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Controlled Release Studies of Immobilized Lysozyme from Polyelectrolyte Multilayer Membrane Sunder Different PH Conditions

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Surface modification, lbl assembly, polyelectrolytes, protein immobilisation, desorption.

Abstract
In the present study, potential application of Chitosan/Polystyrene sulfonate multilayermembrane as reservoirs for releasinga protein in a controlled manner is explored by carrying out the desorption study, especially the time dependent desorption study of lysozyme immobilised on the multilayer at two selected pH 8.8 and 10.6. The desorption studies indicates that the protein is reversibly bound to the polyelectrolyte multilayer for the studied pH, 8.8 and 10.6. It was observed that the desorption of protein increases progressively as the ionic strengthincreases and almost complete desorption occurred when the salt concentration reaches 0.5 M NaCl. The data obtained from UV and FT-IR studies shows that there is difference in the desorption pattern for the lysozyme adsorbed membranes under the two pH conditions. As lysozyme finds medicinal applications, the present work reveals that controlled drug delivery systems can be fabricated using this polyelectrolyte multilayer system.

1. Introduction

The recently developed layer-by-layer (LBL) method involving deposition of cationic and anionic polyelectrolytes alternately on polymeric supports can be used to fabricate nanostructured films of tailored properties. These Nanostructured films are extensively used inthe design of novel functionalities and molecular architectures, biosensing, biomolecule immobilization, controlled drug deliveryetc [1-5]. Multilayers reported in the literature had thickness that ranged between 10 nm and 10 μm [6-8].The properties of polyelectrolyte multilayers (PEM) membranes like thickness, permeability, charge density etc. can be manipulatedby the proper choice of parameters like pH, ionic strength, number of bilayers, nature of polyelectrolyte (whether weak or strong) used for making PEM etc.

Biofunctional membranes can be fabricated by immobilizing biomolecules like proteins, enzymes, amino acids etc. on polyelectrolyte multilayer membranes. There are reports of application of these membranes in various fields including biosensing, catalysis, selective adsorption, affinity separation of synthesized biochemical etc. [9-16].Protein loaded polyelectrolyte multilayer assembly has been recently employed for the fabrication of bioactive surfaces with exceptional properties [17-22]. There have been reports of proteins maintaining their secondary structure when incorporated to the multilayers [23-25] which is a clear indication of retaining the biological function of the protein as a result of immobilization.

The conformational changes of proteins within the multilayer can be followed by the measurement of

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amide bands in the ATR-FTIR spectrum, especially by noting the shape and wave number maximum of amide bandswhich is sensitive to structural changes in the protein [26].The amide I band (1650 cm^{-1}) and amide II band (1540 cm^{-1}) arises from the carbonyl stretching vibrations of the peptide bond andN-H bending and C-N stretching modes of thepeptide chains respectively.

We have fabricated polyelectrolyte multilayer assembly on polyether sulfone support by using Chitosan/Polystyrene sulfonate pair as the polyelectrolytes. The pH dependent transport studies of a model protein, lysozyme was carried out under ultrafiltration conditions.It was observed that a 5 bilayer membrane was capable of rejecting 97.8% lysozymeat pH 8.8 and at pH 10.6, 92% lysozyme wasretained by the membrane [27]. The work revealed that pH has animportant role in the permeation characteristics of the protein through the multilayer membrane. Characterization of the protein filtered membranes using FT-IR,UV reflectance spectrum and SEM confirmed thatthe conformation of the loaded lysozyme is preserved for a wide pH range (5-11). Thus, the study opened up the possible fabrication of bio functional membranes with antibacterial properties [28].

In the present work, the potential application of lysozyme immobilized membranes as controlled release systems is explored. Lysozyme is a protein with antibacterial properties and having an isoelectric point near a pH of 11 and it is positively charged at all pH below 11. The anionic polyelectrolyte PSS is present on the top of the multilayer assembly. As the charge carried by the protein is different at the two studied pH, it is suspected that the protein is loaded on the multilayer surface at pH 10.6 and drawn inside the multilayer at pH 8.8. Desorption studies, especially, time dependent desorption was carried out at the two selected pH using salt of varying concentrations and the protein desorbed multilayer membranes were characterized by UV and SEM.

2. Material and Methods

LbL assembly was made on polyether sulfone microfiltration membranes with 0.45 μm pore diameter purchased from PALL life science.Chitosan,Polystyrene sulfonate and lysozyme were purchased from Sigma Aldrich.The polyether sulfone membrane was first dipped in chitosan solution in waterfor fifteen minutes, whose pH is adjusted to 1.7 with HCl. It was then rinsed with water and then dipped in PSS solution in water at a pH 1.7 for fifteen minutes followed by water rinse to prepare one bilayer on the support. By repeating these dipping steps,required number of bilayers can bemade on the polymer support.

