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Enzyme-Polymer Covalent Conjugates and Hydrogels for Improved Enzymology and ElectroBiocatalysis

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Enzyme-Polymer Covalent Conjugates and Hydrogels for Improved Enzymology and ElectroBiocatalysis

Ananta Ghimire

University of Connecticut, 2018

Abstract

Three dimensional porous protein/enzyme materials such as hydrogels and sponges with larger surface area, tunable hydrophilicity and high permeability are highly desirable for improved electrocatalysis, filtration, adsorption, separation and biomedical applications. The use of enzymes as electrode materials in bioelectronics and biofuel cell (BFC) is mostly limited due to limited enzyme loading, lower percent enzyme electroactive and poor enzyme stability on electrode surface. The primary goal of this thesis will be to design a synthetic method to interlock enzymes on the electrode surface. This method helps overcome three major challenges: increasing the loading of enzyme on electrode surface, reducing leaching of enzyme and increasing the percent enzyme electroactive. The enzyme-polymer matrix and matrix modified electrodes were characterized and evaluated for stability, maximum current density and percent enzyme electroactive in present and absent of mediators. The covalent crosslinking of protein will be extended in the second part of the thesis to design and synthesis of protein based sponges. The proposed technique could serve as a new method to synthesize 3D porous protein based materials for biomedical, biocatalysis and environmental applications. Here in this thesis, the oil-in-water emulsion separation efficiency of BSA-Sponge was explored.
Enzyme-Polymer Covalent Conjugates and Hydrogels for Improved Enzymology and ElectroBiocatalysis

Ananta Ghimire
M.S., Tribhuvan University, 2007

A Dissertation
Submitted in partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2018
Enzyme-Polymer Covalent Conjugates and Hydrogels for Improved Enzymology and ElectroBiocatalysis

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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BFC</td>
<td>Biofuel Cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>Cyt</td>
<td>Cytochrome c</td>
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<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
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<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
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<td>FITC</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEPA</td>
<td>Tetraethylene pentamine</td>
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Chapter 1

Introduction

Redox enzyme based electrodes have attracted increasing interest because of their applications in sensing, biofuel cell and bioelectronics devices.\(^1\) However, a major concern and unmet challenge is the poor stability of enzymes in non-physiological environments such as high temperatures, extreme pHs and strong adsorption onto electrode surfaces.\(^2\) Improved stability will directly enhance shelf-life, possibility for storage at ambient temperatures for extended periods of time, electrocatalysis at high temperatures, and the possibility for producing sterile enzymes by autoclaving. A variety of approaches have been developed in our labs to expand enzymes’ functional and thermal properties by (i) intercalation in layered materials\(^3\)\(^-\)\(^7\) and (ii) introducing multiple covalent attachment points on a protein that can interact with a polymer may lead to restricted unfolding and conformational stabilization.\(^8\),\(^9\)

Our hypothesis is that when the protein molecules are isolated from each other by the polymer matrix, then the corresponding denatured molecules are less likely to aggregate and more likely to fold back to their original, native structures. Covalently attached polymer chains on the surface of the protein prevent the aggregation-induced irreversible denaturation and force the unfolded protein to refold. The goals of this thesis are: (1) Understanding the thermodynamics of protein/polymer interactions; (2) Improving the thermal stability of enzymes by conjugations with synthetic polymers; (3) Design synthesis and characterization of superstable enzymes in protein hydrogels electrodes; (4) Design, synthesis and characterization of protein based sponge for rapid oil-in-water emulsion separation.
1.1 Thermodynamics of Enzyme/polymer interactions

Understanding of the protein-polymer interactions is of current interest, and these interactions play a key role in a number of applications, where polymers are brought in contact with the biological world. The covalent conjugation of proteins with polymers, for example, involves the formation of a physical complex between the two components, prior to chemical bonding, and formation of this complex may be controlled by hydrogen bonding, electrostatic, hydrophobic and a variety of other interactions.\(^\text{10}\) Understanding these interactions is of fundamental value to predict how to design polymers that bind to proteins in a predictable manner. Here, poly(acrylic acid) (PAA) and lysozyme were used as model systems. ITC, Circular Dichroism (CD), Dynamic Light Scattering (DLS) and Zeta Potential were be used to characterize the lysozyme/PAA complex.

