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Research Article

FLUORESCENT STUDIES OF LYSOZYME IMMOBILISED CHITOSAN/POLYSTYRENE SULPHONATE MULTILAYER MEMBRANES

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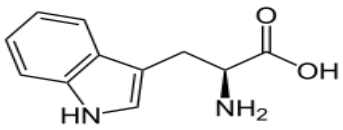
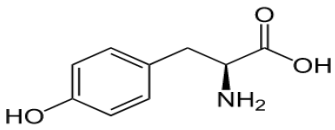
ABSTRACT
The fluorescence emission properties of lysozyme immobilized at selected pH 5, 8.8 and 10.6 on chitosan/polystyrene sulfonate (CHI/PSS) multilayer membranes were investigated. The tryptophanyl fluorescence was selectively excited at 290 nm. The emission maximum and fluorescence intensity were found to be dependent on the pH of lysozyme solution. The fluorescence intensity was highest at pH 8.8. A slight red shift was observed as the pH changes from 5 to 10.6. Quenching of lysozyme fluorescence by iodide was used to study the effect of pH on the adsorption pattern of lysozyme to multilayers. For lysozyme (pH 10.6) adsorbed membrane, the fluorescence intensity was found to decrease progressively with quencher concentration. Almost complete quenching of fluorescence was observed when the iodide concentration was 1 M suggesting full accessibility of quencher to tryptophanyl residues. A linear Perrin plot was obtained suggesting a static mechanism for quenching under the experimental conditions. Fluorescence quenching studies were also carried out with lysozyme (pH 5) adsorbed membranes prepared by normal adsorption methods and also under ultrafiltration conditions. The quenching efficiency was different for the two sets of membranes. The fluorescence quenching studies reveals that adsorption pattern of proteins on multilayers strongly depend on pH and immobilization method.

Keywords: Lysozyme, Immobilisation, Polyelectrolyte multilayers, Fluorescence, Quenching.

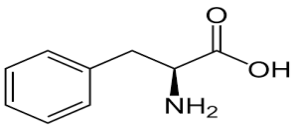
1. INTRODUCTION
Proteins are unique in displaying intrinsic fluorescence, which originates from three fluorescent amino acids-phenylalanine, tyrosine and tryptophan. The fluorescence emission of proteins is dominated by tryptophan, which absorbs at the longest wavelength and displays the largest extinction coefficient. One of the striking features of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. One can frequently observe changes in the emission spectra of tryptophan in response to protein conformational transitions, association, substrate binding, or denaturation, all of which can affect the local environment surrounding the indole ring [1]. The intrinsic fluorescence of proteins can provide considerable information about protein structure and dynamics and is often used to study protein folding and association reactions [2]. The emission maximum of tryptophan in water occurs near 350 nm and is highly dependent on polarity and / or local environment. The excitation at 290-300 nm is frequently used to achieve selective excitation of tryptophan residues. One expects a blue-shifted spectrum for tryptophan in a completely apolar environment and a red-shifted spectrum in polar environment. Thus, following these changes it is possible to monitor the partitioning of proteins between the aqueous solution and membranes [3-5].



Tryptophan



Tyrosine



Phenyl alanine

Change in fluorescence intensity measurements often gives useful insights about the nature of conformational changes in a protein [6-9].

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a compound. Quenching may result from a variety of molecular interactions including excited state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisions [10]. Quenching resulting from collisional encounters between the fluorophore and the quencher is called collisional or dynamic quenching. In the case of static quenching, a complex is formed between the fluorophore and the quencher and this complex is nonfluorescent. Both static and dynamic quenching requires molecular contact between the fluorophore and the quencher.