2.1 Ultrafiltration

Ultrafiltration experiments were carried out at a pressure of 5-10 psi with 400 rotations/minute (rpm) at room temperature (28-30^{0}C) with amicon 8050-ultrafiltration cell (Millipore). The multilayer coated membrane was fixed at the bottom of the cell. A definite volume of the solution to be filtered is taken in the cell, the lid is closed and the cell is pressurized by nitrogen gas.

Protein release studies were conducted with two sets of lysozyme loaded membranes at pH 10.6 and pH 8.8 under UF conditions. Protein release studies were conducted with buffered saline of salt concentration varying from 0.01 to 1 M. Small quantities of the solution were withdrawn for every 15 minutes and UV absorbance at 280 nm was recorded. The membranes were removed after three hours, washed, dried and FT-IR spectra were taken. Buffered saline of 0.5 M NaCl concentration was shaken uniformly in a shaker with lysozyme loaded membranes in time-dependent desorption studies. The membranes were removed after 10 minutes duration till 120 minutes, washed, dried and FT-IR spectra were recorded.

2.2 UV-visible spectroscopy

UV-visible spectroscopy was used to find the amount of lysozyme desorbedby noting the absorbance at 280 nm with a Shimadzu UV-visible spectrophotometer (UV-1700 Pharmaspec).

2.3 Fourier Transform Infra-Red Spectroscopy (FT-IR)

Protein desorbed membranes were characterized by Shimadzu 8400S spectrometer.

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3. RESULTS AND DISCUSSION

Chitosan-Polystyrene sulfonate is selected as the polyelectrolyte pair. The structures of the polyelectrolytes are shown in fig.1. Chitosan is the deacetylated derivative of chitin, which is the second most abundant polysaccharide found on earth next to cellulose. Chitosan is a weak polyelectrolyte and Polystyrene sulfonate is a strong polyelectrolyte. This weak-strong combination of polyelectrolytes results in swollen films which are capable of separating larger analytes like proteins. Furthermore, the process of fabrication is a green method with use of water as solvent.

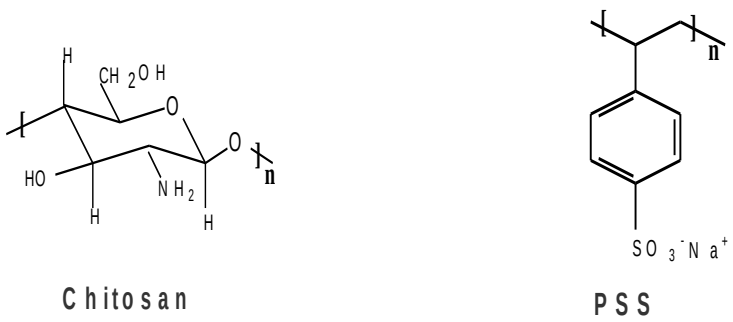

Figure 1: Polyelectrolytes used for coating

3.1 Characterisation of desorbed membranes

The UV absorbance of lysozyme (pH 10.6) adsorbed and desorbed membranes at 280 nm is shown in fig.2. The broad peak at 280 nm which arises due to the adsorbed protein, is found to decrease with increasing salt concentrations and almost vanishes for 0.5 M NaCl concentration.

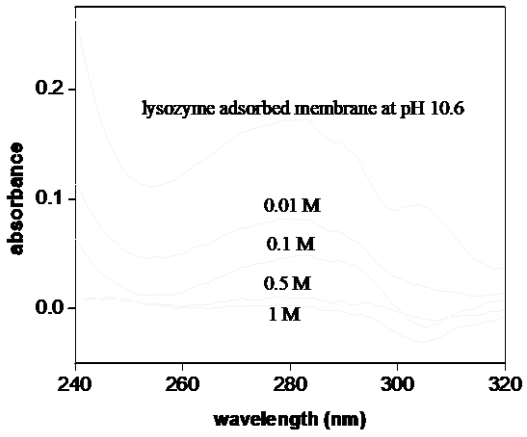

Figure 2: UV absorbance of lysozyme (pH 10.6) adsorbed and desorbed membranes

There is clear evidence for protein desorption in the SEM images of the desorbed membranes (fig.3.). However, a minor structural damage is suspected for the multilayer surface on exposure to strong salt solution. When multilayer is exposed to salt of high concentrations, the polyelectrolyte swells up and if the swelling is sufficiently high, there is a possibility of multilayer decomposition.