Very few studies are reported on the thermodynamics of such interactions\(^\text{11-14}\) and despite these studies, our understanding of factors contributing to these interactions at the molecular level is still rudimentary.\(^\text{15}\) The central question remains: can a universal molecular model be developed to quantitatively predict the best conditions to assist or inhibit the formation of the protein/polymer complex? However, answering this question is a challenging proposition due to the variability in the composition, structure, size and solubility associated with both proteins and polymers, not to mention the role of solvent, ions, temperature and pH on the binding equilibrium. To accelerate progress made in this direction, the study of protein-polymer interactions could be approached using a thermodynamic method in which binding parameters are quantitatively assessed based on physical parameters such as pH, ionic strength or temperature and subsequent analysis of
these data as a function of one variable at a time could reveal a firm molecular basis for these interactions.

Lysozyme is a small protein of mass 14 kDa with an isoelectric point of ~11 and carries a charge of ~ +14\textsuperscript{16} at pH 3.0 and ~ +7 at pH 8.0. PAA is a negatively charged polymer and is fully ionized at alkaline pH and adopts a stretched-out conformation while at acidic conditions it is extensively protonated and collapses into a coil.\textsuperscript{17} Our hypothesis is that electrostatic interactions dominate when the binding partners are oppositely charged and ‘charge neutrality of the protein-polymer interface’ drives binding is tested here. Since pH of the medium determines the net charge on lysozyme and the PAA, the binding interactions are expected to be sensitive to the ionic strength of the medium\textsuperscript{18,19}. We focused on the proton-coupled protein binding where additional changes on the protein surface charges could arise due to protonation-deprotonation accompanying the binding events.

1.2 Protein-polymer nanogels

Enzymes are marvelous catalysts and suitable candidates for many catalytically driven reactions.\textsuperscript{20,21} However many of the applications are hindered by their instability in non-physiological environments of high temperatures, extreme pH’s and organic solvents.\textsuperscript{22,23} Among these wide range of non-physiological conditions that have been used to test the stability of enzymes, steam sterilization is a benchmark of protein thermal stability and most proteins undergo irreversible denaturation under these conditions\textsuperscript{5}. Improved thermal stability also ensures improved shelf-life, possibility for storage at ambient temperatures for extended periods of time, high temperature enzymology, and the possibility for producing sterile enzymes. Enzyme applications in
industry are severely restricted due to their thermal instability, today\textsuperscript{24-26}. Studies addressing this issue are not only extremely limited but also challenging since even thermophilic proteins are deactivated under steam-sterilization conditions\textsuperscript{27}.

We have been interested in enhancing the material properties of enzymes and proteins by covalent conjugation with polymers, such as poly(acrylic acid)(PAA), a soft water soluble flexible polymer extensively used in consumer product\textsuperscript{25,28} Conjugation of enzymes with PAA enhanced the material properties of enzymes and enhanced their room temperature stability by providing a flexible scaffold around it. Here, we show that this approach can be improved further to achieve ultra-high thermal stabilities, using steam-sterilization as a benchmark for stability.

The design and fabrication of soluble protein-polymer nanogels consists of covalent crosslinking of lysine side chains of met-hemoglobin (Hb) with the carboxyl groups of PAA (Scheme 1.1). The protein-polymer nanogel was used to test the stability of protein at high temperatures and steam-sterilization conditions. Previously, we reported the first synthesis of Hb-PAA conjugates and demonstrated the role of PAA (Mw 8k) in improving the room temperature stability of Hb but not at elevated temperatures\textsuperscript{26,29}. Developing along this concept of flexible, protective-shroud around the protein, we used higher molecular weight (Mw 450k) PAA, higher mole ratios of Hb to PAA and further strengthened the polymer matrix around the protein by cross-linking it with polyamines. These modifications endowed the conjugation with high stability toward steam sterilization conditions, and this is achieved by forcing the denatured protein to fold back to the active, native-like state efficiently.
Scheme 1.1: Synthesis of Hb-PAA conjugates
1.3 Enzyme hydrogel as electrode material

There is a need for clean and green methods of electricity production from renewable sources to meet the increased demand globally. In this thesis, enzymes that are superstable and superactive are produced which are then used to catalyze key reactions to test the potential application to generate electrical energy. For example, glucose can be converted to power using enzymatic glucose based biofuel cells (BFC). BFCs are devices that use enzymes as biocatalysts to produce electrical energy from renewable fuels. In BFCs, glucose is oxidized at the anode by the enzyme glucose oxidase (GOx) or glucose dehydrogenase (GDH) while oxygen is reduced at the cathode by enzymes like laccase or bilirubin oxidase (BOD). The output of the BFC depends on enzyme loading and on percent enzyme active on electrode surface, which is limited mostly because of enzyme leaching and denaturation. Several methods have been used to improve loading and prevent the leaching of enzyme from electrode surface. This includes the use of entrapping polymer like nafion, cellulose, covalent immobilization of enzyme to the functionalized carbon electrode and formation of 3D matrix of enzyme on the electrode surface. First two methods have been extensively used in sensing but later method provides a 3D matrix for high loading of enzyme and is useful for BFC. In this thesis, we address the leaching and percent enzyme electroactive on carbon cloth electrode by forming hydrophilic biohydrogel using natural and artificial polymers using carbodiimide (EDC) chemistry. The primary goal of this thesis will be to design a synthetic method to interlock enzymes on the electrode surface. This method helps overcome two major challenges: increasing the loading of enzyme on electrode surface...
and reducing leaching of enzyme. The enzyme-polymer matrix and matrix modified CC electrodes were characterized and evaluated for stability, maximum current density and percent enzyme electroactive in present and absent of mediators.

