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Protein – Metal Ion Interaction: A Spectroscopic Study

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ABSTRACT

This study reports on the spectroscopic interaction studies of mercuric chloride LYZ. The quencher concentration was maintained in the range of 0–6.25 µM. At the studied concentration range (10^{-6} M), LYZ did not have any variation in fluorescence intensity. Increase in LYZ concentration to 10^{-3} M showed a decrease in the fluorescence intensity due to the involvement of nucleophilic functional group. The fluorescence of LYZ was quenched to a large extent depending on the protein solution pH (2.2, 4.5, and 7.4).

At all the studied pH, emission spectra showed blue shift. The onset and the shift varied with solution pH. Stern-Volmer analysis showed a three stage transition At pH 7.4, the presence of $HgCl_2$ did not have much influence on the amide bands. But at pH 4.5, the amide I band showed a shift to lower wavenumber These confirmed that the quenching takes place through both static and energy transfer processes.

Keywords: Protein-Metal ion interaction, LYZ- Lysozyme, CHI-Chitosan, S-V- Stern Volmer Plot, FL-Fluorescence Intensity

1. INTRODUCTION

Layer by Layer assembly (LbL) is a novel way to generate a variety of layered and multilayered structures in nanometer thickness, based on oppositely charged species.[1] Fabrication of such systems faces lots of difficulty because of its incompatible characteristic, nanometer thickness and macroscopic size. But due to their flexibility in tuning the characteristics of the arrangement such as, controlled and step wise formation of multilayer films this method has inspired considerable attention in biotechnology. And promises solutions to many problems in the fields of drug delivery, bio and optical sensors, and micro reactors, as well as in bio separation. [2]Biofunctional thin films can be fabricated by immobilizing protein molecules within nanolayers which finds lots of application in various fields of science. There are reports on the sustainability of biomolecules even after immobilization to nanolayers. [3] These Protein films offer a large potential for sensing applications, since they are thin; they can give fast response time. One major advantage of these biofunctional films is that a wide range of analyte molecules can be detected in milli molar to picomolar level. The selectivity, sensitivity and stability of such sensors depends highly on the nature of indicator (Protein molecule) selected for immobilization. [4] So due to the stabilized structure these biofilms are of top most interest in biosensing areas.

Protein molecules exhibite intrinsic fluorescence property due to the presence of three fluorophoric amino acids tryptophan, tyrosine and phenylalanine. Of these, tryptophan is the dominant fluorophoric

group, highly sensitive to local environment. [5] In the study of the solution structure of multi-tryptophan proteins, selective fluorescence quenching is used to map out the exposed and buried tryptophan (Trp) residues. For the present case lysosyme is selected as the protein molecule for sensing application. Lysosyme contains almost six tryptophanyl residues, of which most of them are located on the active site. Though, Trp 62 and Trp 108 are known to cause a drastic decrease in fluorescence intensity, on interaction with analyte molecules (ref). As, Trp 62 is more exposed to the polarity environment than Trp108, the quenching of Lysosyme fluorescence in most case is from Trp 62. [6]

Lysosyme (LYZ)is an antimicrobial enzyme, also known as N-acetylmuramide glycanhydrolase, widely distributed in nature and is a remarkable bacteriolytic element found in mammalian tissues and secretions, insects, plants, bacteria and viruses. It is also present in cytoplasmic granules of the polymorphonuclear neutrophils and is the major component in soft contact lens deposits. It is a basic globular protein having lots of biomedical properties including anti-inflamatory, anti-histamic, anti-viral,immune modulatory, anti – histamic and anti-tumor activities. In addition to that, it opens a new pathway to drug delivery field as a potent drug carrier. We are focusing on the biosensing ability of Lysosyme, utilizing its intrinsic fluorescence property. It can be used as a model protein as its functionalities are well characterized.

LBL sequential adsorption of the protein, lysosyme was optimized to obtain proteinous nanostructured coatings on composite membranes, Supor-CHI/PSS (Lysosyme- Supor-CHI/PSS) suitable as sensors. Lysosyme was immobilized in different solution pH onto Chitosan(CHI) /polystyrenesulfonate (PSS) nanolayers under normal adsorption as well as under ultra filtration (UF) conditions. Fluorescence studies can be used as a test run to assess the potential change of protein molecular structure and again is a confirmation that the protein yet regains its biological properties in a multilayer environment, making it useful for diagnostic purposes. The successful demonstration of the bio-optical utility of these novel systems suggests an entirely new window of opportunity in environmental and medical sensor platforms that cannot be met with current optical material and device platforms.

