varying the pH of the polyelectrolyte solution, the extent of ionization and hence the degree of multilayer cross-links can be varied. This can affect the permeability and flux of the multilayer membrane. PSS will be more ionized at pH 2.1. Cross-linking may be more and the resulting films are consequently thinner. The pH dependent variation of thickness of PEM coating was also reported by Shiratori et al.[39] They observed that the thickness of PAA/PAH multilayer vary from 5-80A^0 with small variation in pH of the polyelectrolyte solutions. In addition, control over the bulk and surface composition of the resultant multilayer thin films is readily achieved by simple pH adjustments.

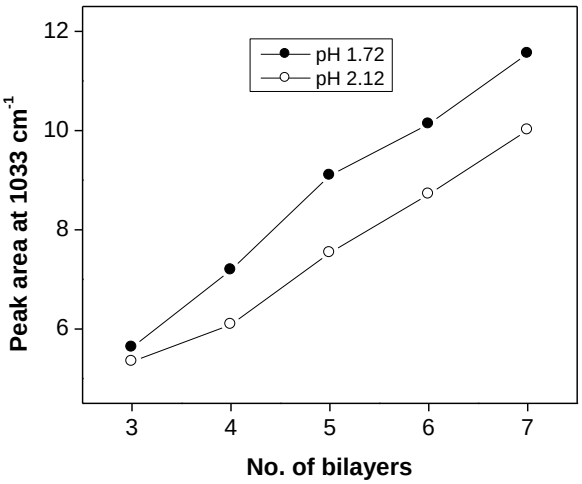


Figure 3.6: Area under the sulfonate peak (1033 cm^{-1}) in the FT-IR spectrum as a function of the number of deposited layers at two different pHs 1.72 and 2.12

Table 3.2: Transport of ovalbumin (0.5 mg/ml), pH 8.8, through CHI/PSS (0.1M NaCl) multilayers at pH 1.72 and pH 2.12

No. of bilayers	CHI/PSS multilayer at pH 1.72		CHI/PSS multilayer at pH 2.12	
	% transmission of ovalbumin	Flux (m^3/m^2day)	% transmission of ovalbumin	Flux (m^3/m^2day)
0	98.1	59.4	98.1	59.4
3	100.3	35.7	104.3	40.1
4	100.8	32.4	106.4	36.7
5	104.2	25.7	106.1	34.2
6	103.5	16.2	103	19.6
7	88.4	1.2	72.4	2.37

Table 3.3: Transport of lysozyme (0.25 mg/ml), pH 8.8, through CHI/PSS (0.1M NaCl) multilayers at pH 1.72 and pH 2.12

No. of bilayers	CHI/PSS multilayer at pH 1.72		CHI/PSS multilayer at pH 2.12	
	% transmission of lysozyme	Flux (m^3/m^2day)	% transmission of lysozyme	Flux (m^3/m^2day)
0	80.7	53.5	80.7	53.5
3	4.2	29.7	3.6	38.2
4	3.1	14.3	2.8	32.4
5	2.2	11.7	3.0	10.7
6	3.0	10.7	3.6	1.71
7	4.0	0.82	5.6	1.07

The transport studies of ovalbumin and lysozyme through the multilayer membranes, prepared under different deposition pH, have been carried out and the results are presented in table 3.2 and table 3.3 respectively. Ovalbumin is completely transported from 3-bilayer membranes onwards up to 7^{th} bilayer. A 5-bilayer membrane at pH 1.7 rejects 97.8% of lysozyme. However, compared to the effect of pH on BSA transport, the influence is less marked in the case of ovalbumin and lysozyme. In the case of these two proteins, slightly better sieving efficiency is observed for membranes coated at pH 1.7 even though flux values are slightly lower. Another striking thing is that going from 6^{th} to 7^{th} bilayer, there is a sharp decrease in the flux value for all the proteins. This may be due to the fact that the multilayer acquires optimum thickness with 7 bl. For further transport studies multilayers were fabricated from polyelectrolytes at pH 1.7.

3.3 The effect of salt addition to polyelectrolytes

The amount of polyelectrolyte adsorbed and the multilayer thickness can be controlled by the addition of salt to polyelectrolyte solutions. Salt addition is employed to adjust the thickness of PEM and the effect is marked for strong polyelectrolytes. In the present multilayer combination, chitosan is a weak polyelectrolyte. So salt is added to PSS solution only. There are a number of reports describing the effect of salt in polyelectrolytes on multilayer thickness. [43-47] Usually as a result of salt addition, the polyelectrolyte layers are coiled and the thickness of the PEM increases. Addition of salt to the polyelectrolyte solution strongly reduces the mutual electrostatic repulsion of the polymer chains, the polymer coils becoming denser and denser so that they are rather adsorbed as coil than in a flat conformation. As a consequence, the thickness of the individual adsorbed layers increases and the salt addition can be used to fine tune the overall membrane thickness.

CHI/PSS multilayers were prepared at pH 1.7 from PSS solutions in the presence and in the absence of salt. The effect of increased thickness on the transport behavior of BSA, lysozyme and ovalbumin under UF conditions were investigated at protein solution pH 8.8. The results obtained in the case of BSA are presented in Table 3.4. Presence of salt in the multilayer has a dramatic influence on the transport of BSA through the multilayer. A 9-bilayer membrane prepared from salt containing PSS rejected 94.7% of BSA with a flux of 0.49 (m^3/m^2 day). PEMs prepared in the presence of salt are thicker with high sieving efficiency even though the flux is slightly lower. When BSA transport studies were conducted through

multilayers fabricated in the absence of salt, 14 bilayers were required for 75.5% rejection of BSA with a flux of 0.63 (m^3/m^2 day).

Table 3.4: Permeation of BSA (0.5 mg/ml) at pH 8.8 through [CHI/PSS (without salt) at pH 1.7] and [CHI/PSS (0.1M NaCl) at pH 1.7]

No. of bilayers	CHI/PSS (0.1M NaCl) at pH 1.7		CHI/ PSS (without salt) at pH1.7	
	% transmission of BSA	Flux (m^3/m^2day)	% transmission of BSA	Flux (m^3/m^2day)
7	28.3	0.73	-	-
8	21.2	0.56	98.4	18.4
9	5.3	0.49	97.7	16.2
10	12.4	0.27	87	7.1
12	-	-	50.2	0.76
14	-	-	24.5	0.63

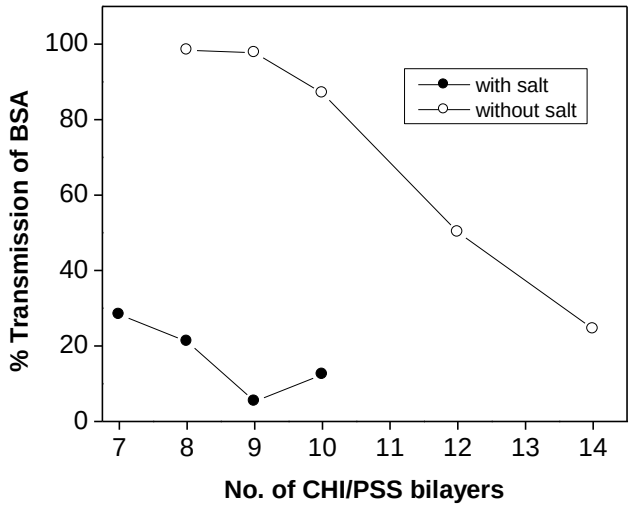


Figure 3.7: Percentage transmission of BSA as a function of number of CHI/PSS bilayers when the multilayers are fabricated in the presence and the absence of salt.

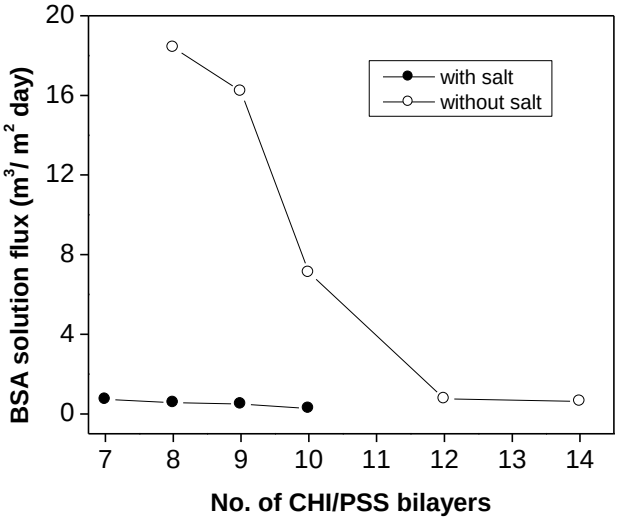


Figure 3.8: BSA solution flux as a function of number of CHI/PSS bilayers when the multilayers are fabricated in the presence and the absence of salt.

If we compare the percentage transmission of BSA for the selected number of bilayers with multilayers fabricated in the absence and in the presence of salt, a wide variation is observed (fig.3.7). Similar trend is observed for the flux values also (fig.3.8). This wide variation in permeability arises due to the presence of salt in the multilayer. So BSA transport studies through the multilayers clearly indicate that thicker multilayers are formed when the multilayer is fabricated in the presence of salt.

As in the case of pH, the addition of salt only slightly modifies the sieving efficiency of ovalbumin and lysozyme (table 3.5 and table 3.6). But there is considerable decrease in the flux values when the transport studies of the two proteins are carried out through multilayers prepared in

the presence of salt. As reported earlier with other multilayer systems, presence of salt in PSS can influence the thickness of CHI/PSS multilayers. The increase in thickness can affect the permeation characteristics of the multilayer. Early reports with ion permeations through multilayer also show that presence of salt in multilayer has a strong influence on the permeation.[13,14,48] It is observed that better sieving efficiency is achieved when multilayers are fabricated in the presence of salt even though flux values are slightly lower. Hence further transport studies of proteins were carried out with PEM deposited from PSS solution containing 0.1 M sodium chloride.

Table 3.5: Permeation of ovalbumin (0.5 mg/ml) at pH 8.8 through [CHI/PSS (without salt) at pH 1.72] and [CHI/PSS (0.1M NaCl) at pH 1.72]

No. of bilayers	CHI/PSS (0.1M NaCl) at pH1.72		CHI/ PSS(without salt) at pH1.72	
	% transmission of ovalbumin	Flux (m^3/ m^2 day)	% transmission of ovalbumin	Flux (m^3/ m^2 day)
3	100.3	35.7	102.9	35.6
4	100.8	32.4	102.5	29.7
5	104.2	25.7	103.2	21.4
6	103.5	16.2	-	-
7	88.4	1.2	108.9	17.8

Table 3.6: Permeation of lysozyme (0.25 mg/ml) at pH 8.8 through [CHI/PSS (without salt) at pH1.72] and [CHI/PSS (0.1M NaCl) at pH1.72]

No. of bilayers	CHI/PSS (0.1M NaCl) at pH1.72		CHI/ PSS(without salt) at pH1.72	
	% transmission of lysozyme	Flux (m^3/ m^2day)	% transmission of lysozyme	Flux (m^3/m^2 day)
3	4.2	29.7	4	32.4
4	3.1	14.3	3.1	29.7
5	2.2	11.7	4.2	26.8
6	3.0	10.7	-	-
7	4.0	0.82	3.4	4.3

The above transport studies with the three proteins through multilayers indicate that pH and ionic strength of polyelectrolytes for multilayer fabrication has a decisive role in the transmission of proteins. This effect is more marked in the case of BSA. These parameters (pH and ionic strength), can vary the thickness of the multilayer. So it can be inferred that BSA sieving through the multilayer is highly dependent on the thickness of the multilayer.

3.4 Effect of molecular weight of polyelectrolyte

Another parameter that may affect the efficiency of multilayer coating is the molecular weight of the polyelectrolytes. Early reports indicate that molecular weight of the polyelectrolyte has some influence, even though, not stronger as compared to pH and ionic strength.[48,49] We have prepared CHI/PSS multilayer assembly on polyether support by using two chitosan samples of different molecular weights (chitosan, medium MW and chitosan, high MW). Two sets of multilayer assemblies, *viz*, CHI (medium MW)/PSS

at pH 1.7 and CHI (high MW)/PSS at pH 1.7, with varying number of bilayers were prepared. The coating efficiency was followed from the FT- IR data. In both cases, the sulfonate peak at 1033 cm^{-1} was found to increase with increasing number of bilayers. It was observed that the coating was more effective with CHI (medium MW)/PSS multilayer assembly. On plotting the peak area at 1033 cm^{-1} in the FT-IR spectrum against the number of bilayers for multilayer system using two sets of chitosan, (fig. 3.9.), it can be seen that there is appreciable variation in the peak area. This study shows that the efficiency of coating is better for medium molecular weight chitosan. Therefore, for further multilayer fabrication, medium molecular weight chitosan was selected.