Fluorescence quenching has been widely studied both as a fundamental phenomenon and as a source of information about biochemical systems [11]. Quenching measurements can reveal the accessibility of fluorophores to quenchers. Collisional quenching of fluorescence is described by the Stern-Volmer equation,

$$F_0 / F = 1 + k_q \tau_0 [Q]$$

where, F_0: fluorescence intensity in the absence of quencher, F: fluorescence intensity in the presence of quencher, k_q: bimolecular quenching constant, τ_0: lifetime of fluorophore in the absence of quencher, Q: concentration of the quencher

The intrinsic fluorescence of proteins can provide considerable information about protein structure and dynamics and is often used to study protein folding and association reactions. Tryptophan (Trp) fluorescence is strongly influenced by the indole side chain and has proved to be a useful tool to monitor conformational changes in proteins [12]. When buried in a hydrophobic environment, Trp fluorescence generally shifts to a shorter wavelength (blue shift in λ_{max}). Other factors such as specific interactions between the indole ring and polar groups on the protein, or the presence of water molecules in the interior of protein may play a part in determining the position of the fluorescence spectrum [13]. Fluorescence intensity and λ_{max} are largely pH dependent [14, 15]. Quenchers decrease the fluorescence intensity through physical contact with the excited indole ring. The ease with which a fluorophore is quenched depends upon its exposure to the quencher. Molecular oxygen and a number of ionic quenchers such as I^-, NO_3^- and Cs^+ have been used quite extensively in quenching studies of proteins. Ionic quenchers, being charged and heavily hydrated, should be able to quench only surface Trp residues [16-18].

Lysozyme (1,4-β-N-acetyl muramidase) is an antimicrobial enzyme widely distributed in nature and is found in mammalian tissues and secretions, insects, plants, bacteria and viruses [19]. Besides its antimicrobial activity, lysozyme has many other functions including inactivation of certain viruses, antitumour activity etc [20, 21]. Lysozyme is a major protein constituent in tear fluids and finds application as a major component in soft contact lens deposits. Incorporation of lysozyme into multilayer has significant practical applications including preparation of antibacterial coatings, controlled drug release etc. For lysozyme adsorbed multilayer to be bioactive, the secondary structure must be preserved.

There are six Trp residues in hen egg white lysozyme, but only two of them, Trp 62 and Trp 108 appear to dominate the fluorescence spectrum [22, 23]. Trp 108 is not quenched by I^-. So Trp 62 appears to be the only tryptophan quenched by I^-. There are various reports of iodide quenching of lysozyme fluorescence in solution [24, 25]. I^- is reported to be an efficient collisional quencher of lysozyme fluorescence.

In our early study of the transport of lysozyme through CHI/PSS multilayer membrane at different pH conditions (5, 8.8 and 10.6) under UF conditions, it was observed that lysozyme is almost fully rejected from the membrane at pH 8.8 (97.8% rejection) and at pH 10.6, 92% lysozyme is rejected from the membrane [26]. The protein filtered membranes (under UF conditions) were washed and the FT-IR spectra were recorded. The FT-IR data clearly indicates the presence of amide I band corresponding to protein. UV reflectance spectrum as well as SEM measurements confirms the presence of lysozyme adsorbed on the membrane with the secondary structure unaltered and we have suggested its potential antibacterial use [27]. Desorption studies of lysozyme also revealed that the secondary structure of the protein is intact even after desorption from the membrane. Desorption kinetics was also found to be pH dependent [27]. When a protein interacts with polyelectrolyte multilayer, two mechanisms may operate- (1) bulk sorption and (2) surface adsorption. Sorption is used to denote a combination of surface adsorption and bulk absorption. Proteins are drawn into the multilayer when the surface is of opposite charge whereas they remain on the surface when it is like-charged. At pH 5, lysozyme is positively charged and due to electrostatic attraction between the positively charged

lysozyme and negatively charged multilayer, the protein may be drawn into the multilayer. In order to get a clear picture of the adsorption mechanism at the two selected pH, 10.6 and 5, fluorescence studies were performed with the protein adsorbed membranes. Lysozyme was immobilized to 5bl CHI/PSS multilayer membranes under UF conditions as well as under simple adsorption conditions for pH 10.6 and 5.