Mercury exposure can be determined through the analysis of a variety of tissues to include blood, urine, finger or toe nails, breast milk and hair. Many studies have measured total Hg in blood without distinguishing the forms of mercury found in the blood. [6] This study was focused on determining the effect of mercury exposure from consumption of processed foods. Inorganic mercury may enter food products during the various manufacturing processes. For example, mercury cell chlor-alkali chemical products are used extensively in food processing and always contain inorganic mercury residues. Vegetable oil products manufactured using the common alkali refining process may present a moderate risk of mercury contamination. The mercury cell chlorine used to bleach flour is expected to contain a small amount of mercury residue. [9] The corn starch used to manufacture the corn sweeteners in the HFCS product line is treated purposely with inorganic HgCl2 as part of the manufacturing process to inhibit endogenous starch-degrading enzymes. It is thus reasonable to suggest that consumers are routinely exposed to non-elemental inorganic mercury (I-Hg) when they consume heavily processed foods, including corn sweeteners. Our justification for adding the support group intervention to help students and community members eliminate corn sweeteners from their diet is based on the concept that consumption of corn sweeteners is both a known and potential source of inorganic mercury exposure and a potential factor in the development of insulin resistance.

Case studies

What is typical about Hg is that it has no role in human metabolism (so far not known) and at the same time body cannot exclude Hg. thus it accumulate in the body fat and reports show that the average 70 kg person has a total burden of 13 mg of Hg. as per the EPA rules the intake of Hg is restricted to 0.1 µg/kg per day. Though it does not interfere in human metabolism it may reduce the antioxidant capacity of various enzymes. This is because of its affinity for –SH groups. –SH groups in amino acids have key roles in protein stabilization, enzyme catalysis, ligand-receptor binding, and play an antagonistic role toward many elements with toxic or eco-toxic behavior, free-radical, and reactive oxygen species. Most importantly –SH groups control the production of free radicals and peroxides and helps in repairing damages caused by them. This detoxification is really important in controlling the free radicals and peroxides that may otherwise cause destruction of cell components such as DNA, lipids and proteins. The indication is that free –SH groups are necessary to control age related diseases, atherosclerosis, cardiovascular diseases, diabetes mellitus, and cancer.

The list of body burden due to both organic and inorganic Hg continues to grow. The worst part about Hg is its potential to cross BBP and BPB. Whereas Hg is not prompt to BBP and BPB but it is known to accumulate by the kidney.

2. MATERIALS AND METHODS

Materials: **P**olyethersulfone (Supor, Pall, 0.45 µm) was selected as the supporting membrane for multilayer build up. Chitosan (CHI, Medium MW, 75-85% deacetylated, Aldrich) the cationic polyelectrolyte and Poly (styrene sulfonic acid) sodium salt (PSS, MW 200,00, 30 wt % in water, Aldrich) the anionic polyelectrolyte, were selected for multilayer fabrication. Lysosyme (MW 14,000 dalton, SRL mumbai) was selected as the protein molecule. A buffer solution covering the pH range 3-5 (citric acid – sodium citrate buffer) and pH 8-11 (tris-HCl and Glycine-NaOH buffer) were prepared in deionized (DI) water. All the other chemicals are purchased from Aldrich and used without further purification.

Figure 1: Crystal structure of LYZ generated using Python molecularviewer from the Protein Data Bank.

