

## Abstract

The *present thesis* discusses the beneficial effects of confining biologically relevant molecules inside porous structures of varying morphology and dimensions. Confinement of a biomolecule such as protein, enzymes drugs leads alteration in structural features and to a significant improvement in its biophysical properties. These properties include electrochemical redox behavior (for electroactive biomolecules) and thermal stability (denaturation temperature) of the concerned biomolecule. Silica (SiO<sub>2</sub>) based materials were primarily used as substrates for confining proteins and drugs. The confinement effects were probed in depth using various electrochemical, spectroscopic, and scattering techniques. The outcomes of confinement were utilized for developing electrochemical biosensors for the protein detection. Confinement of drugs effects their structural properties which gets reflected in their release kinetics studies. Electrochemical sensing was carried out using porous structure modified electrodes. These were used not only for detection of biological analytes but also extended to environmental pollutants. In the thesis, Chapters 2-4 deal with discussions related to electrochemical, spectroscopic, and scattering studies of protein confined inside SiO<sub>2</sub> as well as polymer capsules. In Chapter 5A and 5B, Titania (TiO<sub>2</sub>) based nanotubes were utilized for demonstration of realistic electrochemical biosensors for the detection of myoglobin and a penicillin binding protein. In Chapters 6 and 7, enzyme and inhibitors, drug release kinetics from mesoporous oxides and TiO<sub>2</sub> tubes have been discussed. Using the same approach as in chapter 5, electrochemical sensing of model environmental pollutants using TiO<sub>2</sub> microwires have been discussed in chapters 8A and B. The use of TiO<sub>2</sub> microwires for photocatalytic applications have also been considered in detail. A brief discussion of the contents and highlights of the individual chapters are described below:

**Chapter 1** discusses in detail about the porous substrates extensively used for biomolecular (proteins and drugs) confinement and structural features of these substrates. These substrates include the mesoporous materials of varying pore dimensions and pore arrangement. The alteration in the biophysical properties of the molecules because of confinement within these mesoporous substrates and its effects on related applications such as bio catalysis, drug release rates, electrochemical biosensing have been considered. A brief discussion on the present state of art in the field of drug delivery, enzyme catalysis and electrochemical biosensing have been included. The principles related to electrochemical, spectroscopic and the scattering techniques used to characterize the properties have been discussed in detail in this chapter.

**Chapter 2** include discussions on the investigations on the structure and function of hemoglobin (Hb) confined inside sol-gel template synthesized silica tubes (SiO<sub>2</sub>-tubes) Immobilization of hemoglobin inside SiO<sub>2</sub>-tubes resulted in the facile electron transfer to electroactive heme center leading to an enhanced electrochemical response. The consequences of confinement on

protein structures and activity were further probed via ligand binding and thermal stability studies. Reversible binding of n-donor liquid ligands such as pyridine and its derivatives and predictive variation in their redox potentials were obtained from detailed electrochemical investigations. The results suggested absence of adverse effect on structure and function of Hb confined inside the channels of SiO<sub>2</sub>-tubes. Additionally, the thermal stability of confined Hb was compared to that of free Hb in solution. The melting or denaturation temperature of Hb immobilized inside SiO<sub>2</sub>-tubes increased by approximately 4 °C compared to that of free Hb.

In **Chapter 3A**, the configuration of hemoglobin (Hb) in solution and confined inside silica tubes (SiO<sub>2</sub>-tubes) have been studied using synchrotron small angle x-ray scattering (SAXS) and the consequences were correlated to its electrochemical activity. Confinement inside silica tubes aided in preventing protein aggregation compared to that observed for unconfined protein in solution. In case of confined Hb, the radius of gyration ( $R_g$ ) and size polydispersity ( $p$ ) was considerably lower than in solution. The difference in configuration between the confined and unconfined protein were reflected in their electrochemical response. Reversible electrochemical response (from cyclic voltammograms) were obtained in case of the confined hemoglobin in contrary to only cathodic response for the unconfined protein in solution. This led to the conclusion of difference in orientation of the electroactive heme center. The electron transfer coefficient ( $\alpha$ ) and electron transfer rate constant ( $k_s$ ) were also calculated to further support the structural differences between the unconfined and confined states of the hemoglobin. Thus, absence of any adverse effects on confinement of proteins inside the inorganic matrices such as silica nanotubes opens new prospects for utilizing inorganic matrices as protein “encapsulators” as well as sensors at varying temperatures.