Fluorescence quenching studies using acrylamide were performed to determine the structure and accessibility of lysozyme active site in colloidal complexes formed between lysozyme and different polyelectrolytes (polyacrylic acid, polystyrene sulfonate, dextran sulfate, and chondroitin sulfate) and it is reported that no large structural modification of the active site occurs during complexation [28]. We have employed iodide quenching of lysozyme fluorescence to get an idea about the pH dependence on the adsorption behaviour of lysozyme to the multilayer. Iodide ion was selected as the quencher because it is heavily hydrated and should be able to quench only surface Trp residues.

2. MATERIAL AND METHODS

Chitosan (CHI, medium MW, 75-85% deacetylated) and Poly (styrene sulfonate) (PSS, MW 70,000, 30 wt % in water) purchased from Sigma Aldrich were used as polycations and polyanions respectively. Supor®- 450 (Polyether sulfone) microfiltration membranes of 0.45 μm pore diameter from PALL life science was used as support membrane for LbL assembly. Hen egg white lysozyme (LYZ) was purchased from Sigma Aldrich. Ultrapure water (18.2 MΩ.cm) obtained from Cascada AN™ water purifying system (Pall Corporation) was used for all experiments.

2.1. Preparation of multilayers

Multilayers were coated alternately from 0.01 M solutions of CHI and PSS solutions having pH 1.7. The polyether sulfone support membranes were kept in water overnight before multilayer preparation. pH of the polyelectrolyte solutions was adjusted with 1 M HCl. The support membrane was dipped in CHI and PSS alternatively for 15 minutes with washing in water in between. Filtration experiments were performed in an Amicon-8050 dead-end stirred cell with a total volume of 50 ml and effective membrane area of 13.4 cm^2. Filtration of protein solution was done under constant N_2 pressure of 10 psi (~0.69 bar) with continuous stirring at a rate of 300 rotations/ minute (rpm) at room temperature. The multilayer films were soaked in water for at least 2 hours prior to filtration.

The adsorption of proteins was performed under UF as well as ordinary conditions with multilayer membranes. Lysozyme solutions (0.25 mg/ ml) of pH 5, 8.8 and 10.6 were used for adsorption studies. Simple adsorption method involves dipping of the membranes in 50 ml protein solutions of different pH for 2 hours. After 2 hours, the membranes were taken, washed once with the corresponding buffer to remove unbound protein and dried in air. Protein solutions of pH 8.8 were prepared in tris-HCl buffer and pH 10.6 was prepared in glycine-NaOH buffer. Protein solutions (0.25 mg/ml) of pH 5 were prepared in citrate buffer.

2.2. Fluorescence spectroscopy

Fluorescence measurements were carried out with RF-5301PC Shimadzu Spectrofluorometer having high pressure Xe lamp as the excitation source and a R928 photomultiplier as the photodetector.

2.3. Fluorescence quenching studies

Fluorescence measurements were carried out with RF-5301PC Shimadzu Spectrofluorometer. For quenching studies, lysozyme adsorbed membranes were dipped in KI solutions of varying concentrations from 0.1 M to 1 M. The dipping time was 1 minute. The membranes were dried and loaded into the spectrofluorometer. The excitation wavelength was 290 nm in order to excite the tryptophan residues selectively.

3. RESULTS AND DISCUSSION

3.1. Fluorescence emission spectra of lysozyme adsorbed membranes

Lysozyme solutions of selected pH 10.6, 8.8 and 5 were adsorbed to 5 bl CHI/PSS multilayer under ultrafiltration conditions. The fluorescence spectra of the adsorbed membranes were recorded as shown in fig.1. The excitation wavelength selected was 290 nm to selectively excite tryptophan residues [1, 7]. The fluorescence maxima (λ_{max}) of the observed spectra at pH 5, 8.8 and 10.6 are respectively 316 nm, 318 nm, and 319 nm. The fluorescence maxima of tryptophan residues in native proteins commonly range from 325 to 350 nm depending on the polarity of the solvent. For lysozyme in water, λ_{max} of Trp is reported as 348 nm [5]. So there is a large blue shift in the λ_{max} of lysozyme when it is adsorbed to multilayer system. The fluorescence maximum is usually a measure of the polarity of the environment [4-8]. The blue shift indicates that when lysozyme is incorporated into