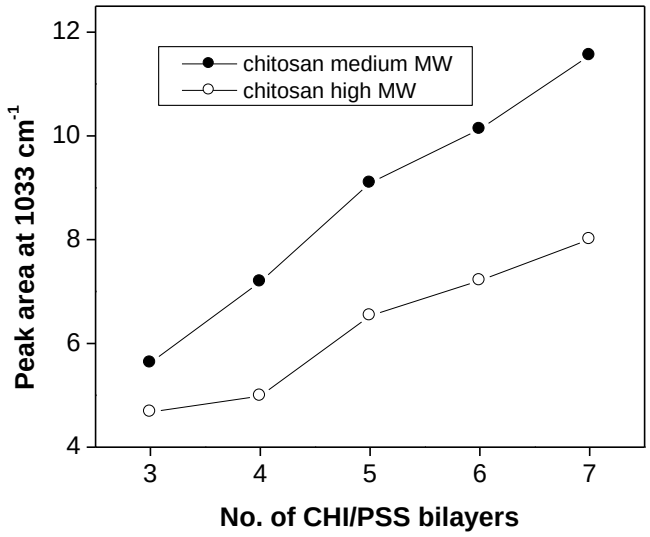


Figure 3.9: The peak area at 1033 cm^{-1} in the FT-IR spectrum as a function of the number of bilayers when multilayers are fabricated from CHI (medium MW) /PSS and CHI (high MW) /PSS

3.5 Effect of protein solution pH on protein transport

Ultrafiltration studies of different proteins using charged membranes demonstrated that the solution pH has marked effect on the permeation properties of proteins.[26,50-52] The change in solution pH can alter the electrical charge on both the protein and the membrane due to the ionization or deionization of various acidic/basic groups on the protein and the membrane surface which can cause either attractive or repulsive interactions depending upon the specific number and equilibrium constants of these charged ligands. According to Pujar and Zydney,[20] an increase in protein charge, increases the effective volume due to the presence of a diffuse ion cloud around the protein. Besides, the conformation of some proteins may be changed by the change in solution pH which can affect the protein diffusion coefficient. We selected three proteins (BSA, ovalbumin and lysozyme) having appreciable size differences and studied their transport properties through the multilayer membrane at different pH (at pI, above pI and below pI). The selected proteins have appreciable size differences as can be seen from table 3.7.

Table 3.7: Properties of proteins used in the study

Protein	MW	pI	stokes radius (nm)	molecular dimensions (nm)	ζ potential at pH 7 (mV)
BSA	67,000	4.8	3.55	-	-16
ovalbumin	44,000	4.5	3.05	7 x 4.5 x 5	-
lysozyme	14,400	10.8-11	2.0	4.5 x 3 x 3	+9

3.5.1 Transport of BSA through chitosan/polystyrene sulfonate multilayer membrane under different pH

The primary objective of this study was to analyze whether a multilayer coating on polymeric support could be used to separate proteins based on size. The transport studies of individual proteins having different molecular sizes were carried out. The pH dependent transport of proteins through composite membranes has been well documented, [21,26,50] but such transport across multilayers has not been reported so far. BSA was chosen as a model protein for the transport studies because it is a well-characterized and easily analyzable protein. The transport was carried out at three pH, 8.8 (above the pI), 4.8 (at pI), and 3.7 (below the pI). BSA was found to permeate almost completely through the bare membrane at pH 8.8, 4.8 and 3.7. BSA flux and percentage transmission are given in Table 3.8. The multilayer starts rejecting BSA from 7 bilayer (% transmission 28.3) and a 9 bilayer rejects 94.7% (% transmission 5.3) with a flux of 0.49 (m^3/m^2 day) at pH 8.8. The addition of each layer reduces flux, but from 7^{th} to 9^{th} bilayer the flux value remains almost similar. At pH > pI the expansion of protein takes place due to intramolecular electrostatic interactions and reduction in the extent of concentration polarization. The hydrodynamic volume is high at this pH since the protein is highly charged. We, thus, assume that this may be the reason that additional numbers of layers are required for rejection of BSA at this pH. At pH 4.8 (i.e., at the pI of protein), the rejection of BSA starts from 5^{th} and a 7^{th} bilayer rejects ~ 98% with a flux of 0.40 (m^3/m^2 day). The protein is neutral at this pH and the sieving is expected to outweigh electrostatic interaction. Sieving is expected to be high due to minimal hydrodynamic volume is at isoelectric point. The protein flux has reduced considerably with the filtration using

bare support itself indicating that unlike at pH 8.8, the protein adsorption takes place at the interface of supporting membrane thereby reducing the flux. This adsorption at the support-multilayer interface results in an early rejection of the protein. Transport studies below the isoelectric point (pH 3.7) also show a protein rejection of 22.5% from fifth bilayer. Here the protein and multilayer has opposite charge. It is observed that when chitosan is at the top layer, ie, for 7.5 bl, the transport of BSA has been considerably increased at pH 8.8 and pH 4.8.

Table 3.8: Transport of bovine serum albumin (0.5 mg/ml) through CHI/PSS (0.1M NaCl) multilayers at pH 8.8, 4.8 (pI) and pH 3.7

No. of Bilayers	BSA transport at pH 8.8		BSA transport at pH 4.8(pI)		BSA transport at pH 3.7	
	% Transmission	Flux (m^3/m^2 day)	% Transmission	Flux (m^3/m^2 day)	% Transmission	Flux (m^3/m^2 day)
0	96.5	42.8	93.6	21.4	94.9	16.2
5	89.2	16.2	48.2	14.1	77.5	11.1
7	28.3	0.73	2.1	0.40	29.7	0.67
7.5	99.7	2.14	40.5	0.23	20.3	0.14
8	21.2	0.56	2.7	0.38	1.25	0.36
9	5.3	0.49	18.9	0.27	13.8	0.28

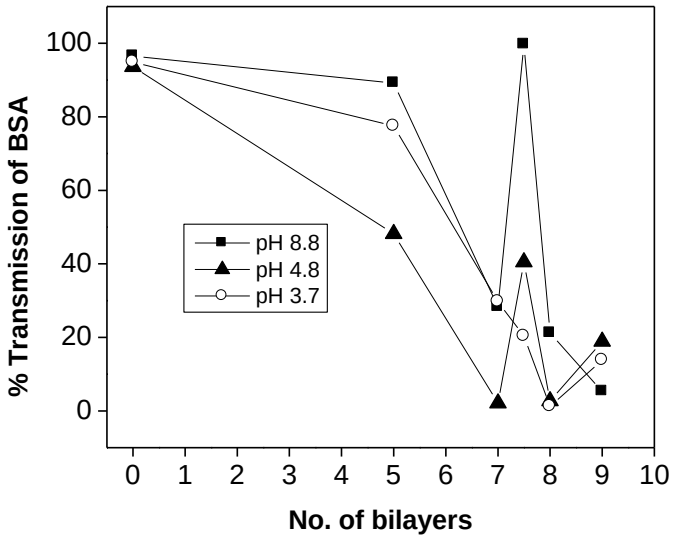


Figure 3.10: Percentage transmission of BSA as a function of the number of bilayers for BSA solutions of different pHs., 8.8, 4.8 and 3.7

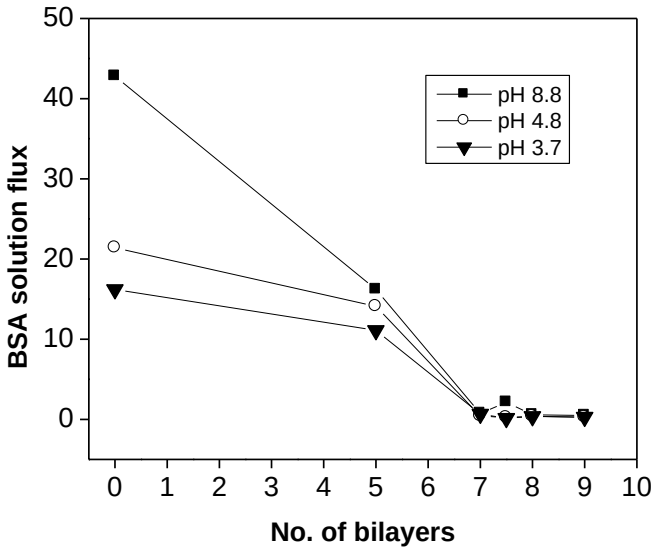


Figure 3.11: BSA solution flux as a function of the number of bilayers for BSA solutions of different pHs., 8.8, 4.8 and 3.7

These results show that even though the transport behavior or the percentage transmission varies with pH, BSA is retained at all pH. The multilayer can retain a protein as large as BSA (67 kDa). This rejection takes place within few multilayer pairs of CHI/PSS itself. These results clearly indicate that proteins can be sieved using deposition of polyelectrolyte multilayers on micro filtration membrane and only deposition of few bilayers are needed for sieving BSA. When we look at the pH dependent transport behavior of BSA, the immediate conclusion can be that the protein is rejected at all pH but at different number of multilayer. To understand the pH dependency of transport, several factors have to be taken into account. The most important are the charge on multilayer, especially the charge of the top layer, influence of inner layers on the top layer and the charge on the protein at the filtered pH. Along with this the variation of hydrodynamic volume with pH also has to be taken into consideration.

With top layer being PSS, the surface layer is always negatively charged. At pH 8.8 the protein carries negative charge and the membrane carries negative charge, and hence we can expect electrostatic repulsion. This condition reduces protein transport and rejection will be favored. We did not observe much variation in the transport characteristics till the multilayer build up was five for BSA transport at the studied pH range. This suggests that polyelectrolyte build up was not sufficient till this layer so that system could sieve a protein as large as BSA. Moreover the protein rejection is expected to be high at the pH where the protein and the multilayer carry same charge if electrostatic interaction alone was the criteria. But interestingly rejections at all pH with difference in the number

of multilayer have been observed. We, thus propose that sieving outweighs the electrostatic interactions and such a system could be used for the separation of proteins based on size. Transport of BSA through multilayers mainly depends on the number of deposited layers.

3.5.2 Transport of ovalbumin through chitosan/polystyrene sulfonate multilayer membrane under different pH

Since the multilayers could be used for the sieving of BSA, we further investigated the transport behavior of ovalbumin and lysozyme, (egg white proteins) through CHI/PSS multilayer. Ovalbumin (MW 45,000, pI 4.5) solutions (0.5 mg/ml) were transported through the membrane (supor/CHI/PSS) at pH 8.8 (in tris-HCl buffer) and 4.5 (in acetate buffer) at 10 psi (400 rpm). At pH 8.8, which is above the isoelectric point of ovalbumin, the protein is negatively charged. Ovalbumin has a low molecular mass compared to BSA and is expected to have higher transmission percentage than BSA. The results are presented in Table 3.9 and fig.3.12 and 3.13.

Interestingly we observed permeate enrichment (negative solute rejection) in the case of ovalbumin at pH 8.8 in the presence as well as in the absence of salt. The permeate enrichment can be explained by taking into account the preferential sorption capillary flow mechanism (involves surface) and modified concentration polarization theory (involves bulk).[32,53]

Table 3.9: Transport of ovalbumin (0.5 mg/ml) through CHI/PSS (0.1M NaCl) multilayers at, pH 8.8 and pH 4.5(pI)

No. of Bilayers	Ovalbumin transport at pH 8.8		Ovalbumin transport at pH 4.5(pI)	
	% Transmission	Flux (m^3/m^2 day	% Transmission	Flux (m^3/m^2 day)
0	98.1	59.4	91.4	82.3
3	100.3	35.7	35.8	42.8
4	100.8	32.4	37	38.2
4.5	102.9	30.9	86.4	21.4
5	104.2	25.7	38.7	28.3
6	103.5	16.2	45	25.9
6.5	104.2	22.9	73.6	12.6
7	88.4	1.2	57	1.34
9	23.3	0.68	-	-

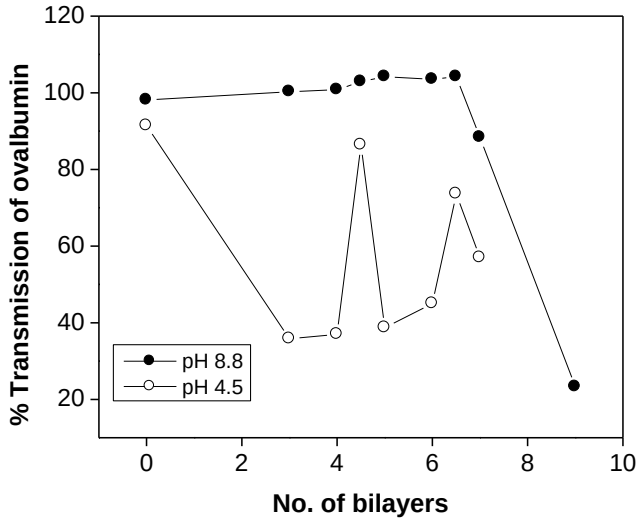


Figure 3.12: Percentage transmission of ovalbumin as a function of the number of bilayers for ovalbumin solutions of different pHs., 8.8 and 4.5

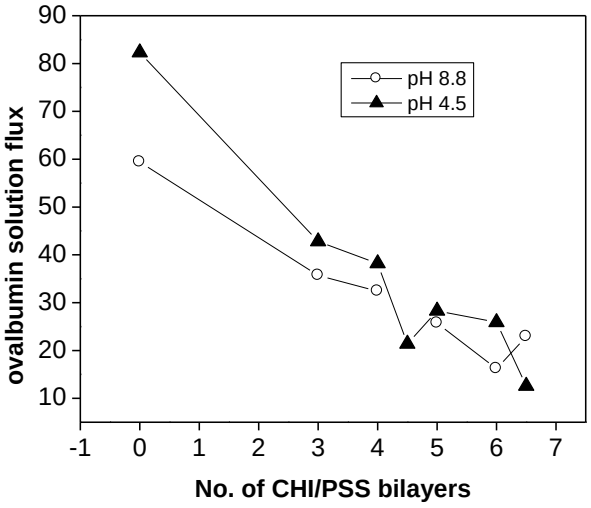


Figure 3.13: Ovalbumin solution flux as a function of the number of bilayers for ovalbumin solutions of different pHs, 8.8 and 4.5

Concentration polarization model can be used to discuss the protein transport through UF membrane.[32] Consider a partially retentive membrane which is in contact with a pressurized feed (fig.3.14).[32] The macromolecules in the feed are transported to the membrane surface by convective flow (JvC). Part of the solute is transported across the membrane and appears in permeate (JvCp) while the rejected solute causes a concentration build up Cm on the membrane surface. Because of the concentration gradient, there is a diffusion flux of the solute from the membrane surface back into the bulk solution (-D dC/dx).