1.4 Protein Sponge for oil/water separation

Three dimensional porous materials such as films and sponges with larger surface area and large volume with high permeability are often used for filtration, adsorption, separation and cleanups.\textsuperscript{36-39} Biocompatible and biodegradable porous materials with shape memory properties are of great importance for biomedical applications such as drug delivery and tissue regeneration.\textsuperscript{40} However, the synthesize of these porous materials often used templates, lyophilization and cryogelation techniques.\textsuperscript{41-43} The present study explains the simple methodology to develop a biodegradable BSA Sponge with shape memory characteristics. The proposed technique could serve as a new method to synthesize 3D porous protein based materials for biomedical, biocatalysis and environmental applications. Here in this chapter, the oil-in-water emulsion separation properties of BSA-Sponge will be explored.

1.5 References


3. Kumar, C. V.; Chaudhari, A., High temperature peroxidase activities of HRP and hemoglobin in the galleries of layered Zr(iv)phosphate. \textit{Chemical Communications} 2002, 0 (20), 2382-2383.


Chapter 2

Proton-coupled Protein Binding: Controlling Lysozyme/Poly (acrylic) acid Interactions with pH

2.1 Abstract

Rational design of protein-polymer composites and their use, under the influence of the stimulus, for numerous applications requires a clear understanding of protein-polymer interfaces. Here, using poly(acrylic acid) (PAA) and lysozyme as model systems, the binding interactions between these macromolecules were investigated by isothermal titration calorimetry. The binding is proposed to require and governed by ‘charge neutralization of the protein/polymer interface’ and predicted to depend on solution pH. Calorimetric data show strong exothermic binding of lysozyme to PAA with a molar $\Delta H$ and $T \Delta S$ values of -107 and -95 kcal/mol, respectively, at pH 7 and room temperature. Both $\Delta H$ and $T \Delta S$ decreased linearly with increasing pH from 3 to 8, and these plots had slopes of -17.7 kcal/mol and -17.5 kcal/mol per pH unit, respectively. The net result was that binding propensity ($\Delta G$) was nearly independent of pH but the binding stoichiometry, surprisingly, increased rapidly with increasing pH from 1 lysozyme binding per PAA molecule at pH 3 to 16 lysozyme molecules binding per PAA molecule at pH 8. A plot of stoichiometry vs pH was linear, and consistent with this result, plot of ln (average size of the protein/polymer complex) vs pH was also linear. Thus, protonation-deprotonation plays a major role in the binding mechanism. ‘Charge neutralization’ of the lysozyme/PAA interface controls the binding stoichiometry as well as the binding enthalpies/entropies in a predictable fashion, but it did not control the
binding affinity ($\Delta G$). The pH dependence of the protein binding by PAA provides a stimuli-responsive system for protein delivery.

2.2 Introduction

Thermodynamics of the interactions of lysozyme with poly(acrylic) acid has been studied by isothermal titration calorimetry, here, and the data show strong coupling of protonation with protein binding.\textsuperscript{1,2} Understanding of the protein-polymer interactions is of current interest, and these interactions play a key role in a number of applications, where polymers are brought in contact with the biological world. The covalent conjugation of proteins with polymers, for example, involves the formation of a physical complex between the two components, prior to chemical bonding, and formation of this complex may be controlled by hydrogen bonding, electrostatic, hydrophobic and a variety of other interactions.\textsuperscript{3} Understanding these interactions is of fundamental value to predict how to design polymers that bind to proteins in a predictable manner.

Very few studies, however, are reported on the thermodynamics of such interactions.\textsuperscript{4,5,6,7} and despite these studies, our understanding of factors contributing to these interactions at the molecular level is still rudimentary.\textsuperscript{8,4,5,6} The central question remains: can a universal molecular model be developed to quantitatively predict the best conditions to assist or inhibit the formation of the protein/polymer complex? However, answering this question is a challenging proposition due to the variability in the composition, structure, size and solubility associated with both proteins and polymers, not to mention the role of solvent, ions, temperature and pH on the binding equilibrium. To accelerate progress made in this direction, the study of protein-polymer interactions could be approached using a thermodynamic method in which binding parameters are
quantitatively assessed based on physical parameters such as pH, ionic strength or temperature and subsequent analysis of these data as a function of one variable at a time could reveal a firm molecular basis for these interactions.