CHI/PSS multilayer, the protein is in a hydrophobic or apolar environment. The λ_{max} of lysozyme is reported as 335 nm when adsorbed to silica surface [8]. The outer layer of the multilayer is preferably hydrophobic due to the presence of PSS. Secondly, in the solid phase, the protein is less flexible compared to solution. The large blue shift in λ_{max} on adsorption to multilayer may be attributed to these two factors. Lysozyme is known to exist in monomeric form in acidic aqueous solutions (pH< 5), as dimer in the pH region from 5 to 10 and as aggregates above pH 10 [5]. The slight red shift in λ_{max} on varying pH can be taken as an indication of the aggregation of lysozyme. From pH 5 to pH 10.6, there is only a marginal red shift (3 nm) indicating slight aggregation of lysozyme at a pH of 10.6. A red shift of ~ 8 nm is observed in the fluorescence spectrum of lysozyme in solution [7]. This result suggests that the aggregation tendency of lysozyme is reduced when it is bound to multilayer.

The fluorescence intensity is found to vary with pH of lysozyme as seen in fig.1. From pH 5 to pH 8.8 the fluorescence intensity increases. But at pH 10.6, the intensity is lowered. A similar trend is observed in the fluorescence spectrum of lysozyme in solution. It is reported that the fluorescence intensity of lysozyme increases from pH 5 onwards, being maximum around pH 7-8 and then decreases beyond pH 8 [6].

It is suggested that decreased intensity at pH 5 is due to protonation of certain carboxyl groups in the acid region. At pH 10.6, the decrease in fluorescence intensity may be due to ionization of the tyrosine residues and subsequent energy transfer from tryptophan [6].

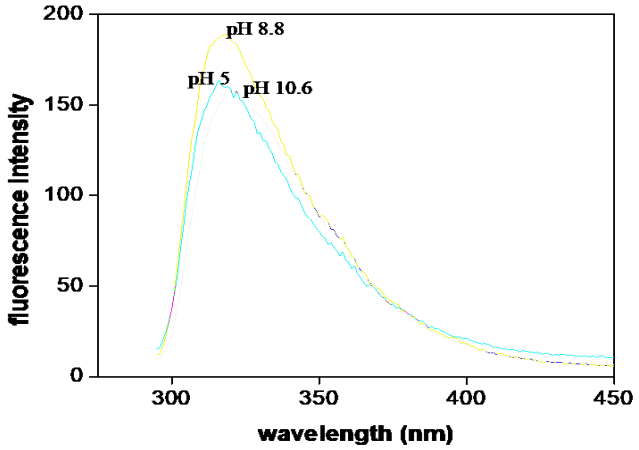
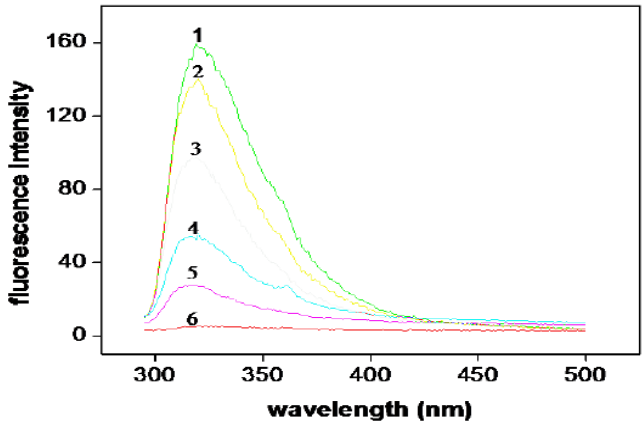


Fig. 1: Fluorescence spectra of lysozyme adsorbed (UF) 5bl CHI/PSS membrane at pHs 10.6, 8.8 and 5