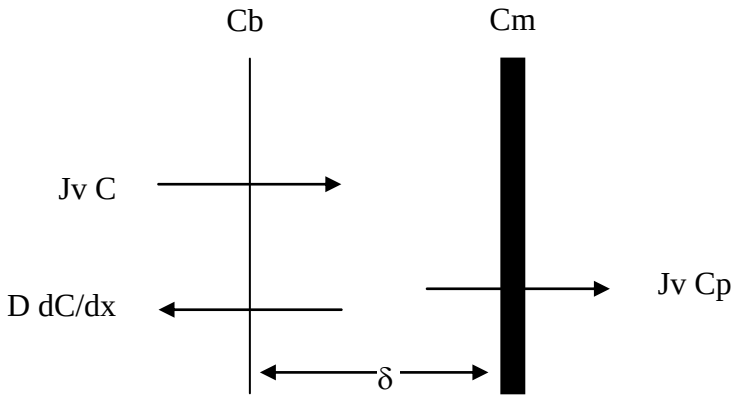


Figure 3.14: Mass balance of solute on the membrane boundary layer [32]

A mass balance on the boundary layer gives:

$$JvC - D\,dc/dx = JvCp \qquad (1)$$

Integrating over the boundary layer of thickness δ and rearranging,

$$\ln \tau_{obs} / 1-\tau_{obs} = Jv/k + \ln \tau / 1-\tau \qquad (2)$$

where $k = D/\delta$ is the mass transfer coefficient, τ: Cp/Cm is the true transmission, and τ_{obs}: Cp/Cb is the observed value of transmission.

To account for protein-membrane interaction, the concentration polarization model may be modified as follows.[32] Apart from protein transport to the membrane surface by convection, an additional transport term Ji is added to account for solute migration due to interaction between the charged protein and the charged membrane surface. The interaction is expected to be predominantly electrostatic in nature and Ji can therefore

be considered to be the electrophoretic transport of protein towards the membrane surface. This interaction continuously pulls the protein towards the membrane even though the feed is getting depleted in the protein. Ji can either be positive or negative depending upon whether the protein-membrane interaction is favorable or unfavorable.

In the present system, the protein is interacting with multilayers (having alternatively charged layers). Hence the interactions of protein with the bare membrane and the multilayers have to be taken into consideration. Modified concentration polarization theory takes into consideration the protein-(charged) membrane interactions too.[22] Protein is transported to the membrane surface by convection and in addition to this, solute migration takes place due to interaction between protein and the charged surface. Balakrishnan et al observed permeate enrichment in case of UF of lysozyme both during permeate recycle and unsteady state transmission.[32,22] The authors noticed negative solute rejection when the protein-membrane interactions were electrostatically favorable. In our system ovalbumin and the surface layers (PSS) are identically charged. If only the interaction of ovalbumin and PSS is taken into account, then the observation would have been reverse since likely charged species would repel and ovalbumin will be rejected. The high permeability here is certainly the result of the interaction of ovalbumin with the multilayers and till 7 bilayer the influence from the interpenetrating layers (the charge density of the top layer PSS is influenced by the inner layers) make these interaction favorable and we have a negative solute rejection. This also explains the unchanged transport behavior even when the top layer is changed to chitosan only at this pH. In this context, the electrophoretic

transport of the protein towards the membrane surface through the multilayers also must be considered in the concentration polarization theory. For 7th layer, the enrichment has slowed down and the transport has reduced to 88%. The flux also reduces considerably. When the number of bilayers was increased to 9, the multilayer rejects appreciable amount of ovalbumin. So it can be inferred that the favorable interaction between ovalbumin and the multilayer exists up to 7th bilayer.

Another plausible explanation for protein transmission greater than 100% is provided by adopting the preferential sorption-capillary flow mechanism put forward for reverse osmosis by Sourirajan.[32,53] Here it is assumed that apart from size separation by sieve filtration, protein UF is also governed by surface/interfacial phenomenon determined by the physical and chemical nature of the membrane as well as the protein. If the surface of a porous membrane has preferential sorption for one of the constituents of a fluid mixture, there is the possibility of a steep concentration gradient and hence the existence of a preferentially sorbed fluid layer at the membrane-feed interface. Consequently, the interfacial fluid layer is enriched in one of the constituents of the bulk solution. A continuous removal of this interfacial layer by flow under pressure through the membrane capillaries results in a product solution whose composition is different from that of the bulk feed solution. In this capillary flow mechanism, it is very important that the thickness of the sorbed layer and the capillary diameter bear a critical relationship. If the capillary diameter is much larger than the sorbed layer thickness one would not see the concentrating effect of the solute in permeate. And, of

course, the capillary diameter cannot be smaller than the solute diameter; otherwise all the solute is retained.

In the UF of ovalbumin solutions at pH 8.8, the protein was probably preferentially sorbed onto the membrane surface as opposed to water. A concentration gradient will come into existence at the membrane-feed interface. The concentration of the protein in the bulk will be different from the interface. With the application of pressure, part of the preferentially sorbed protein on the surface, along with the feed, was transmitted through the pores, thus resulting in a higher concentration of protein on the permeate side. At low pressure, the flux was low and the sorbed protein, therefore, contributed significantly to the total protein transported across the membrane. The membrane surface remains same though there is an appreciable change in bulk since the addition of each layer changes the bulk density. When the surface layer was terminated with chitosan instead of PSS, there was no change in the protein transmission behavior for ovalbumin at this pH (pH 8.8). But for BSA and lysozyme (Table 3.8. and 3.10. respectively) the percentage transmission showed a sudden increase with the change in surface layer.

This increase can only be seen at pH 4.5 (Table 3.9.) in the case of ovalbumin. In all these cases the transmission was high compared to the previous layer terminated with PSS. This indicates that irrespective of the top layer, at pH 8.8, ovalbumin had maximum transmission through the multilayers till the deposition of 7 bilayers. Ovalbumin starts getting rejected from multilayer from 7^{th} bilayer onwards.

A sharp fall in flux value was also noted on going from 6th to 7th bilayer. The transmission maximum away from the isoelectric point for proteins has also been discussed by Ricq et al in their studies of streaming potential and transmission in the ultrafiltration of β-lactoglobulin and lysozyme.[26] The observed transmission maximum was close to proteins steric transmission value, and not at its pI .

A change in the solution pH can affect the protein multilayer interactions.[54] At isoelectric point of protein, it is no longer preferentially adsorbed onto the surface. The protein transmission remains within ~35-57% with different number of deposited layers. The flux is quite high indicating less interaction between the protein and the multilayers till 7th bilayer. At the pI as the protein is neutral, sieving outweighs electrostatic interactions. And if the transport behavior of BSA and ovalbumin are compared at pI (Tables 3.8. and 3.9.), size based sieving is prominent.

3.5.3 Transport of lysozyme through chitosan/polystyrene sulfonate multilayer membrane under different pH

The transport of lysozyme (MW 14, 000, pI 10.8) solutions (0.25 mg/ml) through the multilayer membranes was investigated at three pH's (10.6, 8.8 and 4.5) at 10 psi (400 rpm). There is considerable adsorption of lysozyme by the supporting membrane especially at the isoelectric point. At 10.6 (near pI of lysozyme) the bare membrane rejects about 33% of the protein, then from 3rd bilayer onwards the protein gets rejected and the rejection is within 82-88% (Table 3.10). The value of the flux reduces with the addition of each

bilayer. At pH 8.8 the bare membrane rejects around 19% protein (% transmission 80.7) and more than 95% lysozyme is rejected from 3rd bilayer. More than 97% rejection is observed at 5^{th} bilayers (% transmission 2.2). The rejection of protein by the bare membrane is reduced to 11% at pH 4.5 and here we observed low rejection of lysozyme as can be seen from Table 3.10. The rejection is less than 50% with high flux values.

Table 3.10: Transport of lysozyme (0.25mg/ml) through CHI/PSS (0.1M NaCl) multilayers at pH 10.6, pH 8.8 and pH 4.5

No. of bilayers	Lysozyme transport at pH 10.6		Lysozyme transport at pH 8.8		Lysozyme transport at pH 4.5	
	% Transm	Flux (m^3/m^2 day)	% Transm	Flux (m^3/m^2 day)	% Transm	Flux (m^3/m^2 day)
0	66.7	66.8	80.7	53.5	89.9	59.4
3	18.7	32.4	4.2	29.7	47.1	53.5
4	12.8	30.2	3.1	14.3	30.8	42.8
5	9.1	5.3	2.2	11.7	46.3	36
6.5	-	-	15.5	12.9	89.9	18.8
7	12	2.7	4.0	0.82	72.6	2.92

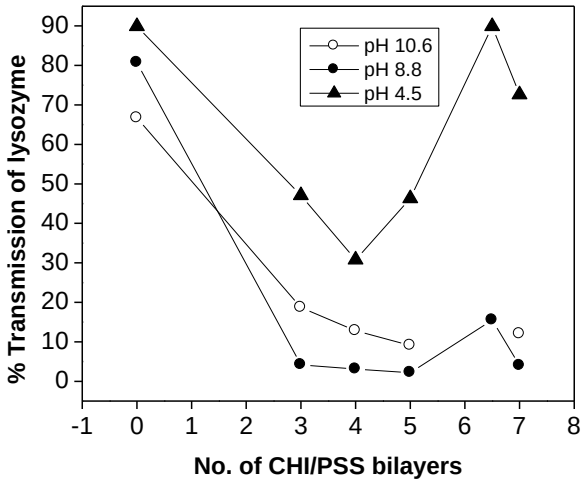


Figure 3.15: Percentage transmission of lysozyme as a function of the number of bilayers for lysozyme solutions of different pHs., 10.6, 8.8 and 4.5

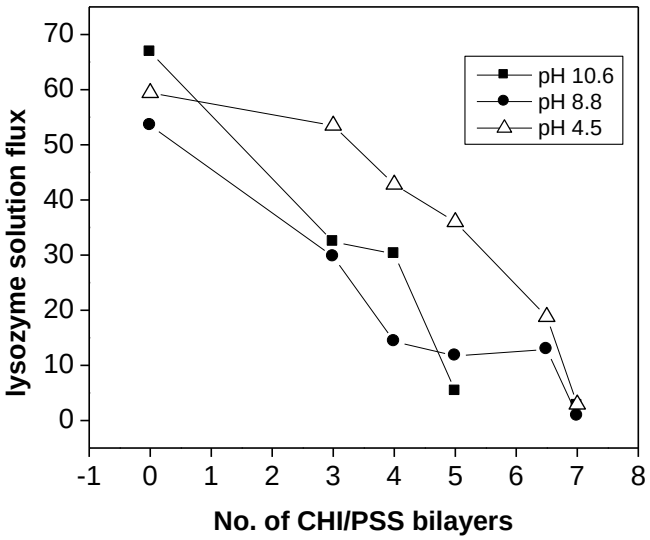


Figure 3.16: Lysozyme solution flux as a function of the number of bilayers for lysozyme solutions of different pHs., 10.6, 8.8 and 4.5

The molecular size of lysozyme is less than that of ovalbumin and we expect higher transmission of lysozyme through the membrane. But the bare membrane itself shows affinity towards lysozyme (Table 3.10.). Chitosan contains around 20% of chitin which has N-acetyl-D-glucosamine units in its structure which can bind lysozyme.[55] Moreover, lysozyme in the pH range 5 to 10, form dimers and aggregates which could yet be another reason for its exclusion.[56] Furthermore, there is a possibility of adsorption of positively charged lysozyme on negatively charged membrane. The experimental pH (i.e., 8.8) is below the pI of lysozyme (pI 10.8). At pH 8.8, lysozyme is positively charged and the membrane is negatively charged which would result in an electrostatic attraction of lysozyme by the membrane and this will facilitate its transport across the multilayers. However the pH is closer to pI and the positive charge on lysozyme is not that high to get attracted towards the membrane surface. Normally a cent percent transmission of the protein can be achieved when the pH is significantly below the pI. On the contrary, we observed 97.8% rejection of lysozyme. Thus, this behavior leads to a reversal of selectivity. This means that even though lysozyme has a low molecular mass than ovalbumin, its transfer across the membrane at this pH is very low. This makes the separation of lysozyme-ovalbumin at this particular condition very viable. The single protein transmission profile of BSA, ovalbumin and lysozyme at pH 8.8 (fig.3.17) shows that with 5 bl CHI/PSS system, lysozyme-ovalbumin as well as BSA-lysozyme separations can be attempted.