The pH of the medium provides a good handle to evaluate the role of protonation and de-protonation that might occur prior to or during the binding event. It influences the interaction of proteins and polymers by changing the net charge on the protein, such as lysozyme, and the net charge on ionizable polymers such as poly(acrylic) acid (PAA). Therefore, pH provides a good handle in evaluating the roles of electrostatic interactions and the protonation-deprotonation equilibrium on the binding process.

Using the thermodynamic approach, the binding of PAA to met hemoglobin (Hb), for example, was examined by isothermal titration calorimetry (ITC), where binding is driven by increase in entropy.\(^9\) Approximately, 14 Hb molecules bind to 1 molecule of PAA (MW 450,000), and direct electrostatic interactions between weakly negatively charged Hb with strongly negatively charged PAA are unfavorable. Therefore, other interactions were thought to be responsible for the binding,\(^10\) but metal ions present in the solution were found to assist ‘charge neutralization’ of the protein-polymer interface and promote the binding of these similarly charged partners. These observations led to the metal ion-coupled protein binding, which appears to be more widely applicable than originally proposed.\(^11\)

In contrast to Hb, the binding of a positively charged protein such as lysozyme with the negatively charged PAA (Chart 1) may be dominated by favorable electrostatic interactions, especially under alkaline pH conditions rather than under acidic pHs. This
simple electrostatic model was tested here by examining the binding of lysozyme with PAA by ITC and the binding thermodynamics evaluated as a function of pH.

Lysozyme is an enzyme with antibacterial properties and it is capable of hydrolyzing bacterial cell walls. It is used as a food preservative, as an additive in infant milk formula to resemble protein composition of human milk, and as an analgesic for cancer patients.\(^ {12}\) It is a small protein of mass 14 kDa with an isoelectric point of \(~\text{11}\) and carries a charge of \(~\text{+14}\)\(^ {13}\) at pH 3.0 and \(~\text{+7}\) at pH 8.0. The active site of the enzyme is lined with two key carboxyl groups while the enzyme surface has several positively charged residues for interactions with anionic substrates such as the rigid cell walls.

PAA is a negatively charged polymer and its degree of ionization increases with pH as 20, 50, 65, 90 and 100% at pH values of 3.0, 4.5, 5.5, 7.0 and 8.0, respectively.\(^ {14}\) PAA is fully charged at alkaline pH and adopts a stretched-out conformation while at acidic conditions it is extensively protonated and collapses into a coil.\(^ {15}\) Therefore, the charge on PAA changes drastically with pH 3-8, while the charge on lysozymes changes only marginally over this range.

The hypothesis that electrostatic interactions dominate when the binding partners are oppositely charged and ‘charge neutrality of the protein-polymer interface’ drives binding is tested here. Since pH of the medium determines the net charge on lysozyme and the polyelectrolyte, the binding interactions are expected to be sensitive to the ionic strength of the medium.\(^ {16,17}\) We focused on the proton-coupled protein binding where additional changes on the protein surface charges could arise due to protonation-deprotonation accompanying the binding events. To our surprise, the data show that ‘charge neutrality’ controls the binding stoichiometry of the protein/polymer complex,
and the binding affinity was nearly independent of pH. The data provide a predictable model to explain the pH dependence of the binding equilibrium, the size of the protein/polymer complex as well as its stoichiometry. Our observations are enumerated below.
Chart 2.1. (A) 3D structure of lysozyme, (B) Distribution of the cationic (blue) and anionic (red) residues on the surface of lysozyme, and (C) Poly acrylic acid (MW 8,000). Lysozyme structure was from the Protein Data Bank (hydrodynamic radius, 18.9 Å).
2.3 Experimental

2.3.1 Materials. Chicken egg white lysozyme and poly(acrylic acid) (PAA, MW 8000, aqueous solution of 45% by wt.) were obtained from Sigma Aldrich (St. Louis, MO). Phosphate buffers (PB, 10 mM) with pH adjusted to 3.0, 4.5, 5.5, 7.0 and 8.0 were used to keep the same type of ions in the solution. Phosphate buffer of pH 7.0 was prepared, for example, by dissolving 0.5836 g/liter of \( \text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \) and 1.5466 g/liter of \( \text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O} \) in deionized water (DI). The pH of the solution was adjusted with phosphoric acid or sodium hydroxide, as needed.