3.2. Quenching studies of lysozyme adsorbed membranes

The fluorescence quenching studies of lysozyme adsorbed membranes were carried out using iodide as the quencher. Different lysozyme (pH 10.6) adsorbed 5 bl CHI/PSS membranes were prepared. The membranes were dipped in KI solutions of varying concentrations from 0.1 M to 1 M, dried and fluorescence spectra were recorded. Fig.2 shows the fluorescence spectra of lysozyme (pH 10.6) adsorbed membranes after dipping in KI of increasing concentration. The variation of fluorescence intensity of lysozyme (pH 10.6) adsorbed membrane with iodide ion concentration is depicted in fig.3. There is a progressive decrease in lysozyme fluorescence with increase in iodide ion concentration. At high concentration (1 M), iodide ion is capable of quenching the lysozyme fluorescence almost completely.



1: 0 M, 2: 0.1 M, 3: 0.2 M, 4: 0.4 M, 5: 0.6 M, 6: 1 M

Fig. 2: Fluorescence spectra of lysozyme (pH 10.6) adsorbed (UF) 5bl CHI/PSS membranes with increasing concentration of iodide ion

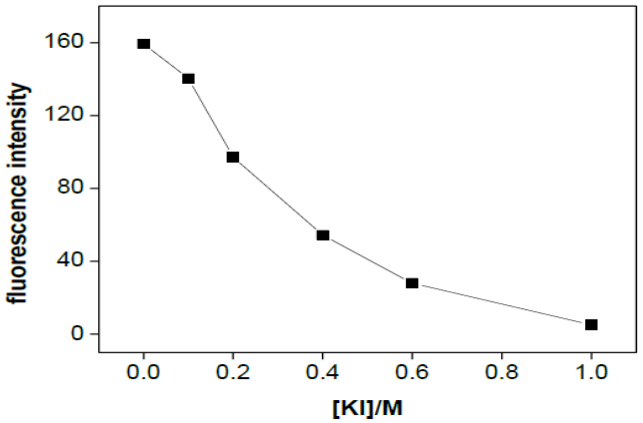


Fig. 3: Variation of fluorescence intensity of lysozyme (pH 10.6) adsorbed (UF) 5bl CHI/PSS membranes with quencher concentration

At pH 10.6, it is assumed that lysozyme is preferably adsorbed at the surface of the multilayer. Almost complete quenching of fluorescence means that the quencher, iodide ion is accessible to all the fluorophore in lysozyme. As iodide ion is heavily hydrated, it is not expected to penetrate the multilayer membrane. So this quenching study suggests that lysozyme is located at the surface of the multilayer membrane at pH 10.6.

Quenching data are typically presented as plots of F_0/F versus [Q] (Stern-Volmer plots). Stern-Volmer plots are having an intercept of 1 on the Y-axis and a slope equal to $k_q\tau_0$ known as Stern-Volmer quenching constant. A linear Stern-Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to quencher. If two fluorophore populations are present, and one class is not accessible to quencher, the Stern-Volmer plot deviate from linearity towards the X-axis.

Static quenching results from the formation of a non-fluorescent fluorophore–quencher complex, formed in the fluorophore's ground state. When this complex absorbs light, it immediately returns to the ground state without emission of a photon. The dependence of the fluorescence intensity upon quencher concentration for static quenching is easily derived by considering of the association constant for complex formation.This constant is given by

$$Ks = [F\text{-}Q] / [F] [Q]$$

Where, [F-Q] is the concentration of complex, [F] is the concentration of the uncomplexed fluorophore and [Q] is the quencher concentration. Here also the dependence of F_0/F on [Q] is linear and is identical to that observed for dynamic quenching except that the quenching constant is the association constant.