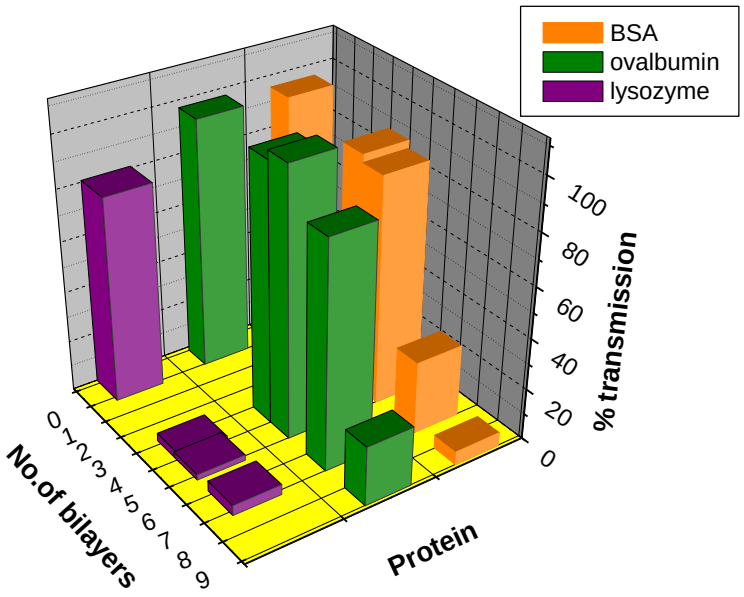


Figure 3.17: Single protein transmission profile of BSA, ovalbumin and lysozyme at pH 8.8.

3.6 Effect of salt concentration in protein solution on protein transport

The transport of BSA, lysozyme and ovalbumin through the composite multilayer membrane (supor/CHI/PSS) was studied by varying the concentration of salt in protein solutions. The pH dependent transport studies (section 3.5) show that at pH 8.8, 9 bl CHI/PSS multilayer system rejects 94.7% BSA. With 5 bl CHI/PSS system, lysozyme showed 97.8% rejections whereas ovalbumin showed solute enrichment in permeate (percentage transmission 104.2).[57] The pH of protein solutions was kept as 8.8 at which BSA and ovalbumin were negatively charged and lysozyme was positively charged. At this pH the preparation of ovalbumin free lysozyme seems to be

viable since ovalbumin permeated completely through the system. The ionic strength dependence on protein transport through multilayer was studied with 9 bl for BSA because in the absence of salt, BSA showed maximum rejection with 9 bl membrane. Ovalbumin transport through the multilayer was studied with 5 bl and 9 bl membranes. Transport studies were conducted through 9 bl membranes in order to have a comparison of the ionic strength dependence of BSA and ovalbumin transport through the multilayer membrane. The percentage transmission of BSA was found to increase from 5.3 to 115.6 when the salt concentration was increased from 0 to 1 M. With 5 bl membranes, ovalbumin transmission had been increased from 104.2% to 118.1% when the salt concentration in the protein was varied up to 0.05 M NaCl. On further increase in salt concentration in the protein from 0.1 M onwards, the percentage transmission remained more or less the same. For 9 bl membranes, the percentage transmission of ovalbumin was found to increase from 23.3 to 104.7 when the salt concentration was increased from 0 to 0.01 M. Here also, maximum permeate enrichment was observed for a salt concentration of 0.05 M as with 5 bl system. Lysozyme transmission was found to increase from 2.2% to 33.3% as the salt concentration in the protein was increased from 0 M to 0.25 M NaCl. Thus it is seen that ionic strength of protein has a decisive role in determining the transmission of proteins through multilayer membranes.

A number of complex phenomena are involved in the flow of charged species like proteins through a charge-carrying multilayer membrane. The fluid flowing through the membrane pores is influenced by the existence of electrical double layer inside the pores. Under ultrafiltration conditions, pressure- driven flow through the pores leads to the generation of

a streaming potential across the charged membrane which can cause a drag on the solution moving through the charged pores. The net effect is a diminished flow of liquid in the forward direction. This causes an apparent increase in the viscosity of the liquid and is referred to as electroviscous effect or counter electro-osmosis.[23,58] The permeability of a charged membrane is usually lower than the value expected based on the membrane pore size as a result of electroviscous effect. The magnitude of counter electro-osmotic flow depends on both the solution ionic strength and membrane charge. At high salt concentration, the permeability increases due to decrease in electroviscous effect. The surface potential, which is a measure of the membrane charge, decreases with increase in salt concentration as a result of compression of the double layer.

3.6.1 Transport of BSA through chitosan/polystyrene sulfonate multilayer membrane under different salt concentration

Ultrafiltrations were carried out with 9 bl CHI/PSS multilayer membrane using BSA solutions (0.5 mg/ml, pH 8.8) having different salt concentrations (0.01 M, 0.025 M, 0.05 M, 0.1 M, 0.25 M, 0.5 M, 1M NaCl). The investigations on pH dependent transport of BSA at three pH, 8.8 (above pI), 4.8 (at pI), and 3.7 (below pI) showed retention of BSA at all pH even though the percentage transmission varied with pH (section 3.5.1).

Table 3.11: Effect of ionic strength on the percentage transmission and flux of BSA (0.5 mg/ml, pH 8.8) and ovalbumin (0.5 mg/ml, pH 8.8) through 9 bl CHI/PSS membranes

NaCl concentration in protein (mg/ml)	% Transmission of BSA (pH 8.8) through 9bl membrane	BSA flux (m^3/m^2 day)	% Transmission of ovalbumin (pH 8.8) through 9 bl membrane	Ovalbumin flux (m^3/m^2 day)
0	5.3	0.49	23.3	0.68
0.01	30.5	0.76	104.7	0.82
0.025	53.2	1.18	120.5	1.30
0.05	73.6	1.33	125.8	2.49
0.1	103.5	1.78	113.3	6.06
0.25	106.3	2.14	117.9	6.71
0.5	108.8	3.06	119.3	7.08
1	115.6	5.35	118.9	10.1

Like pH, ionic strength is another factor that may influence the transport behavior of proteins through multilayers. Since BSA was rejected at all pH with difference in the number of multilayers we had proposed that sieving along with electrostatic interaction was the criteria for the protein separation. It would be interesting to investigate the factors that influence the transport profile of proteins through multilayers at different salt concentrations.

At pH 8.8, a 9 bilayer membrane permeated only 5.3% BSA with a flux of 0.49 (m^3/m^2 day). As the salt concentration in protein solution increases, BSA flux and percentage transmission were found to increase as

can be seen from Table 3.11.and fig.3.18. A concentration of 0.1 M NaCl in BSA is sufficient enough to permeate all the BSA. Further increase in salt concentration in the protein results in negative solute rejection (percentage transmission greater than 100). This may be due to solute enrichment in permeate. When the salt concentration was 1 M, the transmission approached 115%. The percentage transmission increases almost linearly up to 0.1 M, further addition only enhances the solute enrichment. In the present system there is again alternating layers of polyanion (PSS) and polycation (CHI) on porous substrate and had already observed permeate enrichment in case ovalbumin at pH 8.8 with high flux till 6^{th} bilayers (section 3.5.2). This was explained with the help of preferential sorption capillary flow mechanism and modified concentration polarization theory. Different models have been used for the discussion of protein transport through ultrafiltration membranes.[32] Here we have fixed number of bilayers and protein solution of increasing ionic strength have been filtered through the membrane. This means at each filtration the surrounding to which the multilayer have been exposed is different. The permeate enrichment can be explained with the help of modified concentration polarization theory. According to concentration polarization theory the macromolecules are transported to the membrane surface by convective flow. Part of the solute is permeated while partly it is rejected that causes a concentration build up on the membrane surface. This concentration builds up causes a back diffusion too. In the modified concentration polarization theory along with this, electrostatic interaction between the membranes and the protein can also be accounted. In the present system transport of charged solute is taking place through alternatively charged multilayers and electrostatic interaction between the two take places. The electrophoretic transport of

protein to the membrane surface is a function of pH and ionic strength. The observed transmission profile will depend on the kind of protein membrane interaction (favorable or unfavorable). If the interaction is favorable then the observed transmission would be higher.

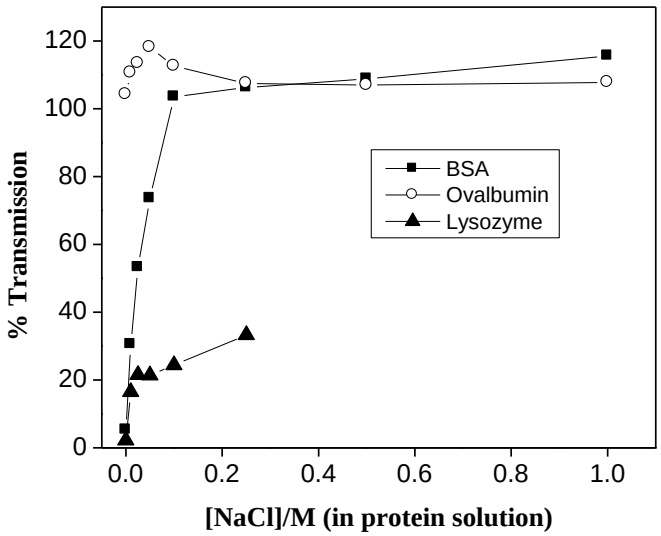


Figure 3.18: Effect of ionic strength on the percentage transmission of BSA (0.5 mg/ml, pH 8.8), ovalbumin (0.5 mg/ml, pH 8.8) and lysozyme (0.25 mg/ml, pH 8.8) through CHI/PSS multilayer membranes. BSA transport studies were carried out with 9 bl system; ovalbumin and lysozyme transport studies were carried out with 5 bl CHI/PSS system

In the beginning at zero ionic strength the electrostatic interaction is unfavorable since both the top layer and protein are negatively charged. Hence at low ionic strengths, the multilayer rejects BSA and as the ionic strength increases protein is transported across the system.

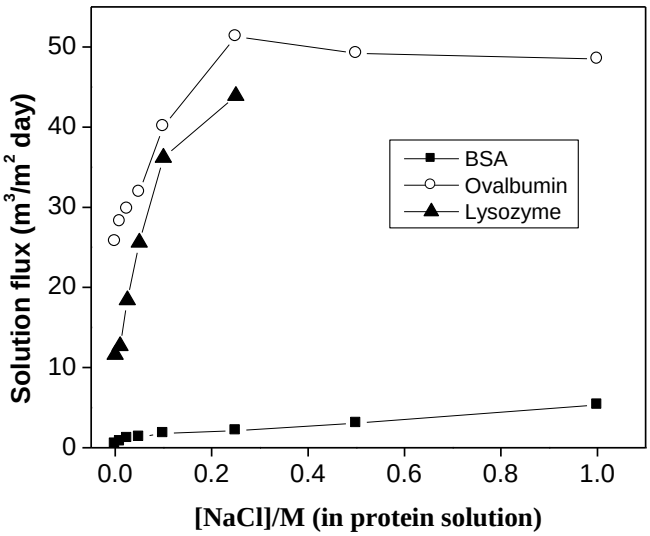


Figure 3.19: Effect of ionic strength on the solution flux of BSA (0.5 mg/ml, pH 8.8), ovalbumin (0.5 mg/ml, pH 8.8) and lysozyme (0.25 mg/ml, pH 8.8) through CHI/PSS multilayer membranes. BSA transport studies were done with 9 bl system; ovalbumin and lysozyme transport studies were done with 5 bl CHI/PSS system

The high rejection of BSA from the membrane surface in the absence of salt is due to the repulsion between the negatively charged BSA and the negatively charged surface. At pH 8.8, which is above the pI (4.8), BSA is negatively charged and it experiences coulombic repulsion from the negatively charged PSS outer layer. Consequently it exists in an enlarged conformation and gets rejected from the multilayer. In the presence of salt, this enlarged conformation gets changed to a more compact conformation due to ionic shielding.[24,27] As the salt concentration in the protein is increased, unfavorable protein-membrane interaction is decreased causing increased protein transmission. This is due to a decrease in the thickness of the electrical double layer (double layer compression) within the pore

associated with the increase in salt concentration leading to an effective shielding of the electrostatic interactions.[58,59] Furthermore, there is a possibility of conformational changes in charged multilayer surface on exposure to the protein solutions of varying salt concentrations. It is reported that when multilayers fabricated were kept in contact with solutions of varying ionic strengths, their porosity and thereby the permeability can be varied.[60-62] Multilayers are usually hydrated strongly even in the dry state and they swell when exposed to water.[63] The extent of swelling is strongly dependent on the ionic strength of the solution. Salt ions can penetrate into the multilayers where they compete with the polyion charges for binding sites. This result in the release of a fraction of polyion bonds which leads to a more flexible layer arrangement. In addition, the surface morphology of the multilayers can be varied on changing the ionic strength of the solution in contact. A decrease of surface roughness of multilayer is reported on increasing the concentration of salt solution.[64] The wide variation in BSA transmission (5.3% to 115.6%) with increase in salt concentration up to 1 M NaCl can be attributed to the combined effect of conformational change in protein and the multilayer surface. BSA solution flux is found to increase progressively with increase in salt concentration (Table 3.11 and fig.3.19). As the salt concentration increases, Debye length, which is a measure of the double layer thickness, decreases. Consequently, the effective pore size increases and it approaches the real pore size. This results an increase of solution flux.[59] Another factor for increasing flux with ionic strength is the conformational change in the multilayer. Increase in salt concentration leads to more flexible multilayers which may result in higher solution flux.