Fabrication of biofunctional film (Lysosyme- Supor-CHI/PSS): Chitosan (CHI)/ polystyrenesulfonate (PSS) multilayers were fabricated on polyethersuulfone (Supor) substrate at the deposition pH 1.7 as reported earlier (ref). Lysosyme (pI 10.6) solutions (0.25 mg/ ml) of pH 5 was prepared in citrate buffer, pH 7.2, 8.8, and 10.6 were prepared in tris - HCl and glycine - NaOH buffer respectively. Lysosyme solution prepared at all these selected pHs were immobilized onto CHI/PSS nanolayers in a normal adsorption way

and using ultra filtration conditions, which is a pressure driven process, with amicon 8050-ultrafiltration cell (Millipore) according to the published procedures.[6]

Characterization and sensing ability: Lysosyme immobilized CHI/PSS nanolayers were characterized using FTIR, SEM analysis**.** Layer-by layer build up and protein immobilization were monitored under areas of characteristic bands (sulfonate, υ (SO_3^-), at 1033 cm^{-1} and amide I band at 1650 cm^{-1} . Surface morphology was studied using SEM (JSM-840, Scanning Electron Microscope) Sensing ability was investigated from fluorescence emission spectra obtained using a spectrofluorometer (LS 55, Perkin–Elmer spectrofluorometer). For fluorescence measurements, lysosyme immobilized nanolayers were placed in 1 cm quartz cuvette filled with deionized water. Quencher molecules prepared in various concentrations are injected through a micro syringe to the cuvette and emission is recorded. The excitation wavelength was kept as 290 nm, to excite tryptophanyl residues selectively. The emission spectral data were obtained in the range 300-500 nm.

3. RESULTS AND DISCUSSION

Chitosan / polystyrenesulfonate (CHI/PSS) nanolayers (up to 5 bilayers) were fabricated on polyethersulfone substrate according to the published procedure . [7]

SEM IMAGES

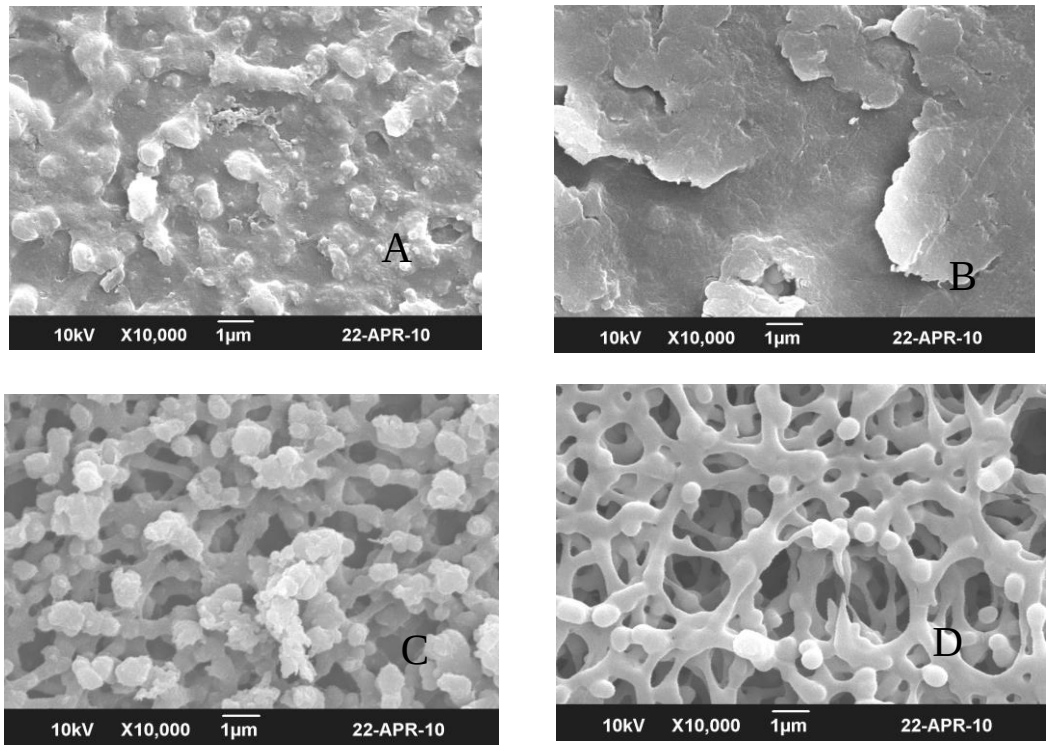
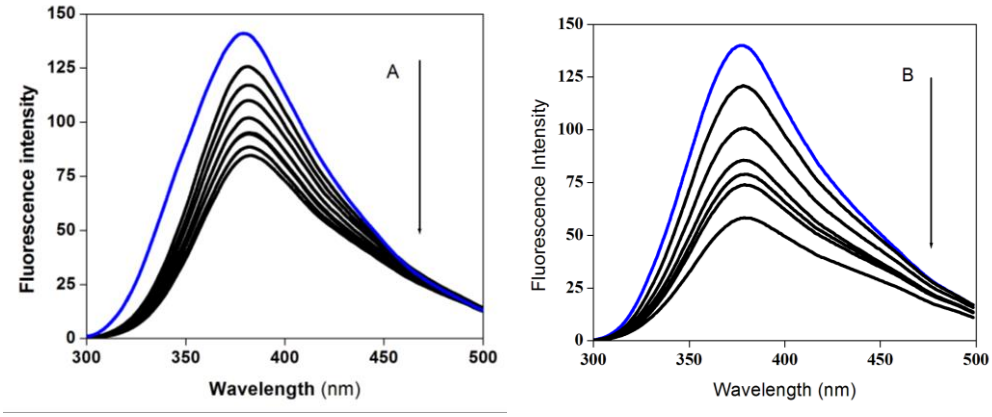