The total concentration of the fluorophore, is given by

$$F_0 = [F] + [F\text{-}Q]$$

We can substitute fluorescence intensities for the fluorophore concentrations, and rearranging the above equation

$$F_0 / F = 1 + Ks [Q]$$

A characteristic for this type of quenching is that increasing quencher concentration decreases the fluorescence intensity or quantum yield but does not affect the fluorescence life time. An important feature of static quenching is its decrease with increasing temperature, as the stability of the fluorophore-quencher ground state complexes is lower at higher temperatures.

The Stern-Volmer plots were found to be non-linear for the present system. When Stern-Volmer plot is not linear, the mechanism of quenching is more complex, and static quenching may become important at high quencher concentrations. In such a case, Perrin plot for static quenching is applicable according to which log F_0/F against quencher concentration [Q] is a linear plot.Perrin plot for lysozyme (pH 10.6) adsorbed 5bl CHI/PSS membranes under UF conditions is shown in fig.4. The plot is almost linear suggesting that static quenching is taking place with the present system.

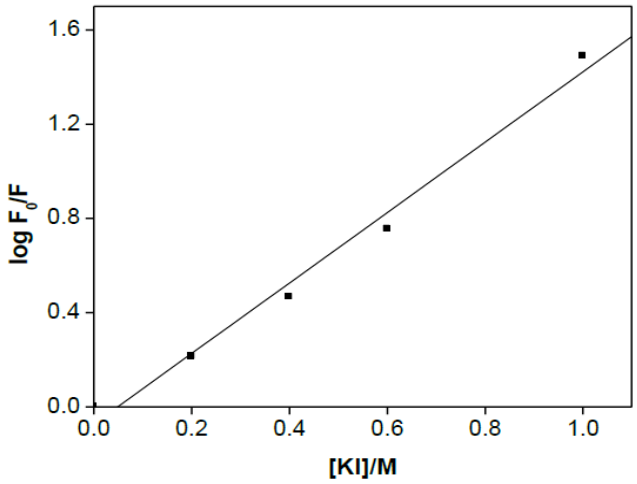


Fig. 4: Perrin plot for lysozyme (pH 10.6) adsorbed (UF) 5bl CHI/PSS membranes

The present fluorescence quenching study is conducted in the solid phase. In solid phase, the mechanism by which quenching takes place may be different from collisional quenching. Quenching studies were also performed with lysozyme (pH 5) adsorbed CHI/PSS membranes. Lysozyme adsorbed membranes were prepared under UF conditions and simple adsorption conditions. Here also the quenching study was conducted in the solid state. Perrin plot for lysozyme (pH 5) adsorbed 5 bl CHI/PSS membrane under UF condition is shown in fig.5. A linear plot is obtained which indicates a static quenching. Perrin plots of lysozyme adsorbed membranes under UF conditions at two different pH, 10.6 and 5 as shown in fig.4 and fig.5 respectively shows that iodide quenching is more efficient for lysozyme adsorbed membranes at pH 10.6.

Perrin plot for lysozyme (pH 5) adsorbed 5 bl CHI/PSS membrane under simple adsorption condition is shown in fig.6. Here also the Perrin plot is almost linear. On comparing Perrin plots (fig.5 and fig.6) for lysozyme (pH 5) adsorbed under UF and simple adsorption conditions respectively, it can be seen that iodide quenching is more efficient for lysozyme (pH 5) adsorbed membranes under simple adsorption conditions.