3.6.2 Transport of ovalbumin through chitosan/polystyrene sulfonate multilayer membrane under different salt concentration

Ultrafiltrations were carried out with 5 bl and 9 bl CHI/PSS multilayer membranes with ovalbumin solutions (0.5 mg/ml, pH 8.8) of different salt concentrations (0.01 M, 0.025 M, 0.05 M, 0.1 M, 0.25 M, 0.5 M, 1 M NaCl). Ovalbumin had no appreciable interaction with the polyether sulfone supporting membrane. [51]

Table 3.12: Effect of ionic strength on the percentage transmission and flux of ovalbumin (0.5 mg/ml, pH 8.8) and lysozyme (0.25 mg/ml, pH 8.8) through 5 bl CHI/PSS membranes.

NaCl concentration in protein (mg/ml)	% Transmission of ovalbumin (pH 8.8) through 5 bl membrane	Ovalbumin flux (m^3/m^2 day)	% Transmission of lysozyme (pH 8.8) through 5 bl membrane	Lysozyme flux (m^3/m^2 day)
0	104.2	25.72	2.2	11.6
0.01	110.6	28.2	16.5	12.7
0.025	113.4	29.8	21.5	18.4
0.05	118.1	31.9	21.4	25.6
0.1	112.6	40.1	24.4	36.2
0.25	107.5	51.3	33.3	43.9
0.5	107.0	49.2	-	-
1	107.8	48.5	-	-

Transport studies were performed with 5 bl membranes because our earlier studies showed that 5 bl multilayer membranes may be used to

produce ovalbumin free lysozyme. Since the actual separation requires the addition of salts to reduce the electrostatic attraction between ovalbumin and lysozyme it is beneficial to study the influence of salt on ovalbumin transport through the membrane. Ovalbumin showed negative solute rejection (permeate enrichment of solute) at all selected salt concentrations ranging from 0-1 M NaCl as can be seen from Table 3.12 and fig.3.18.

It is observed that the percentage transmission of ovalbumin increased gradually and reached a maximum of 118% for a salt concentration of 0.05 M. Further increase in salt concentration caused a decrease in the percentage transmission up to 0.25 M. The percentage transmission remained more or less the same beyond 0.25 M salt concentration. The ovalbumin flux has also been increased steadily with increase in salt concentrations up to 0.25 M, but it remained more or less same thereafter (fig.3.19). Even though ovalbumin was negatively charged (pI 4.5) at the experimental pH 8.8, it showed high permeation even in the absence of added salt in protein solution (section 3.5.2). Negative solute rejection can be explained by taking into account the preferential sorption capillary flow mechanism (this involves surface) and modified concentration polarization theory (this involves bulk).[22,32,53,57] The protein was preferentially adsorbed onto the membrane surface as opposed to water and a concentration gradient was operating at the membrane-feed interface. The high permeability is attributed to the interaction of ovalbumin with multilayers and the influence from the interpenetrating layers makes this interaction favourable. The presence of salt further favours the passage of ovalbumin. It seems that presence of salt has not much influence in ovalbumin transmission beyond 0.25 M.

This may be due to the fact that salt concentration beyond 0.25 M may cause reduction in the favourable membrane-protein interaction. Ovalbumin solution flux is found to be almost leveled off beyond a salt concentration of 0.25 M.

The effect of salt on ovalbumin transport was studied through 9 bl membranes in order to compare the influence of salt on BSA and ovalbumin transport through the same number of bilayers. The results are presented in Table 3.11 and fig.3.20. With 9 bl membrane, in the absence of salt, the percentage transmission of ovalbumin has reduced to 23.3%. With 5 bl system, ovalbumin showed negative solute rejection in the absence of salt and it was observed that the transmission value dropped to 88.4% with 7 bl system (section 3.5.2). It is observed that as the salt concentration in ovalbumin reaches 0.01 M, all ovalbumin permeated through the multilayer membrane. Thus salt has a marked influence on ovalbumin transport especially with 9 bl membrane. For BSA, 0.1 M NaCl was required for complete permeation through the membrane. The percentage transmission of ovalbumin increases from 23.3 to 125.8 as the salt concentration increases to 0.05 M. Thereafter the percentage transport of ovalbumin decreases and remains almost steady beyond 0.25 M. On comparing the solution flux of BSA and ovalbumin, there is appreciable difference as seen in fig.3.21. If we compare the effect of ionic strength of ovalbumin through 5 bl and 9 bl membranes, the transmission pattern is almost the same as can be seen in fig.3.22. But there is considerable difference in ovalbumin solution flux on going from 5^{th} to 9^{th} bilayer (fig.3.23). This is not unexpected because the multilayer thickness increases considerably when we go from 5^{th} to 9^{th} bilayer. In the case of

BSA, higher salt concentration was required for complete permeation. If the percentage transmission of the two proteins through 9 bl membranes in the absence of salt were compared, it can be seen that ovalbumin permeates more compared to BSA (fig.3.20).

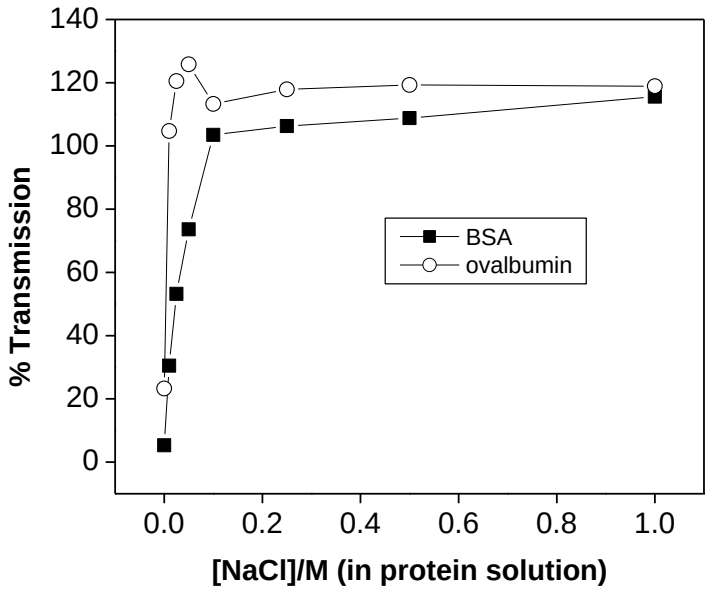


Figure 3.20: Percentage transmission Vs NaCl concentration for BSA and ovalbumin with 9 bl CHI/PSS membrane

The low transmission of BSA is consistent with the size difference between the two proteins. The transmission pattern of the two proteins is almost similar on varying salt concentration. The only difference is that the negative solute rejection shown by ovalbumin is larger compared to BSA.

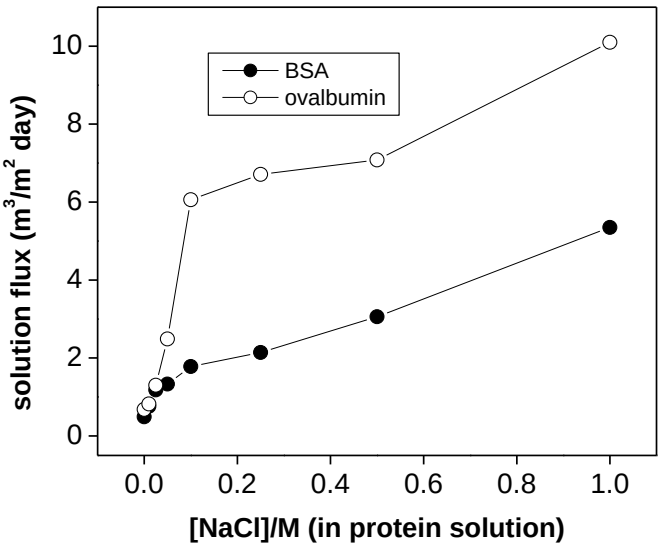


Figure 3.21: Solution flux Vs NaCl concentration for BSA and ovalbumin with 9 bl CHI/PSS membrane

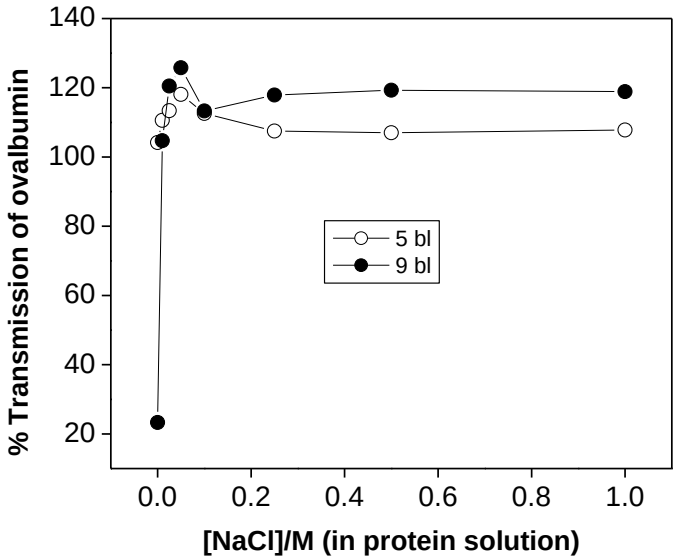


Fig.3.22: Comparison of percentage transmission and NaCl concentration for ovalbumin through 5 bl and 9 bl CHI/PSS membranes

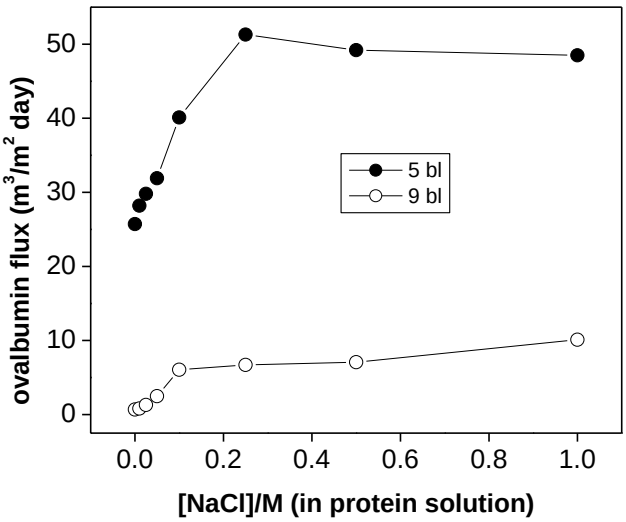


Figure 3.23: Comparison of flux and NaCl concentration for ovalbumin through 5 bl and 9 bl CHI/PSS membranes

A comparison of protein solution flux shows that ovalbumin flux is appreciably higher compared to BSA (fig.3.21). This may be due to the size difference between the two proteins. The permeation studies through 9 bl membranes show that higher salt concentration is required for complete permeation of BSA (0.1 M NaCl) compared to ovalbumin(0.01 M NaCl). This indicates that the major factor controlling the transport of proteins through the multilayer is the change in multilayer conformation with ionic strength.

3.6.3 Transport of lysozyme through chitosan/polystyrene sulfonate multilayer membrane under different salt concentration

Transport properties of lysozyme (0.25 mg/ml, pH 8.8) under UF conditions through 5 bl CHI/PSS multilayer was studied by varying the salt concentration in the protein. Lysozyme transmission is increased from 2.2% to 33.3% as the NaCl concentration in the protein is varied up to 0.25 M as seen in Table 3.12 and fig.3.18. Lysozyme solution flux also increases with increase in salt concentration (fig.3.19). From the earlier experiments it was observed that 5 bl CHI/PSS system is capable of rejecting 97.8% lysozyme whereas ovalbumin, even though larger in size compared to lysozyme, permeated completely at pH 8.8. This system is expected to be useful in ovalbumin-lysozyme separations. In order to make lysozyme-ovalbumin separation viable with this system, it is necessary to minimize the possible ionic interaction and complexation between negatively charged ovalbumin and positively charged lysozyme at this selected pH. (pI of lysozyme is 10.8 and pI of ovalbumin is 4.5). Usually this is achieved by the addition of salt. Lysozyme, which is positively charged at pH 8.8, may get adsorbed on to the negatively charged membrane surface resulting in low transmission in the absence of salt. The unexpectedly high rejection of lysozyme is due to affinity adsorption of lysozyme on PES supports.[57] Chitosan contains around 20% of chitin which has N-acetyl-D-glucosamine units in its structure which can bind lysozyme.[55] Moreover, lysozyme in the pH range 5 to 10, form dimers and aggregates which could yet be another reason for its exclusion. Furthermore, there is a possibility of adsorption of positively charged lysozyme on negatively charged membrane. The presence of salt

in the protein provided ionic shielding causing weakening of strong protein-membrane attractions. The fact that lysozyme transmission was not much influenced by the presence of salt indicates that in addition to electrostatic attraction, other factors are also involved in its binding to the membrane. So we can infer that lysozyme is strongly bound to the polyelectrolyte multilayer which is in agreement with early reports. [54] When the weight of the membranes was taken before and after filtration with lysozyme, a weight gain of 1.1 mg was observed for lysozyme filtered membrane. Furthermore, there is clear evidence of lysozyme adsorption in the FT-IR spectrum of lysozyme adsorbed membranes. When UF was performed with lysozyme solution in 0.5 M and 1 M NaCl, during filtration, turbidity appeared which may be either due to denaturation of the protein or partial dissolution of the polyelectrolyte from the multilayer.