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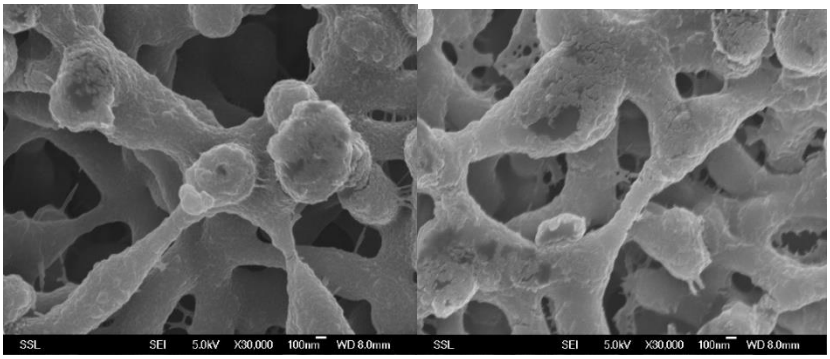

a **b**

Figure 3: SEMimages oflysozyme desorbed membranes with 0.5M NaCl at two selected pH 8.8(a)and10.6 (b)

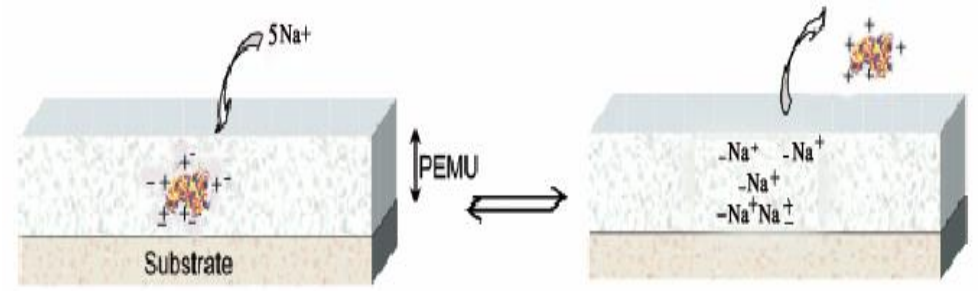

Figure 4: Displacement of loaded proteinsfrom the multilayer, where salt counter ionsreplace adsorbed proteins in high salt concentration.

Salt induced release of adsorbed proteinsfrom polyelectrolyte multilayers can be understood in terms of reduced electrostatic attraction between the adsorbed protein and the multilayer surface. With increasing ionic strength, charge screening effect by salt causes increased desorption.When the protein is drawn into the multilayer, the salt induced releasecan be thought of an exchange between Na+ ions of salt solution with the positively charged lysozyme from the multilayer membrane which is depicted in fig. 4.

3.2Time-dependent desorption studies of lysozyme adsorbed membranes

Time-dependent release studies were conducted with two sets of proteinincorporated multilayer membranes (lysozyme loaded at pH 10.6 and pH 8.8). Protein loaded membranes were shaken uniformly in a shaker with buffered saline of 0.5 M NaCl concentration. The membranes were taken after different time intervals of 10, 20, 40, 60 minutes etc. were washed, dried and FT-IR spectra were recorded. The amide I band in the FT-IR spectrum of lysozyme loaded at pH 8.8 in the time dependent release studies is shown in fig. 5. It can be seen that even after 60 minutes a considerable fraction of lysozyme remains undesorbed and it takes about 120 minutes for almost complete desorption. When the absorbance at 1654 cm^{-1} is plotted as a function of time, (fig.6), a straight line graph is obtained. This means that release of lysozyme loaded at pH 8.8 from the multilayer membrane follows first order kinetics.

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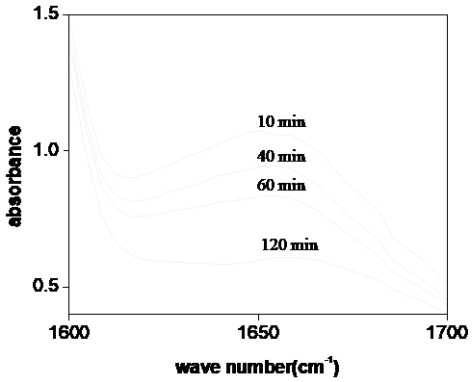

Figure 5: Amide I band in the FT-IR spectrum for time-dependent release of lysozyme at pH 8.8 from 5 bilayerchitosan/polystyrene sulfonate membrane with 0.5 M NaCl.