2.3.2 Isothermal Titration Calorimetry (ITC). Thermodynamic parameters were obtained using a nanocalorimeter (VP-ITC from Microcal Inc, Piscataway, NJ) by following methods established in our laboratory.\(^{20}\) Lysozyme solution was prepared by dissolving 4.41 mg/mL of lysozyme in DI water. Stock solution of PAA (0.5 mM) was prepared by dissolving 11.55 mg/mL in DI water, as described earlier.\(^{21}\) All solutions were dialyzed against desired phosphate buffers of appropriate pH (Table 2.1).
Table 2.1: Concentration of lysozyme and PAA used in ITC at different pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Lysozyme (µM)</th>
<th>PAA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>20</td>
<td>350</td>
</tr>
<tr>
<td>4.5</td>
<td>70</td>
<td>220</td>
</tr>
<tr>
<td>5.5</td>
<td>75</td>
<td>95</td>
</tr>
<tr>
<td>8.0</td>
<td>56</td>
<td>75</td>
</tr>
</tbody>
</table>
Calorimeter was thermally equilibrated for 1 h and the sample cell was loaded with lysozyme (1.46167 mL) while the syringe has been filled with PAA solution (300 µL). Titration was carried out by adding 18 µL of PAA solution in successive injections to lysozyme solution at 300 s time intervals between injections, at a stirrer speed of 260 rpm. Prior to the titration, both solutions were degassed approximately for 30 minutes and changes in power required to maintain the same temperature between the sample and reference cells recorded and plotted. The corresponding changes in the power for the dilution of the protein and the polymer solutions were measured separately and both subtracted from the protein/polymer titration data.

The observed change in power data were converted to integrated enthalpy changes as a function of protein to polymer concentrations (Origin software v. 5.0, Microcal Inc., Piscataway, NJ). Heat released or absorbed (Q) was calculated with equation 1,

\[
Q = \frac{nM_t \Delta H V_o}{2} \left[ 1 + \frac{X_t}{nM_t} + \frac{1}{nK_bM_t} - \sqrt{1 + \frac{X_t}{nM_t} + \frac{1}{nK_bM_t} - \frac{4X_t}{nM_t}} \right]
\]

--- Equation 1

The experimental isotherms were fitted to the ‘single set of non-interacting, identical’ binding site model, which consistently gave satisfactory fits for data collected at all pHs. The value of binding constant (K_b), binding stoichiometry (n), enthalpy change (ΔH) and entropy change (ΔS) were obtained from the best fits to equation 1. All experiments were at 298 K and have been repeated at least twice to estimate errors.
2.3.3 Dynamic light scattering (DLS). Hydrodynamic radii of lysozyme and lysozyme/PAA complexes were measured using CoolBatch+ dynamic light scattering apparatus, with a Precision detector (Varian Inc. Palo Alto, CA). Samples were prepared at stoichiometric ratios obtained from ITC studies. Samples were then centrifuged and filtered using 0.2-micron syringe filter to remove the larger aggregates, if any, and were kept in 0.5 x 0.5 cm² square cuvette for DLS studies. Laser source of 658 nm wavelength at 90° geometry was used to excite the sample and all samples were equilibrated for 100 s at 25 °C and four trials were averaged at constant temperature. Precision Elucidate Version 1.1.0.9 and Deconvolve Version 5.5 from the manufacturer were used to estimate the corresponding average hydrodynamic radii of the protein/polymer complexes at 298 K.

2.3.4 Circular Dichroism (CD). Protein structural changes, if any, upon complexation of lysozyme with PAA, at particular pH values, were monitored using JASCO J-710 Spectropolarimeter in 0.05 cm path length cuvette from 200 to 260 nm at a scan speed of 50 nm/minute. Average of four accumulations collected for each sample, and data plotted using Kaleidagraph 4.1.3 (Synergy Software, Reading, PA). The spectra were normalized with respect to protein concentration and path length, and data analyzed by methods well established.24

2.3.5 Zeta Potential Measurements. A ZetaPLus (Brookhaven Instruments, Holtsville NY) with SR-516 type electrode was used to measure the zeta potentials of samples prepared in 10 mM PB (4 ml) at stoichiometric ratios determined from ITC. Three runs were averaged for each sample and zeta potentials were calculated using Smoluchowski fits with software provided by the manufacturer.
2.4 Results and Discussion