The above studies clearly show that ionic strength of protein solution has a decisive role in protein transport through multilayer membranes. In the case of ovalbumin filtration through 9 bl multilayer, only 0.01 M NaCl in the protein solution is capable of overcoming the repulsive interaction between the protein and the multilayer whereas for BSA through the same system, 0.1 M NaCl was required. When the salt concentration in lysozyme solution is increased up to 0.25 M, only 33% lysozyme permeated through the membrane. Thus lysozyme is strongly bound to the multilayer. According to Ricq et al about 0.2 M salt concentration in lysozyme is enough to make the streaming potential zero.[26] So this study reveals that lysozyme-ovalbumin separation may be achieved with 5 bl CHI/PSS membrane at pH 8.8 by adding up to 0.2 M

NaCl solution to the protein mixture in order to overcome the possible complexation between the two proteins (at pH 8.8, lysozyme is slightly positive whereas ovalbumin carries a net negative charge). The strong binding of lysozyme and the absence of interaction of ovalbumin with the multilayer may facilitate lysozyme-ovalbumin separation.

The results with BSA and lysozyme indicate that the separation of large molecular weight protein and small molecular weight protein is also possible with this multilayer system. Because of the strong affinity with the multilayer, lysozyme, though smaller compared to BSA, gets rejected by the multilayer whereas BSA permeates almost completely in 0.1 M NaCl solution. Even though BSA-lysozyme separation is not having any practical application, it still opens the possibility of exploring the various forces operating in protein multilayer interactions. The transport behaviour of proteins through multilayer membranes is consistent with that of proteins through charged UF membranes. This supports the fact that a uniform multilayer coating on microfiltration membrane imparts it the characteristics of a charged UF membrane.[57]

3.7 Effect of protein concentration on protein transport

In order to study the effect of protein concentration on the transport of proteins through multilayer membranes, BSA was selected as the model protein. The transport studies of BSA of varying concentrations, (0.25, 0.5, 1 and 2 mg/ml) were carried out through the multilayer membranes and the results are given in Table 3.13. The multilayer could sieve BSA at all the studied concentrations. As the protein concentration was increased, more number of bilayers was required for the rejection of

BSA from the multilayer. For the transport studies of BSA (0.25 mg/ml) solution through 6-10 bilayers of CHI/PSS membrane, the highest rejection (% transmission 14.1) were obtained with 9 bl membrane with a solution flux of 1.13 m^3/m^2 day. The transport studies with BSA (0.5 mg/ml) through 5-10 bilayers of CHI/ PSS membrane showed highest rejection (% transmission 5.3) with a 9 bilayer membrane with a solution flux of 0.49 m^3/m^2 day. Where as transport of BSA (1 mg/ml) through 6-11 bilayers of CHI/PSS membranes, the highest rejection (% transmission 6) of BSA resulted with a 10 bilayer membrane having a solution flux of 0.41 m^3/m^2 day. Further increase in concentration of BSA to 2 mg/ml shifted the highest rejection to (% transmission 5.7) 12^{th} bilayer with a solution flux of 0.36 m^3/m^2 day.

Table 3.13: Transport of BSA solutions (pH 8.8) of varying concentrations (0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml) through CHI/PSS multilayer membranes.

No. of bilayers	BSA (0.25mg/ml)		BSA (0.5mg/ml)		BSA (1mg/ml)		BSA (2mg/ml)	
	% Transm.	Flux	% Transm.	Flux	% Transm.	Flux	% Transm.	Flux
5			89.2	16.2				
6	82.5	5.35			98	14.2		
7	30	1.34	28.3	0.73	94.5	7.13		
8	24.4	1.19	21.2	0.56	18	1.34	61.4	1.78
9	14.1	1.13	5.3	0.49	8.4	0.60	16.3	0.63
10	32.7	1.07	12.4	0.27	6.0	0.41	11.1	0.35
11					6.8	0.46	9.2	0.40
12							5.7	0.36

Percentage transmission of BSA decreases from 14.1 to 5.3% with an increase in concentration to 0.5 mg/ml. On further increase in concentration, the percentage transmission remains more or less same even though more number of bilayers was required for highest rejection. When we closely examine the percentage transmission and flux values of BSA at two different protein concentrations (0.25 mg/ml, 0.5 mg/ml), we can see that there is a sharp decrease in transmission and flux value from the 7^{th} bilayer onwards. This sharp fall in percentage transmission and flux values were shifted to 8^{th} and 9^{th} bilayers for next two concentrations, 1 mg/ml and 2 mg/ml respectively. Thus it seems that as the protein concentration increases, more bilayers are necessary to hold the charged protein. The hydrodynamic volume at this pH is high since the protein is highly charged. Thus electrostatic repulsion between the negatively charged protein and negatively charged multilayer surface is mainly responsible for the high rejection of BSA from the membrane surface at the experimental pH 8.8.

When we take a particular multilayer, say, 8 bl, 9 bl, or 10 bl (Table 3.13 and fig.3.24) and compare the percentage transmission values, it is seen that the percentage transmission decreases as BSA concentration increases up to 1 mg/ml and then increases sharply at a concentration of 2 mg/ml especially for 8^{th} bl. The BSA solution flux value also exhibits a similar trend as seen in fig.3.25. There is a decrease in transmission profile up to a feed concentration of 1 mg/ml, followed by an increase in transmission at highest concentration of protein under investigation. Here the pHs of the protein solutions are maintained above pI so that self aggregation would be low so as to decrease the transmission. Then another

possible reason could be concentration polarization. Concentration polarization refers to an increase in concentration of rejected species with decreasing distance from the membrane. The rejected species form a 'gel layer' which consequently covers the surface of the membrane and offers hydraulic resistance to solvent flux which may alter the net sieving properties of the membrane. As the bulk concentration increases, thickness of the gel layer also increases, thereby lowering the flux according to the equation,

$$J = k \times \ln (Cg/ Cb)$$

Where k is the mass transfer coefficient which is a measure of the efficiency with which the rejected solute is transferred from the membrane surface back into the bulk fluid.[29] A decrease in transmission with increase in feed concentration was also reported earlier in UF studies of lysozyme and ovalbumin of varying concentrations through hydrophilic polyacrylonitrile membranes.[22] Another reason for the reduced transmission is the increased viscosity of protein solution with increase in feed concentration thereby reducing the diffusivity. Only the feed concentration is varied and the number of multilayer is fixed. And hence the solution being filtered has more number of solute molecules which cause an increased solute-solute interaction, thereby decreasing the protein multilayer interaction again resulting in low transmission. The protein-multilayer interaction (interaction between the solute and the alternatively charged polyelectrolyte layers) up to a concentration of 1 mg/ml seems to be an unfavorable one to cause a decrease in transmission.

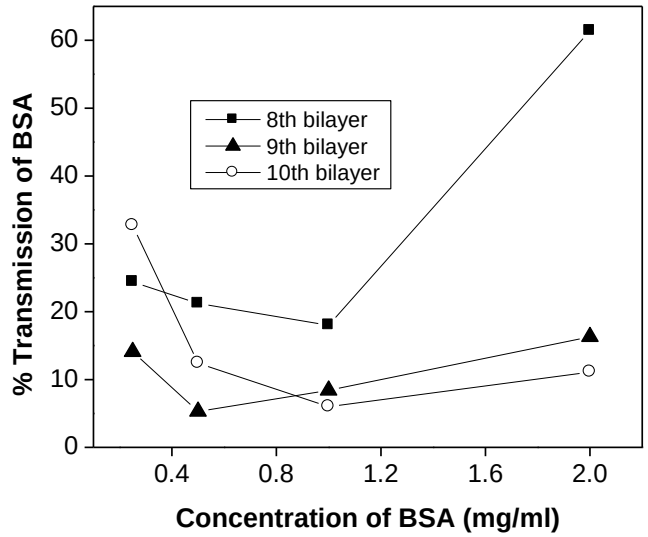


Figure 3.24: Percentage transmission of BSA Vs Concentration of BSA (CHI/PSS 8^{th}, 9^{th}, 10^{th} bilayer)

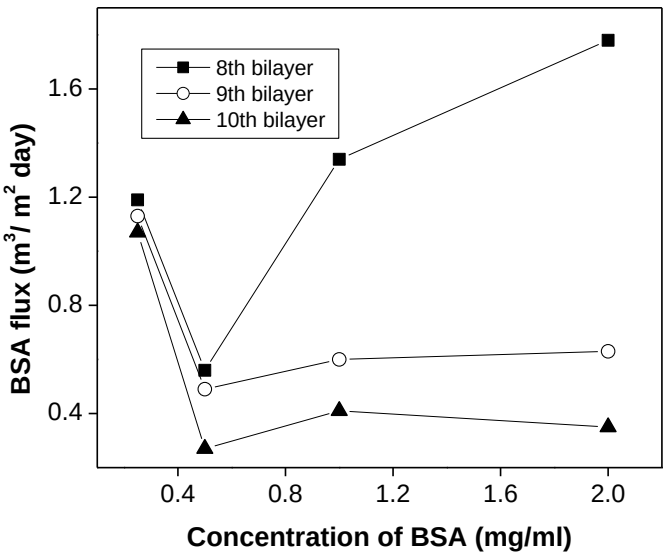


Figure 3.25: BSA flux Vs Concentration of BSA (CHI/PSS 8^{th}, 9^{th}, 10^{th} bilayer)

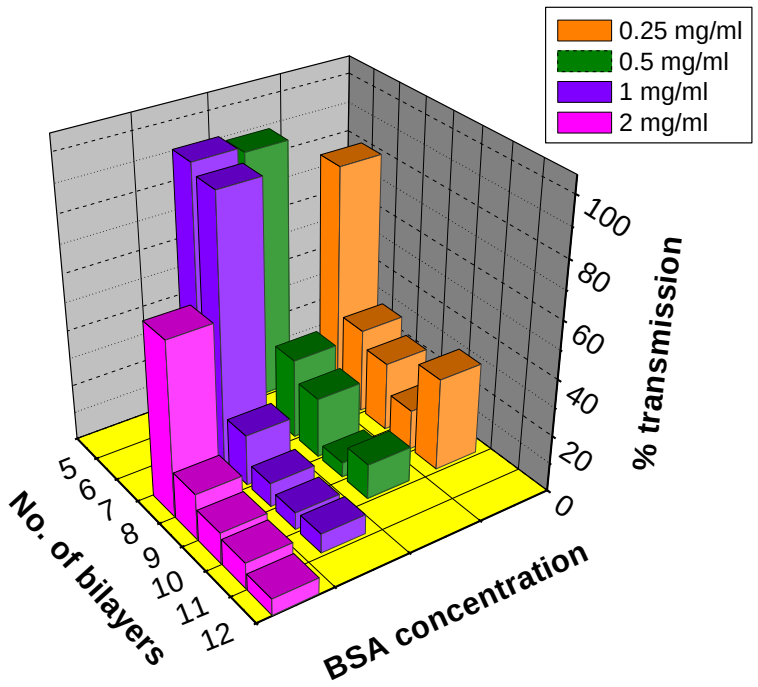


Figure 3.26: Transmission profile of BSA of different concentration through CHI/PSS multilayers

There is an increase in transmission of BSA through 8 bl and 9 bl systems for a concentration of 2 mg/ml. So it can be inferred that at high concentration, the multilayer thickness has an appreciable role in the percentage transmission of proteins. This increase in transmission with change in protein concentration, which is observed with the same multilayer membrane having identical thickness, may be due to an increasing passage of macromolecules due to their deformation in the convergent flow through the pore entrances. The percentage transmission has increased from 18 to 61.4 for the 8th bilayer followed by the same

trend in the consecutive bilayers. It looks that the solute multilayer interaction is more favorable so as to enhance the transmission. And this electrostatic interaction seems to be more favorable (the change in solution environment with higher concentration and the alternatively charged bilayers, the solutes and the bare membrane) in case of the 8th bilayer so that the difference in transmission with previous concentration, 1 mg/ml is quite high. This enhanced solute repulsion promotes increased concentration near the periphery of the pores irrespective of whether solutes and pores are uncharged or of like charge.[65] Several authors have reported on increase in protein transmission with high feed concentration during the UF of proteins through conventional UF membranes.[30,65-68] The influence of polymer concentration on the rejection of PEG from IRIS 3042 membrane was studied by Nguyen et al under UF conditions. They observed high rejection (low percentage transmission) at low concentration and beyond certain concentration, rejection decreased sharply.[30] A similar result was also observed in the UF of BSA through track- etched membranes.[68]

The flux values remained more or less similar at higher concentration compared to lower concentrations. We have small flux values as can be seen from Table 3.13. Under conditions of relatively low flux, diffusion outweighs convection so that during filtration of macromolecules, the distance between the macromolecules becomes considerably greater inside the pores than in bulk solution. Again when there is no protein adsorption to the membrane as in our case there will be an increase in bulk concentration which results in higher penetration of macromolecules through the pores which will lead to an increase in

transmission. FT-IR spectrum of BSA filtered multilayer membrane had no characteristic peak in the amide I and amide II band regions which confirms that there is no adsorption. This immediately rules out the possibility of pore plugging. Hence the observed higher transmission at higher concentration is possibly due to concentration polarization from an increased bulk concentration alone.