**Chapter 3B** discusses the implications of host dimensions on the protein structure. This is a very important parameter as it considerably influences the protein properties under confinement. This study probes the structure of same Hb molecules, confined inside silica tubes of pore diameters varying by one order in magnitude: ~ 20-200 nm. The confinement effect on structure was probed vis-à-vis the protein in solution. Small angle neutron scattering (SANS), which provides information on the protein tertiary and quaternary structures, was employed to study the influence of tube pore diameter on confined protein structure and configuration. Depending on the SiO<sub>2</sub>-tubes pore diameter, confinement significantly influenced the structural stability of Hb. High radius of gyration ( $R_g$ ) and polydispersity ( $p$ ) of Hb in case of the 20 nm diameter SiO<sub>2</sub>-tubes indicated that Hb undergoes significant amount of aggregation. However, for SiO<sub>2</sub>-tubes with pore diameters > 100 nm,  $R_g$  of Hb was found to be in very close proximity to that obtained from the protein data bank (PDB) reported structure. This strongly indicated that the protein has a preference for the more native like non-aggregated state when confined inside tubes of diameter ~ 100 nm. Further insight in to the Hb structure was obtained from distance distribution

function,  $p(R)$  and ab-initio models calculated from the SANS patterns. These also suggest that the size of SiO<sub>2</sub>-tubes is a key parameter for the protein stability and structure.

In **Chapter 4** we have introduced an organic substrate to investigate the effect of confinement on structure of hemoglobin (Hb). Like as discussed in chapters 3(A and B), Hb transformed from an aggregated state in solution to non-aggregated state when confined inside the polymer capsules. Synchrotron small angle x-ray scattering (SAXS) studies directly confirmed this fact. The radius of gyration ( $R_g$ ) and polydispersity ( $p$ ) of the proteins in the confined state were smaller compared to that in solution. In fact, the  $R_g$  value was very similar to theoretical values obtained using protein structures generated from protein databank. The  $R_g$  value was almost constant in the temperature range (25-85 °C,  $T_m = 59$  °C), for the confined Hb. This observation is in contrary to the increasing  $R_g$  values obtained for the free Hb in solution suggesting higher thermal stability of confined Hb inside the polymer capsule. Protein functions gets significantly altered as a result this. It resulted in an enhancement of the electroactivity of confined Hb. While Hb in solution showed dominance of the cathodic process ( $Fe^{3+} \rightarrow Fe^{2+}$ ), efficient reversible  $Fe^{3+}/Fe^{2+}$  redox response is observed in case of the confined Hb. This again gave an indication of the difference in orientation of electroactive heme group resulting it to reside in a chemically different environment compared to when it is in solution. This has important implications on protein functional properties and related applications. Thus, in this chapter we get a detailed overview of how confinement orients different groups' viz., electroactive heme center to take up positions that makes it favorable to participate in biochemical activities such as sensing of analytes from small to macromolecules and controlled delivery of drugs. The conclusions derived from the studies in previous chapters have been utilized in chapters 5A and 5B for developing a realistic electrochemical biosensor. Since the sensing based on electrochemical response largely depends on the location of heme group, the location of the heme center was altered in a controlled manner using chemical treatment.

**Chapter 5A** deals with an alternate antibody-free strategy for the rapid electrochemical detection of cardiac myoglobin (having heme center) using hydrothermally synthesized TiO<sub>2</sub> nanotubes (TiO<sub>2</sub>-NT). In this strategy, myoglobin was unfolded using denaturants to expose deeply buried electroactive heme center into the solution very close to the electrode. This leads to an efficient reversible electron transfer from protein to electrode surface. The sensing performance of the TiO<sub>2</sub>-NT modified electrodes were compared *vis á vis* commercially available titania and GCE electrodes. The tubular morphology of the TiO<sub>2</sub>-NT led to facile transfer of electrons to the electrode surface which eventually provided linear current response (obtained from cyclic voltammetry) over a wide range of Mb concentration. The sensitivity of the TiO<sub>2</sub>-NT based sensor was remarkable and was equal to 18 A/ mg ml<sup>-1</sup> (detection limit= 50 nM). This coupled with the rapid analysis time of few tens of minutes (compared to few days for ELISA)

demonstrates its potential usefulness for the early detection of the acute myocardial infarction (AMI).

**Chapter 5B** comprises of a discussion of employing a rapid electrochemical detection method of proteins without any electroactive center. The protein was transformed to an electrochemically active protein via metal tagging ( $\text{Fe}^{3+}$  in this case). This biosensor was also based on titania ( $\text{TiO}_2$ -NT) nanotubes which was used to modify the working electrode. To reduce the detection volumes drastically, screen printed carbon electrodes (SPCE) was introduced in this detection. It was possible to detect as low as  $1 \text{ ng l}^{-1}$  of protein in very small sample volumes (as low as 30 l). The feasibility of this method for the detection of PBP2a, a marker for methicillin resistant *Staphylococcus aureus* (MRSA) was demonstrated here. This biosensor could effectively detect PBP2a in whole cell lysate samples. To mimic the practical detection conditions, the selectivity and efficiency was also validated using other non-selective proteins such as PTP10D, a protein tyrosine phosphatase, and bovine serum albumin (BSA). As already mentioned, this electrochemical detection strategy could reproducibly detect protein samples within minutes compared to standard ELISA methods (3-4 h) or a modified ELISA protocols (FAST-ELISA; 30 mins) excluding the time taken for sample preparation. These observations suggest the potential of the titania nanotube based electrochemical biosensor in both clinical and community settings for the detection of infectious pathogen