Understanding the details of protein-polymer interactions is important in designing advanced materials for various applications but only few studies have been conducted and these are yet to be fully understood.\textsuperscript{25} The simple electrostatic model predicts that the interaction requires ‘charge neutralization’ of the protein/polymer interface. This hypothesis was tested here using lysozyme and PAA by ITC, circular dichroism and light scattering studies as a function of solution pH.
**Figure 1.1.** (A) Schematic representation of lysozyme binding to PAA. (B) Addition of a solution of the polymer (56 µM, in syringe) to the protein solution (65 µM, in cell) in the calorimeter. (C) Change in power vs time plot for the titration (black curve) and the corresponding heat of dilution data are given by the red curve, recorded in PB pH 7.0 and 25 °C. (D) Enthalpy change vs [PAA]/[lysozyme]. Black line is the best fit to the experimental data according to equation 1.
2.4.1 ITC studies at pH 7. The binding of lysozyme to PAA in 10 mM phosphate buffer at pH 7.0 was carried out by adding a solution of 56 µM PAA solution in the syringe to a solution of lysozyme (65 µM) in the calorimeter (Figure 1.1B). A total 15 injections of 18 µl each were made and the heat produced during each injection has been recorded as the change in power (Δp) vs time (Figure 1.1C). The integrated area under each peak, therefore, is the heat produced during each injection.

Subsequent to each injection, the amount of the free protein available to bind to PAA progressively decreases, and the magnitude of the heat released also decreases progressively. The residual heat effects observed after the binding is saturated, seen after 11 injections in Figure 1.1C, originate from the dilution of PAA, Lysozyme/PAA complex and the mechanical effects. The enthalpy of dilution of PAA, measured during the control experiment, was subtracted from the observed titration data while the enthalpy of dilution of lysozyme was found to be negligible, under these conditions. There was no aggregation or precipitation during or after the titration, and the net heat produced during the titration was plotted as function of the molar ratio of PAA to lysozyme (Figure 1.1D). The thermodynamic parameters were obtained from the best fit to the experimental data using equation 1, which describes ligand binding to a single, identical, non-cooperative binding site. The goodness of the fit was routinely tested using a number of criteria provided by the software and best fits are chosen to extract the binding parameters.

The binding stoichiometry (n) deduced from the best fits from Figure 1.1D was that approximately 14 lysozyme molecules bind to 1 PAA molecule, or 8 monomers of PAA are occupied by 1 lysozyme. The protein packs around the polymer as beads-on-a-
string, at this pH, and this value of n approximately corresponds to the mid point of the titration curve, as expected. This agreement confirms validity of the best fit to the experimental data and evaluation of the binding parameters were then conducted.

The affinity constant ($K_b$) of lysozyme for PAA at pH 7 was found to be $1.9 \pm 0.3 \times 10^7$ M$^{-1}$ which is orders of magnitude larger than the affinity constants noted for lysozyme binding to PAA ($5.1 \times 10^4$ M$^{-1}$) or poly(vinyl) sulfonic acid ($2.7 \times 10^3$ M$^{-1}$). The previously reported binding constant of lysozyme for PAA, however, was measured at 50 mM PB which is much higher than 10 mM PB used here and this increase in affinity at lower ionic strength suggests the strong role of electrostatic interactions in the binding mechanism. We tested this hypothesis by measuring the transmission percentage (T) of lysozyme/PAA complex using uv/vis spectrophotometer, at different concentration of lysozyme/PAA and the ionic strength (pH 7.0). At lower concentration of lysozyme and PAA, the complex formed is soluble and T > 90% in 10 mM PB. At higher concentration lysozyme (100 µM) with PAA (n=7.7), the complex formed precipitate with T < 4%. The T (%) increases with ionic strength and reaches to 99% in 50 mM PB, indicating binding is greatly inhibited by the ionic strength (Figure 2.2). This decrease in affinity at higher ionic strength is consistent with previously reported theoretical studies.$^{14,16}$
Figure 2.2: Change in T (%) of lysozyme/PAA complex (100 μM lysozyme, PAA n=7.7) as a function of ionic strength (PB, pH 7.0).
The binding was strongly exothermic and the corresponding $\Delta H$ and $T\Delta S$ values estimated from the above data were $-107 \pm 1$ and $-95\pm1$ kcal/mol, respectively. The binding was entirely driven by enthalpy at an entropic penalty. In contrast, the binding of Hb to PAA was driven entirely by entropy at an enthalpic penalty. The two proteins differ in terms of their net charge, positive vs negative, and favorable electrostatic interactions between lysozyme and PAA drive this process against entropy losses. The entropy loss could be due to decreased flexibility of the polymer backbone in the complex, loss in its rotations, and losses in the rotational motions of the amino acid side chains of the protein that are present at the protein/polymer interface. Nevertheless, the simple electrostatic model appears to be adequate in describing the binding scenario.