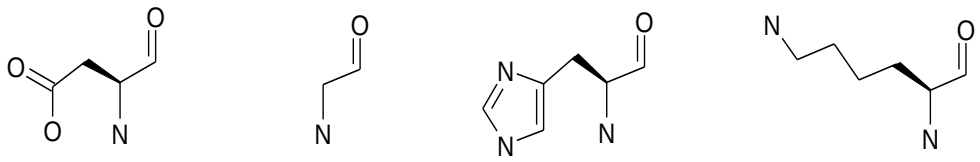
For all the studied BSA concentrations, BSA solution flux was found to decrease with increase in number of bilayers and corresponding to sharp decrease in percentage transmission, there is a sharp decrease in flux value also(Table 3.13 and fig.3.25). Even though the percentage transmission increased from the bilayer of highest rejection onwards the flux value continued to decrease with increase in the number of bilayers. As the multilayer thickness increases with the addition of each bilayer, it can cause barrier in fluid flow.

3.8 Permeation studies of amino acids

Amino acid separations are usually achieved by nanofiltration methods. There are reports of employing polyelectrolyte multilayer on suitable substrates as the thin layer for NF membranes.[33,34] We have fabricated a novel composite membrane with high flux and good selectivity by modifying the surface of a microfiltration membrane (supor 450, 0.45 μm pore size, and polyethersulfone membrane) with a uniform coating of chitosan (CHI)/polystyrene sulfonate (PSS) polyelectrolyte multilayer.[57] Due to the presence of charged multilayers as skins, the microfiltration membrane acquires the characteristics of a charged UF membrane and the possible application of this multilayer membrane in

protein separations has been discussed in section 3.5. Protein transport studies reveals that the size factor and steric factor are the important parameters controlling the permeation of proteins through the multilayer. Size factor was prominent in BSA transport whereas the protein charge was the major factor in ovalbumin and lysozyme transport through CHI/PSS multilayer. In the present study, the permeation of amino acids (neutral, acidic and basic) is investigated under different pH conditions through the composite membranes under ultrafiltration (UF) conditions. UF is a milder method as the applied pressure is low. Our objective was whether amino acid separation is possible in a cheaper way with this system. The amino acids selected were histidine, glycine, lysine and aspartic acid. The transmission behaviour of single amino acids through the multilayer has to be accounted before starting the actual separation. Another part of interest of this work is to better understand transport through polyelectrolyte multilayer films by studying the movements of small molecules like amino acids. In order to evaluate the charge effect on the transport characteristics of amino acids, two basic, one acidic and one neutral amino acid were taken for the transport studies. The amino acid concentrations were determined by the ninhydrin method.[69]

Early works with nanofiltration membranes show that solution pH has a decisive role in the permeation characteristics of amino acids.[35, 36] In this work, we have selected neutral, basic and acidic amino acids to investigate the pH dependence of amino acid solution on the permeation through multilayer membrane. It is expected that basic and acidic amino acids may experience more rejection from the multilayer membrane when the pH of the solution is away from their isoelectric points.



L- Aspartyl Glycyl L- Histidyl L- Lysyl

Figure 3.27: Structure of amino acids used for permeation study

Table 3.14: Physical properties of the amino acids selected for the study

Amino acid	Stokes radius	MW	pI
Histidine	4.45	155	7.6
Lysine	4.01	146	9.5
Aspartic acid	3.72	133	2.7
Glycine	2.56	75	6

Amino acid concentration in permeate was determined spectrophotometrically at 570 nm (ninhydrin method). Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha-amino acids and yields an intensely coloured bluish purple product which is colorimetrically measured at 570 nm.

The percentage transmission was calculated using the following relationship

$$\% \text{ Rejection} = [(C_{feed} - C_{permeate})/ C_{feed}] \times 100$$

$$\% \text{ Transmission} = 100 - \% \text{ rejection}$$

The amino acid solution flux was determined by measuring the time necessary to collect a known volume of permeate.

3.8.1 Permeation studies of histidine through multilayer membrane

Histidine (2-amino-3-(4-imidazolyl) propionic acid), a basic amino acid with isoelectric point 7.6 was selected as one of the amino acids because of its potential therapeutic applications. This amino acid is a precursor for histamine and carnosine biosynthesis. The imidazole side chains and the relatively neutral pKa, 6, of histidine mean that relatively small shifts in cellular pH will change its charge. For this reason, this amino acid side chain finds its way into considerable use as a coordinating ligand in metalloproteins, and also as a catalytic site in certain enzymes. The imidazole side chain has two nitrogens with different properties; one is bound to hydrogen and donates its lone pair to the aromatic ring and as such is slighty acidic, whereas the other one donates only one electron pair to the ring so it has a free lone pair and is basic. These properties are exploited in different ways in proteins. The permeation studies of histidine solution (0.5 mg/ml) through the multilayer membrane [0 bl (uncoated supor membrane) to 8 bl] was carried out at three selected pHs, 10.6 (above pI), 7.6 (pI), 5 (below pI). The results are summarized in table 3.15.

Table 3.15: pH dependent transport studies of L-histidine through the CHI/ PSS multilayer membrane

No.of bilayers(bl)	Histidine pH10.6		Histidine pH 7.6		Histidine pH 5	
	% transm.	flux	% transm.	flux	% transm.	flux
0	98.5	35.6	42.9	4.6	87.9	6.2
3	97.6	21.2	40.2	2.1	75.4	5.7
5	97.0	14.1	39.9	1.2	69	4.9
7	95.8	6.7	37.8	0.65	46.8	2.4
8	96.7	4.9	41.2	0.71	-	-

The pH dependent transport studies of histidine through the multilayer membrane shows that the presence of multilayer on the support has not much influence on the percentage transmission of histidine except for pH 5. At pH 10.6, when both the multilayer surface and the amino acid were negative, there was no appreciable influence on the percentage transmission of amino acid with the number of bilayers. Even after 8 bilayer deposition, the percentage transmission is above 90%. The flux value decreases with the addition of each bilayer. Probably, the negative charge carried by histidine is not sufficient to get rejected from the multilayer. But at pH 5, when the multilayer surface is negative and the amino acid is positive, it appears that multilayer has some influence on the permeation of the amino acid. The percentage transmission decreased from 87.9 to 46.8 when the number of bilayers was increased from 0 to 7. At pI (pH 7.6), the percentage transmission of histidine got decreased to 42.9% on permeation through the bare membrane. The percentage

transmission remained more or less the same up to 8 bl membrane. The transmission data at this pH indicates that CHI/PSS multilayer has not much influence on the permeation of histidine (fig.3.28). The amino acid may get adsorbed to the polyether sulfone support at pH 7.6 (usually, at the isoelectric point, membrane fouling is highest). This is also reflected from the relatively lower flux values at this pH.

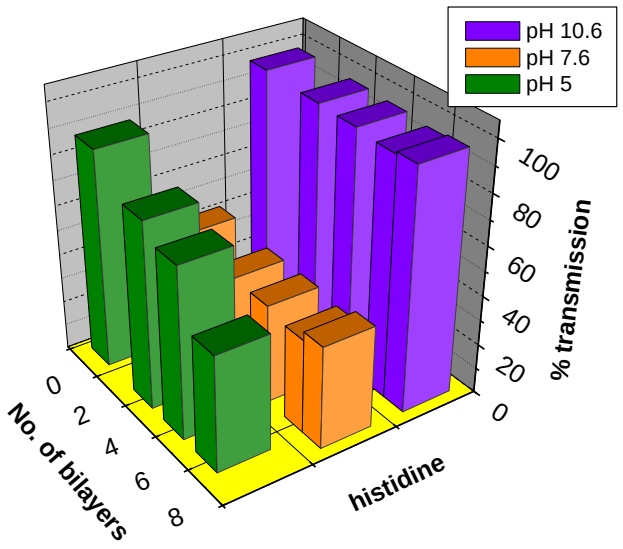


Figure 3.28: Transmission profile of L-histidine through CHI/PSS multilayer membrane

3.8.2 Permeation studies of glycine through multilayer membrane

The effect of pH on the transport properties of neutral amino acids was studied by taking glycine as an example. The transport properties were studied at pH 6 (pI) and pH 3 (below pI). The results are

summarized in table 3.16. If we compare the percentage transmission of glycine through the multilayer under the two pH conditions, no appreciable rejection was seen at both the pH values. This is not unexpected in the case of neutral amino acids for which the net charge attained on pH variation is small compared to acidic or basic amino acids. However, there is slight higher rejection at pH 3 when glycine is positively charged. The change in percentage transmission on varying the number of bilayers is also minimal.

Table 3.16: pH dependent transport studies of glycine through the CHI/PSS multilayer membrane

No.of bilayers(bl)	Glycine pH 6		Glycine pH 3	
	% transm.	flux	% transm.	flux
0	97.5	10.6	99.4	32.4
3	97.2	10.2	94	28.4
5	95.4	8.5	95.1	7.2
7	93.6	0.49	88.5	0.59
8	88.2	0.42	86.2	0.52

The flux value is less for glycine solution at its isoelectric point. For both the pH values, the solution flux decreased progressively with increase in number of bilayers. The permeation studies of glycine through CHI/PSS multilayer system shows that neutral amino acids like glycine cannot be sieved using this system.

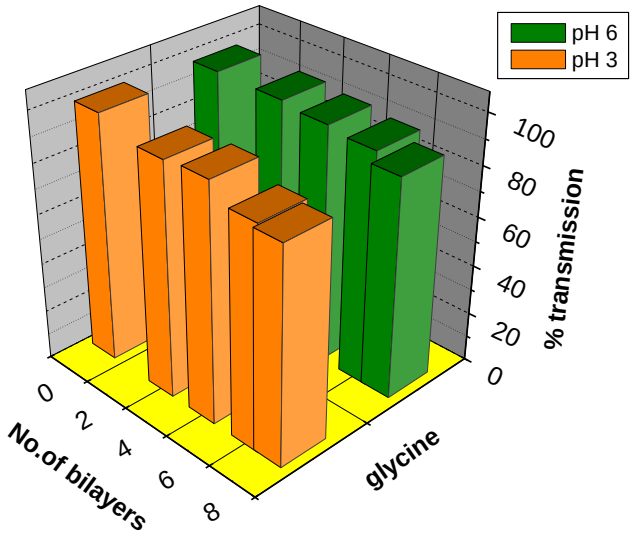


Figure 3.29: Transmission profile of glycine through CHI/PSS multilayer membrane

3.8.3 Permeation studies of lysine through multilayer membrane

The transport studies of L-lysine were conducted at two selected pHs, 9.5 (pI) and 5 (below pI) and the results are presented in table 3.17.

Table 3.17: pH dependent transport studies of L-lysine through the CHI/PSS multilayer membrane

No.of bilayers(bl)	Lysine pH 9.5		Lysine pH 5	
	% transm.	flux	% transm.	flux
0	98.9	9.2	99.5	11.4
3	95.1	6.1	76.7	8.2
5	45.7	2.6	37.8	4.6
7	32.2	0.48	3.6	0.6

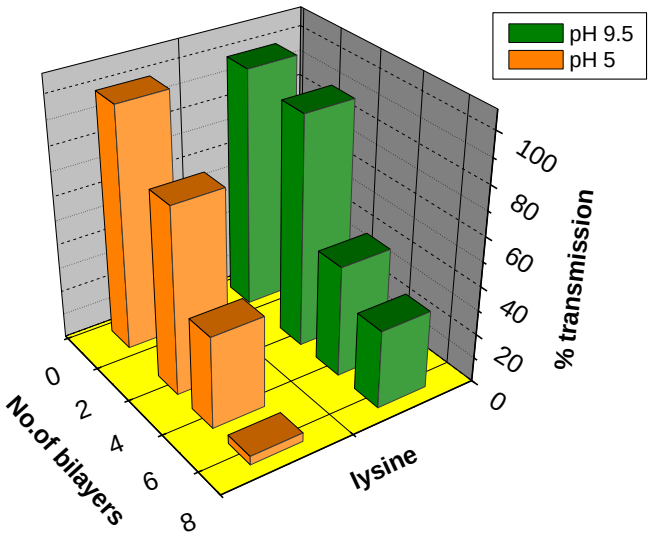


Figure 3.30: Transmission profile of L-lysine through CHI/PSS multilayer membrane

At the isoelectric point of lysozyme (pH 9.5), the percentage transmission decreases from 98.9 to 32.2%. The solution flux also decreases with increase in number of bilayers. A very high rejection of lysine occurred at pH 5 with 7^{th} bilayer.