In **Chapter 6**, the feasibility of utilizing mesoporous matrices of alumina and silica for inhibition of enzymatic activity have been presented. These studies were performed on a protein tyrosine phosphatase by the name chick retinal tyrosine phosphatase-2 (CRYP- 2), a protein that is identical in sequence to the human glomerular epithelial protein-1 and involved in hepatic carcinoma. The inhibition of CRYP-2 is of tremendous therapeutic importance. Inhibition of catalytic activity was examined using the sustained delivery of para nitrocatechol sulfate ( $p\text{NCS}$ ) from bare and amine functionalized mesoporous silica (MCM-48) and mesoporous alumina ( $\text{Al}_2\text{O}_3$ ). Amine functionalized MCM-48 was found to exhibit the best release of  $p\text{NCS}$  among the various mesoporous matrices studied and hence inhibition of CRYP-2 was maximum in this case. The maximum speed of reaction,  $v_{\text{max}}$  ( $= 160 \pm 10 \mu\text{mols min}^{-1}\text{mg}^{-1}$ ) and inhibition constant,  $K_i$  ( $= 85.0 \pm 5.0 \mu\text{mols}$ ) estimated using a competitive inhibition model were found to be very similar to inhibition activities of protein tyrosine phosphatases using other methods.

In **Chapter 7**, we have demonstrated another very attractive application of the  $\text{TiO}_2$ -NT which have been already used for protein sensing application. Due to the porous nature of the surface and its other attractive features,  $\text{TiO}_2$ -NT has a great potential in drug delivery applications. The  $\text{TiO}_2$ -NT mimicked the pore channels of the mesoporous substrates that have been used in the previous chapter. The drug release from these  $\text{TiO}_2$ - NT exhibited a completely different

sigmoidal release profile compared to our previous reports from the group. Additionally, the effect of surface functionalization and solution pH on drug release profile have also been considered during our studies. The release profiles were modelled with theoretical Hill equation to extract several physical parameters to explain the extent of drug substrate interactions. These results further supplemented the unique nature of the release profile.

In **Chapter 8A** we have again demonstrated an electrochemical detection strategy but this time for chemical pollutants. Commercial textile industry effluents such as dyes were chosen for the model studies. Mesoporous anatase titania microwires synthesized via an optimized polyol method were used for sensing and photocatalysis of these dyes. Using spectroscopic investigations, we have showed that these titania microwires preferentially sense cationic (e.g. methylene blue, Rhodamine B) over anionic (e.g. Orange G, Remazol Brilliant Blue R) dyes. It was observed that variation in microwire dimensions and pH of dye solution, led to an increase in the concentration of the adsorbed dye. These findings were later corroborated with much faster electrochemical sensing. The effect of microwire length on electrochemical detection sensitivity have also been accounted in these studies. The photochemical performance of these titania microwires have been compared with the commercial P25-TiO<sub>2</sub> nano powders. The photochemical performance was also studied as a function of exposure times and pH of dye solution. Excellent sensing ability and photocatalytic activity of the titania microwires was attributed to increased effective reaction area of the controlled nanostructured morphology. This makes them an attractive substrate for commercial sensing applications

In **Chapter 8B**, anatase TiO<sub>2</sub> microwires used in previous chapter were chemically modified to silver (Ag) decorated TiO<sub>2</sub> microwires (Ag-TiO<sub>2</sub>). This was done with an aim to improve the detection sensitivity and photodegradation performance. The Ag-TiO<sub>2</sub> microwires were synthesized via polyol synthesis route followed by a simple surface modification and chemical reduction approach for attachment of silver. The electrochemical sensing performance of Ag-TiO<sub>2</sub> microwires have been subsequently compared with the base TiO<sub>2</sub> microwires in the detection of cationic dye such as methylene blue. The superior performance of the Ag-TiO<sub>2</sub> composite microwires was attributed to improved surface reactivity, mass transport and catalytic property because of decorating the TiO<sub>2</sub> surface with Ag nanoparticles. Further studies were also carried out to compare its photocatalytic activity with TiO<sub>2</sub> microwires at constant illumination protocols and observation times. As demonstrated the improved photocatalytic performance of Ag-TiO<sub>2</sub> composite microwires was attributed to the formation of a Schottky barrier between TiO<sub>2</sub> and Ag nanoparticles leading to a fast transport of photogenerated electrons to the Ag nanoparticles.