If ‘charge neutrality’ of the protein/polymer interface is important for the binding then these parameters reflect on the enthalpy changes due to the charge neutralization and other contributions such as desolvation, H-bonding, van der Waals interactions etc. From the known charge of $+8$ on lysozyme, $-8$ on the PAA segment occupied by each lysozyme molecule at pH 7, the enthalpy change per unit charge of PAA has a minimum of $-107/(8)$ or $-13$ kcal/mol per charge. As the pH of the medium is decreased, the charge on PAA diminishes and charge on lysozyme increases. Therefore, the electrostatic contributions to $\Delta H$ are expected to decrease due to the decrease in the degree of ionization of PAA as well as lysozyme when the pH is lowered. We systematically examined the binding thermodynamics as a function of pH, from a value well below the isoelectric point of PAA to alkaline pH where PAA is fully ionized.

2.4.2 The pH dependence study. The isoelectric point of lysozyme is 11.0 and it carries $+8$ charge at pH 7 and its charge increases marginally to $+14$ at pH 3. On the other hand,
the degree of ionization of PAA decreases with decrease in pH, from 8 to 3. If electrostatic interactions are important in the binding mechanism, then the binding affinities, enthalpies, entropies and stoichiometry should vary systematically, in a predictable manner, over this pH range. Therefore, we carried out a number of titrations at pH 3.0, 4.5, 5.5, and 8.0 (Figure 2.3), and the reproducibility of the data are established in multiple runs.

The ITC curves were corrected for dilution of PAA, using the corresponding control data sets, there have been no precipitation or aggregation during or after the titrations, and the resulting data have been fitted to equation 1. All data sets were fitted satisfactorily to this model, and this fact further supports validity of the binding model. Qualitative examination of the data in Figure 2.3A shows that the binding is exothermic at all pHs examined here (pH 8.0, 7.0, 5.5, 4.5 and 3.0), while the magnitude of ΔH decreased. Quantitative values for the binding parameters were deduced from the best fits to these data sets (Kₘ, ΔH, ΔS and n), which are compiled in Table 2.2.
Figure 2.3: (A) Enthalpy change vs [PAA]/[lysozyme] at pH 3.0, 4.5, 5.5, 7.0 and 8.0. Solid lines are the best fits according to equation 1. (B) $\Delta H$ as a function of pH for lysozyme/PAA interactions at 25 °C.

Table 2.2. Values of $K_b$, $\Delta H$, $\Delta G$, $\Delta S$ and n for PAA binding to lysozyme at different pHs and the corresponding estimated electrostatic charges on PAA and lysozyme.$^{14,15}$

<table>
<thead>
<tr>
<th>pH</th>
<th>$K$ ($\times 10^7$)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$T\Delta S$ (kcal/mol)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>n</th>
<th>Charge on PAA</th>
<th>Charge on Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>1.7±0.7 x 10$^7$</td>
<td>-34 ± 1</td>
<td>-24</td>
<td>-107 ± 1</td>
<td>129.0</td>
<td>-22</td>
<td>+14</td>
</tr>
<tr>
<td>4.5</td>
<td>6.4±0.2 x 10$^7$</td>
<td>-51 ± 1</td>
<td>-43</td>
<td>-117 ± 1</td>
<td>35.2</td>
<td>-55</td>
<td>+11.5</td>
</tr>
<tr>
<td>5.5</td>
<td>2.1±0.1 x 10$^7$</td>
<td>-93 ± 2</td>
<td>-83</td>
<td>-108</td>
<td>-101</td>
<td>-72</td>
<td>+9.5</td>
</tr>
<tr>
<td>7.0</td>
<td>1.9±0.4 x 10$^7$</td>
<td>-107 ± 1</td>
<td>-95</td>
<td>-11 ± 1</td>
<td>7.7</td>
<td>-100</td>
<td>+8</td>
</tr>
<tr>
<td>8.0</td>
<td>1.3±0.1 x 10$^6$</td>
<td>-117 ± 1</td>
<td>-108</td>
<td>-10 ± 1</td>
<td>7.0</td>
<td>-111</td>
<td>+7</td>
</tr>
</tbody>
</table>
The \( \Delta H \) changed from -34 to -117 to kcal/mol when pH increased from 3 to 8, respectively, which demonstrated a strong contribution of electrostatic interactions to the binding. The \( \Delta H \) became more and more negative with increase in pH, plot of \( \Delta H \) vs pH was linear with a slope of -17.7 kcal/mol per unit pH (Figure 2.3B). As the polymer charge increased with pH, the binding is driven more and more by enthalpy, and binding is more enthalpically favorable at higher pH than at lower pH. At pH 8.0, where PAA is completely ionized and stretched out, the electrostatic interaction and the corresponding magnitude of binding enthalpy is maximized. At pH 3.0, due to extensive protonation of the carboxylate groups of PAA, the magnitude of \( \Delta H \) decreased, even when the charge on lysozyme was increased to +14.