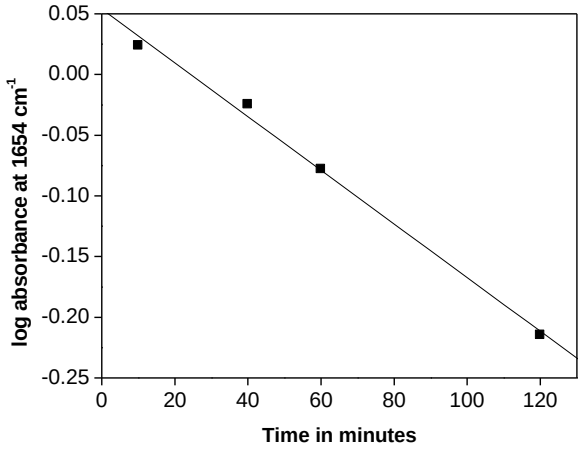

Figure 6: Time dependent release oflysozyme at selected pH 8.8 from 5 bl chitosan/ polystyrene sulfonate membrane with 0.5 M NaCl. The logarithm of amide I band peak area in the FT-IR spectrum at 1654 cm^{-1} is plotted as a function of time

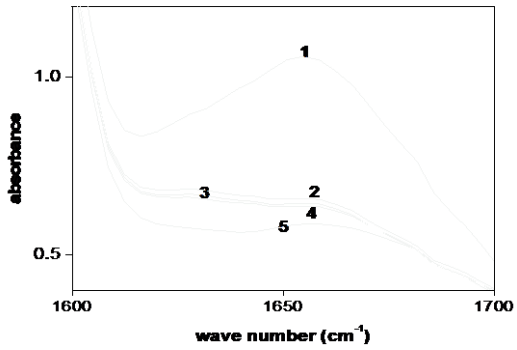

Figure 7: Amide I band in the FT-IR spectrum for time-dependent release of lysozyme pH (10.6) from 5 bl membrane with 0.5 M NaCl. **[1:** lysozyme (pH 10.6) loaded membrane; desorbed membranes **2:**after 10 min; **3:**after 40 min; **4:**after 60 min; **5:**after 120 min**]**

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The amount of lysozyme desorbing from the multilayer membrane can be varied by adjusting the salt concentration. If a particular salt concentration is selected, the amount of lysozyme desorption can be varied by changing the time. This means, controlled lysozyme delivery systems can be fabricated from lysozyme adsorbed membranes.

From fig.7, it is clear that an appreciable amount of lysozyme gets desorbed from the pH 10.6 membranes just after 10 minutes of uniform shaking with 0.5M NaCl. There is a marked decrease in the peak after 10 minutes. Whereas even after 60 minutes of shaking a good quantity of lysozyme remained undesorbed from the pH 8.8 membrane as can be seen from fig.5. The time dependent desorption studies of lysozyme adsorbed membranes under the two pH conditions (8.8 and 10.6) also reveals that lysozyme at pH 8.8 is more strongly bound to the membrane than at pH 10.6. The rate of desorption is high for lysozyme adsorbed at pH 10.6. This also points out that the adsorption modalities of lysozyme on the membrane at the two studied pHs are different.

3. Conclusions

The desorption studies of lysozyme indicates that the protein is reversibly adsorbed to the polyelectrolyte multilayer for the studied pH, 8.8 and 10.6. It is observed that the amount of protein desorbed depends on the salt concentration and it increases with increase in ionic strength and almost complete desorption occurred for a salt concentration of 0.5 M NaCl. The data obtained from UV and FT-IR studies shows that there is difference in the desorption pattern for the lysozyme adsorbed membranes under the two pH conditions. This study also demonstrates that at pH 8.8, when lysozyme and the multilayer surface carries opposite charges, lysozyme is somewhat sorbed into the bulk of the multilayer system whereas at pH 10.6, more surface accumulation of the protein occurs. Time-dependent desorption studies of lysozyme loaded membranes at pH 8.8 indicates that lysozyme can be desorbed from the multilayer in a controlled way by adjusting the salt concentration. As lysozyme is an antibacterial, it opens up a possibility in the controlled drug delivery systems. Furthermore, lysozyme is incorporated into a comparatively nontoxic environment. As lysozyme is an enzyme, there is a chance of loss of enzyme activity (either partial or complete) upon adsorption into multilayer membrane. Therefore, future activity measurements are required to understand the applicability of these systems as controlled delivery agents.

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