The percentage transmission of lysine was found to decrease to 3.6 with 7^{th} bilayer (fig.3.30). That means a 7 bl CHI/PSS coating on the polyether sulfone support membrane rejects 96.4% lysine having a pH of 5. The uncoated membrane allows 99.5% permeation for lysine at this pH. Eventhough the solution flux has decreased considerably, the very high rejection of a small molecule like lysine from the multilayer membrane has great significance.

Usually amino acid separation and purification requires nanofiltration conditions requiring high applied pressure. In the present system, a few CHI/PSS bilayer coatings on a microfiltration membrane can change its sieving properties to the extent that it is capable of sieving molecules of the nanofiltration range. Lysine is a basic amino acid having two amino groups. At pH 5, the two amino groups are protonated resulting in a high positive charge density/molecule. When the solution pH is 5, lysine carries a high positive charge and the multilayer surface is negatively charged. As a result, the amino acid gets attracted and adsorbed to the membrane surface. Adsorption of amino acid on the membrane surface was revealed from the modification of the FT-IR spectrum of the amino acid filtered membrane in the 1400-1700 cm^{-1} region (fig.3.31). The very high rejection shown by lysine at pH 5 clearly indicates the fact that in the permeation of charged species, charge factor outweighs size factor. The solution flux was found to decrease with increase in the number of bilayers. The highest rejection was shown by the 7^{th} bilayer. If we examine closely the permeation behavior of amino acids, it is seen that from 5^{th} to 7^{th} bilayer, there is a sharp decrease in the flux values also. Probably when the multilayer reaches 7 bl, an optimum thickness is reached. So the permeation studies of lysine through CHI/PSS multilayer membrane reveals that charge factor outweighs size factor. Moreover, there is potential scope for a 7 bl CHI/PSS membrane to be used for lysine-glycine separation at pH 5.

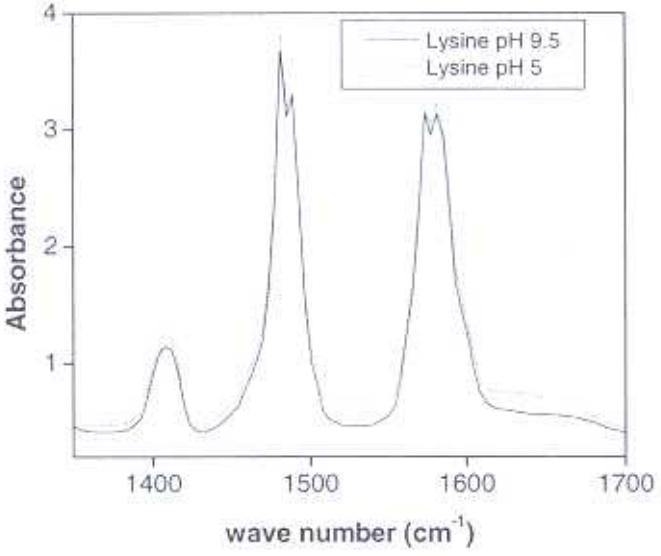


Figure 3.31: FT-IR spectrum of lysine adsorbed 5 bl CHI/PSS membranes

3.8.4 Permeation studies of aspartic acid through multilayer membrane

Aspartic acid (2-aminobutanedioic acid) is non-essential in mammals, being produced from oxaloacetate by transamination. In plants and microorganisms, aspartic acid is the precursor to several amino acids, including four that are essential: methionine, threonine, isoleucine, and lysine. Aspartic acid is also a metabolite in the urea cycle and participates in gluconeogenesis. Aspartate (the conjugate base of aspartic acid) stimulates NMDA receptors. It serves as an excitatory neurotransmitter in the brain and is an excitotoxin. The transport properties of aspartic acid through the multilayer membrane was studied at pH 2.7 (pI) and pH 5 (above pI) and the results are presented in table 3.18. Strikingly in the case of aspartic acid also, a high rejection was observed at pH 5, for the 7^{th} bilayer. The percentage

transmission had decreased from 98.1 to 6.6 on varying the number of bilayers from 0 to 7. Aspartic acid is an acidic amino acid containing two carboxyl groups. At pH above the isoelectric point, it is negatively charged. The high charge build up in the molecule is sufficient to get rejected from the multilayer. In this case, the amino acid is negatively charged and the multilayer surface is also negatively charged. So the rejection of amino acid takes place due to mutual repulsion between the negatively charged amino acid and the negatively charged multilayer surface. This was confirmed from the fact that no modification was observed in the FT-IR spectrum of the amino acid filtered membrane in the 1400-1700 cm^{-1} region.

Table 3.18: pH dependent transport studies of aspartic acid through the CHI/ PSS multilayer membrane

No.of bilayers(bl)	Aspartic acid pH 5		Aspartic acid pH 2.7	
	% transm.	flux	% transm.	flux
0	98.1	31.6	96.8	11.9
3	71.5	7.5	94.4	4.6
5	58.9	2.3	95.7	1.8
7	6.6	0.67	28.5	0.53

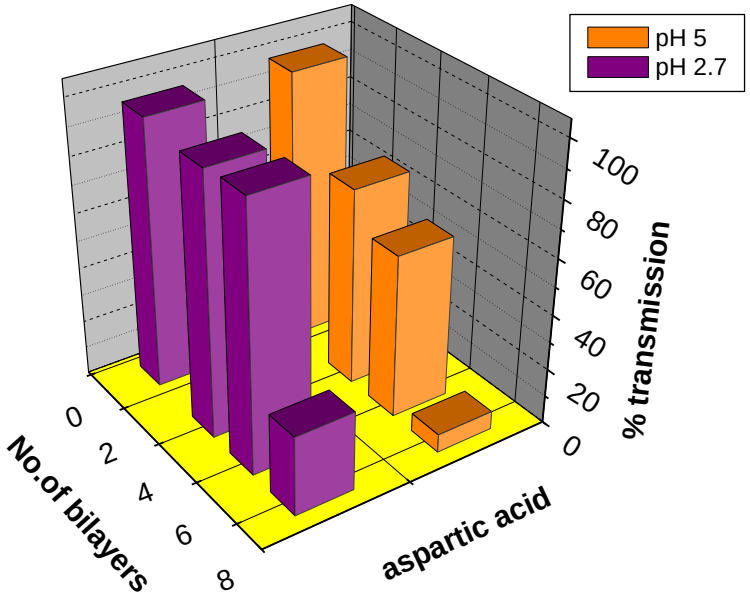


Figure 3.32: Transmission profile of L-aspartic acid through CHI/PSS multilayer membrane

The percentage transmission of aspartic acid was found to decrease from 96.8 to 28.5% on varying the number of bilayers from 0 to 7 at the isoelectric point (pH 2.7). The solution flux decreased progressively with increase in number of bilayers. As in the case of lysine, from 5^{th} to 7^{th} bilayer, there is considerable change in percentage transmission and flux. Another thing is that in the case of lysine and aspartic acid, even at the isoelectric point, the multilayer has influence on its transport properties.

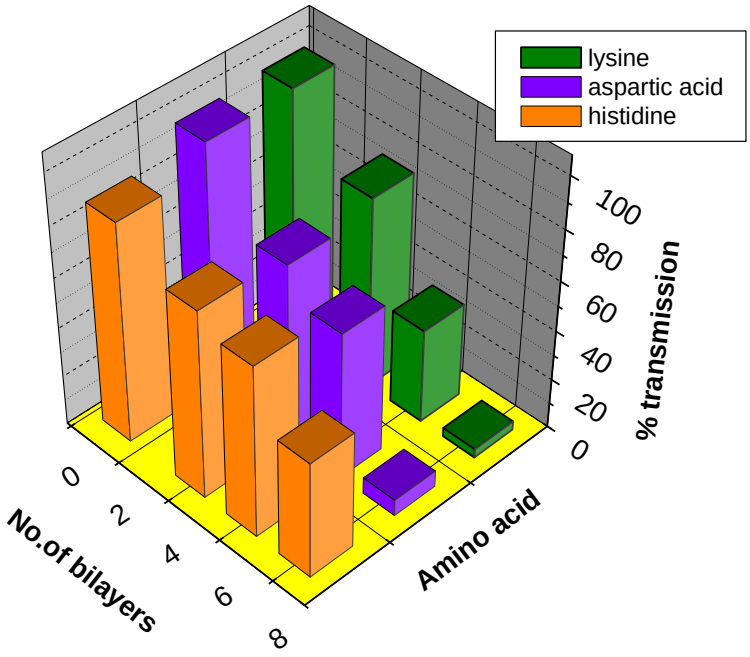


Figure 3.33: Transmission profile of lysine, aspartic acid and histidine through CHI/PSS multilayer membrane at pH 5

The transmission profile of lysine, aspartic acid and histidine at pH 5 are shown in fig.3.33. It can be seen that at this pH there is considerable change in percentage transmission of the amino acids with the number of bilayers. The transport studies of amino acids through CHI/PSS multilayers fabricated on supor microfiltration membranes show that nanofiltration properties can be achieved with this system.

Conclusions

In summary, the sieving characteristics of microfiltration membranes have been modified to render it ultrafiltration characteristics,

by forming a few polyelectrolyte multilayers on it. We observed for the first time that deposition of a few bilayers on a polymeric support can be used for the sieving of protein as large as BSA from its solution. The permeation characteristic of proteins through the polyelectrolyte multilayer depends on pH and the number of deposited layers. BSA can be retained by the multilayer at all pH and the percentage rejection varies with the number of bilayers at different pH. Addition of salt to the polyelectrolyte solution has a strong influence on the number of bilayers for the rejection of BSA. When we compare the percentage transmission values of BSA and ovalbumin at their respective isoelectric points, sieving of BSA and ovalbumin is size dependent. In the transport of ovalbumin and lysozyme, charge factor appears to outweigh the size factor. The presence of salt in the polyelectrolyte did not show much influence on the transport of these proteins.

The transmission percentages for the proteins under study at pH 8.8 through the multilayer membranes deposited from salt solution (at pH 1.7) are shown in the fig.3.17. At bilayers 5 and 6, ovalbumin is completely transported and can be recovered in permeate where as 97.8% of lysozyme is rejected by the system. Lysozyme is an important protein, which finds applications in biomedical field, and its main source is egg white. There have been many studies related to its purification. The major difficulty is to combine selectivity and flux in a single membrane. A two-step procedure which involves filtration with a highly selective membrane followed by filtration by a high flux membrane is generally used for overcoming this difficulty. In this context the present procedure combines the two properties (high selectivity and high flux) in a single

membrane. In addition to this, we have started with a microfiltration membrane, which is rather cheap and could provide ultrafiltration properties to it by an environment friendly method of fabrication. It is, therefore, concluded that the present procedure can be a highly viable method for the preparation of ovalbumin free lysozyme.

The data presented in this study also provides experimental evidences that along with solution pH, ionic strength and protein concentration can strongly influence the transport of proteins through polyelectrolyte multilayers. It was observed that ionic strength of protein solution has a decisive role in the percentage transmission and flux on the transport properties of BSA, ovalbumin and lysozyme through CHI/PSS multilayer membranes. There is a drastic change in the percentage transmission of BSA with ionic strength. 0.1 M NaCl in BSA is sufficient to permeate all the BSA. With 9 bl membrane, the percentage transmission of BSA is found to increase from 5.3 to 115.6 when the salt concentration was varied from 0 to 1 M. The percentage transmission of ovalbumin through 9 bl membrane was found to increase from 23.3 to 125.8 when the salt concentration in the protein was increased from 0 to 0.05 M. Only 33% lysozyme was transported through the multilayer membrane even at a salt concentration of 0.25 M suggesting that lysozyme is strongly bound to the multilayer membrane. BSA was found to be rejected from the multilayer membrane at all the studied concentrations. More number of bilayers was needed for rejection of protein as the feed concentration increased. This opens the possibility to understand the interaction of proteins with the multilayers. This study also reveals that lysozyme-ovalbumin separation may be achieved with

this system at pH 8.8 by adding up to 0.2 M NaCl solution to the protein mixture in order to overcome the possible complexation between the two proteins. BSA-lysozyme separation may also be achieved with this multilayer system.

The pH dependent transport studies of basic, neutral and acidic amino acids were carried out through CHI/PSS multilayer coated membrane under ultrafiltration conditions. The percentage transmission and flux values were found to change with the number of bilayers and pH conditions for each amino acid. The effect was more marked in the case of basic and acidic amino acids. A very high rejection was observed for lysine at pH 5 (% transmission 3.6) and aspartic acid at pH 5 (% transmission 6.6). The high rejection of small molecules like amino acids from the multilayer surface indicates that in the transport of amino acids across the multilayer membrane, charge factor outweighs size factor which is in agreement with the transport behavior of amino acids through conventional nanofiltration membranes. By the proper selection of the pH, this multilayer membrane may be used for the separation of amino acid mixtures in a cheaper, less tedious and environmental friendly way.

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