Figure 2: SEM images of lysosyme immobilized (under ultra filtration) 5 bl CHI/PSS nanolayer as a function of pH, A: (pH5), B: (pH7.2), C: (pH 8.8), D: (pH 10.6)

These nanolayers were characterized using FTIR, UV-Vis and SEM as reported earlier. [6] well studied antimicrobial protein i.e. lysosyme was selected for the immobilization procedures. From the transport profile studies of lysosyme, through CHI/PSS nanolayers, it was observed that maximum protein rejection is from 5bl CHI/PSS. [8] So we have fixed 5 bl CHI/PSS as the basis of our immobilization studies. Lysosyme is a basic globular protein having a relatively rigid structure, with an isoelectric pH 10.6. Because of its numerous appealing biomedical properties, it can be used as a potent sensor for both environmental and medical studies. It was (Lysosyme, 0.25mg/ml) immobilized onto CHI/PSS nanolayers through simple adsorption as well as through ultra filtration (UF) the pressure driven process. Lysosyme was immobilization under all selected protein solution pH (pH 5, 7.2, 8.8 and 10.6). These biofunctional films are again characterized using FTIR and surface morphological studies are obtained from scanning electron microscope image (SEM).

Emission spectral datas were measured from lysosyme immobilized nanolayers at an excitation wavelength 290 nm. Emission maximum is observed at 374 nm. Solution state emission studies of lysosyme got an emission maximum at 349 nm. Compared to that a relevant red shift is observed when immobilized onto nanolayers, indicating conformational change during immobilization . [9] Lysosyme is a multi tryptophanyl enzyme. It consists of six tryptophanyl residues per monomer unit. [10] Shift in fluorescence emission suggesting that all the tryptophanyl residues are equally exposed to the solvent than in free state. Fluorescence emission fron lysosyme immobilized CHI/PSS nanolayer is shown in figure 3. These confirmational changes help in selective sensitization studies.

Fluorescence quenching properties of lysosyme in free state is a well studied area and fluorescence quenching is the standard parameter used in sensing studies for most of the indicator molecules. We have mercuric chloride, to study the sensing ability of this biofunctional film.. Quencher concentrations kept as 10^{-6} mol/l through out the experiment. In addition to biosensing, the same nanolayers can be used for chemical, optical and environmental sensing. The sensing unit is based on the useful intrinsic fluorescence property of lysosyme, immobilized within CHI/PSS nanolayers