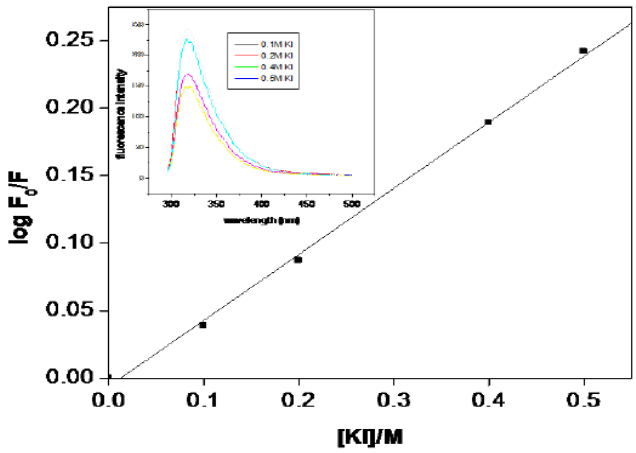


Fig. 5: Perrin plot for lysozyme (pH 5) adsorbed (UF) 5bl CHI/PSS membranes

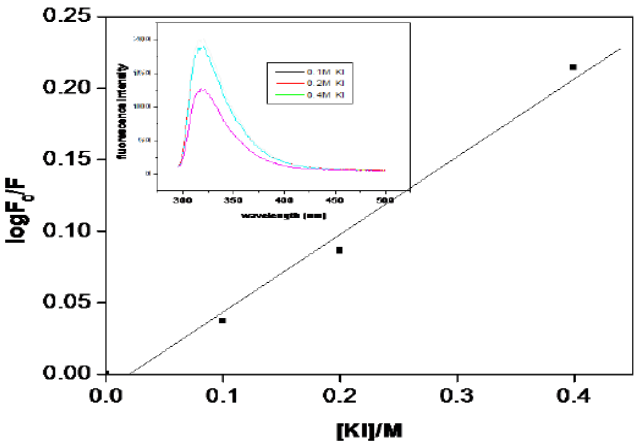


Fig. 6: Perrin plot for lysozyme (pH 5) adsorbed (simple adsorption) 5bl CHI/PSS membranes

When lysozyme (pH 5) is incorporated into multilayer under UF conditions, the quenching efficiency of iodide ion seems to be decreased as can be seen from fig.5. The fluorescence intensity is decreased to 36% when the iodide concentration is 0.4 M. For multilayers prepared under simple adsorption conditions, a 41% decrease in fluorescence intensity is observed for an iodide concentration of 0.4 M. It may be noted that with lysozyme (pH 10.6) adsorbed membrane, the fluorescence intensity is decreased to 66% at an iodide concentration of 0.4 M. This is due to the fact that at pH 5, lysozyme is positively charged and it may be drawn into the multilayer. It is assumed that lysozyme adsorbed on the surface of the multilayer is selectively quenched by iodide ion. The fraction of lysozyme penetrated into the multilayer may not be accessible to the quencher. It may be recalled that iodide ion is heavily hydrated and is not able to penetrate into the multilayer. Thus, fluorescence quenching studies give a qualitative idea about the pH dependence on the adsorption pattern of lysozyme on the multilayer.

4. CONCLUSION

The fluorescence spectra of lysozyme adsorbed membranes show that the tendency of aggregation of lysozyme is lowered when it is incorporated into CHI/PSS multilayer. Also, a high blue shift in λmax is observed for lysozyme on immobilisation into CHI/PSS multilayer indicating a hydrophobic environment surrounding the protein. Fluorescence quenching studies of lysozyme adsorbed multilayer membranes reveal that pH of lysozyme has a significant role in determining the adsorption pattern of lysozyme in the multilayer. This supports the earlier reports suggesting that when the multilayer surface and the protein carry identical charge, the protein preferably gets adsorbed on the multilayer surface [9, 10]. If they carry opposite charges, the protein may get adsorbed on the surface as well as penetrate into the bulk of the multilayer. Fluorescence quenching is an ideal probing technique from both an interpretive and experimental point of view. The results obtained from this study shows that fluorescence quenching studies can give qualitative information regarding adsorption pattern of lysozyme immobilized on multilayer systems.

One striking feature is that the secondary structure of the proteins remains unaltered on adsorption to CHI/PSS multilayer membranes. This opens up the possibility of fabricating bioactive coatings with the protein adsorbed multilayer membranes. The fact that the secondary structure of adsorbed lysozyme is preserved for a wide pH range (5-11), it is a good indication that bio functional membranes with antibacterial properties can be fabricated upon adsorption of lysozyme into CHI/PSS multilayer membranes.

5. ACKNOWLEDGEMENTS

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Conflicts of interest

None

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