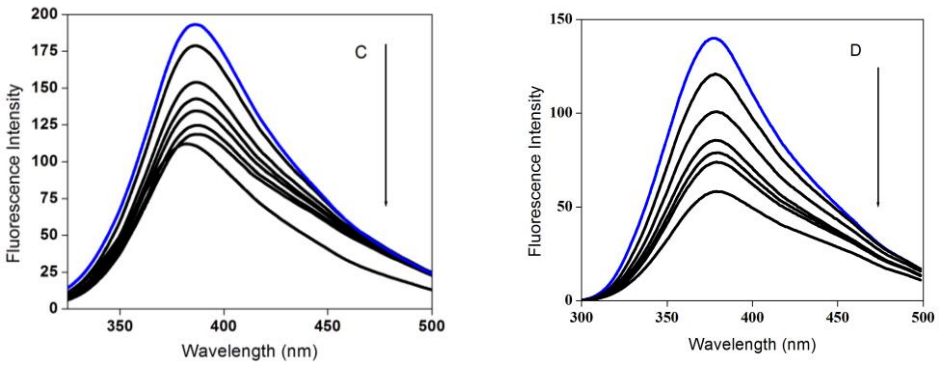


Figure 3: Fluorescence emission spectrum of LYZ-CHI / PSS nanolayer in presence of mercuric chloride (10^{-6} mol L^{-1}) . A: pH 3.0, B: pH 4.5, C: pH 8.8, D: pH 10.6

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Fluorescence emission from Lysosyme immobilized CHI/PSS nanolayers are investigated as a function of quencher concentration and observed that fluorescence intensity decreases with increase in quencher concentration. This decrease in fluorescence intensity is believed to be due to some interactions between the fluorophore (indole ring of tryptophan) and quencher molecule. In lysosyme, the is But we observed some deviations in Stern-Volmer plot (S-V plot) from linearity as a function of pH and also various with the immobilization condition. The nanolayers exhibit collective properties, that are sensitive to very minute perturbations. Sensitivity is quantified using the Stern-Volmer equation,

$$I_o/I = 1 + K_{sv}[Q]$$

Where I_o and I are the fluorescence intensity of the indicator molecule in the presence and absence of quencher, K_{sv} is the bimolecular quenching constant and [Q] is the quencher concentration. A linear stern –volmer plot generally indicates a single class of fluorophores, all of which are equally accessible to the analyte. But deviations from linearity can happen with herogenous systems like polymer films suggesting normal Stern-Volmer equation does not explains the result . Deviations are of two types; positive and negative. Positive deviation is explained using the equation

$$F_o / F = (1 + K_D[Q]) \quad (1 + Ks[Q])$$

This is the modified form of Stern- Volmer equation, which is second order with [Q], accounts for upward curvature. [13]

Some time there is the possibility of static quenching along with normal dynamic one, when the extent of quenching is large. In such case we have to consider the association constant for complex formation between the quencher and the fluorophore. And in such circumstances the S-V plot shows a pronounced upward curvature, towards the y-axis. Even though this is the case, there is no appreciable red shift the emission spectrum of lysosyme when it is in contact with the quencher molecule indicating no structural denaturation. But there is the possibility of structural confirmation as a function of the deposition pH suggesting deviation. In some case only light variation from linearity is observed towards y-axis, suggesting the inaccessibility of all the tryptophanyl residued at first, but as the concentration increases more molecules are avilable for interaction.

Negative deviation from linearity indicating the presence of different fluorophoric environment.[14] If different fluorophoric populations are present, and if only one class is accessible to analyte species, then the S-V plot deviates from linearity towards x-axis. In most cases we observed good negative deviation and is likely to happens in the case of multitryptophanyl systems like lysosyme.

These kinds of deviations are observed also as a function of immobilization conditions, indicating the role of conformational change in deviation. [15]

CONCLUSION

Therefore in the present study, we investigated the binding interaction of mercuric chloride, with lysozyme using steady state spectroscopic methods. The quenching of fluorescence of lysozyme was used to monitor the nature of binding and number of binding sites for the protein-ligand system. Conformational investigation lysozyme structure upon binding with the said ligands are carried out to understand the stability/activity after complexation. Apart from that, self assembled polyelectrolyte multilayer was developed using the layer by layer assembly method. Which recently attracted considerable attention having applications in environmental, industrial, clinical, medical and biological fields. The sensitivity of a sensor that detects analytes by interacting with molecules on the surface requires large surface area per unit mass. The layer by layer assembly technique is a novel and cost effective approach for fabricating large surface area membranes for a variety of sensor applications. The large amount of available surface area of these self assembled membranes has the potential to provide unusually high sensitivity and fast response time in sensing applications.

KEY WORDS

LYZ- Lysozyme
CHI-Chitosan
PSS-Polystyrenesulfonate
S-V- Stern Volmer Plot
FL- Fluorescence Intensity

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