Binding was enthalpy driven and achieved at an entropic penalty, under all pH conditions, and the magnitude of \( \Delta S \) increased with an increase in pH (Figure 3A). The corresponding T\( \Delta S \) plot was linear with a slope of -17.5 kcal/mol and this clearly shows a strong compensation for a strong decrease in \( \Delta H \). The large increase in the magnitude of \( \Delta S \) with increase in pH cannot be explained solely by considering that entropy changes are due to the two partners coming together, as this happens at all pH values examined. An alternate source for this large negative slope of the T\( \Delta S \) v pH plot could be due to protonation of functional groups at the protein/polymer interface, particularly at low pH values.
Figure 2.4. (A) Change in entropy as a function of pH and (B) plot of $\ln(n)$ as a function of pH for lysozyme/PAA interactions at 25°C.
As the pH drops, the likelihood of protonation of remaining carboxylate groups on PAA increases, and such protonation will result in the loss of entropy due to the transfer of the proton from the bulk to the lysozyme/PAA interface. Proton-coupled protein binding can explain the large negative slope of $\Delta S$ vs pH plot, and this explanation is also applied to our previous study of Hb binding to PAA, where the two partners are negatively charged. The binding was promoted by protonation of the functional groups at the protein/polymer interface and this explanation was supported by the observed increase in binding stoichiometry (n) discussed below.

The n values obtained from the best fits of the above data indicated an exponential decrease with increase in pH. That is, the number of monomers of PAA occupied by 1 lysozyme molecule decreased from 129 at pH 3.0 to 7 at pH 8.0. Note that the charge on the binding partners changes as a function of pH but at pH 3, lysozyme carries a net charge of only +14 while the net charge on PAA is much larger, -22. At the observed stoichiometry of 1 lysozyme binding to 129 monomers of PAA at pH 3, the protein/polymer complex should have excess negative charge which can destabilize it. This excess negative charge can be neutralized if carboxylate groups at the protein/polymer interface are protonated, as promoted by the negative charge field of the polymer. A plot of ln n as a function of pH is linear (Figure 2.4B) with a negative slope, which relates the increase in the binding stoichiometry with decrease in pH.
**Scheme 2.1.** Formation of distinct protein/polymer complexes at high and low pHs

![Diagram showing the formation of complexes at high and low pHs]
The above explanation is in agreement with the observed stoichiometric charge ratio, that is the ratio of ‘observed stoichiometry multiplied by the expected degree of ionization of PAA’ to ‘expected charge on lysozyme, the a given pH’. This gives the charge ratio of the lysozyme/PAA complex formed at that particular pH, and it will be equal to ‘~1’ if charge neutrality is to be maintained in the complex. The Zeta potential of lysozyme/PAA at pH 7.0 and 8.0 is nearly zero (Figure 2.5A) supporting the assertion that the negative and positive charges cancelled each other and charge neutrality is maintained. Thus binding proceeded till complete charge neutralization occurred. But this doesn’t preclude protonation and deprotonation at the protein/polymer interface. To test this possibility, we examined the change in pH when lysozyme solution (1 mM in DI, pH 7.0) was mixed with a solution of PAA (0.4 mM in DI, pH 7.0) in the stoichiometric ratio (n=7.7). The net pH increased by 0.7 units, when these two samples of equal pH were mixed together, indicating the depletion of bulk H⁺ ion concentration. Thus, protein binding and proton absorption at the interface is perhaps more significant for these two systems.

As pH decreased, stoichiometric charge ratio increased and reached 1.8 at pH 3.0. That is, a larger number of PAA monomers bind per lysozyme than anticipated from the estimated charges on the individual binding partners. As suspected, the excess negative charge of PAA in the complex (pH 3.0) can be reduced by protonating its remaining carboxylate groups. This explanation is in agreement with the observed Zeta potential (+2) of lysozyme/PAA complex (pH 3.0) prepared under the 1:1 charge stoichiometric ratio condition (equivalent to 1:1.6 molar ratio of lysozyme to PAA) in 10 mM phosphate buffer (Figure 2.5B). Thus, protonation of the polymer and its binding might be
interconnected. This interpretation is supported by the reported stoichiometric charge ratio of 1.4 for lysozyme binding to poly (sodium (sulfamate-carboxylate) isoprene).\textsuperscript{26} Essentially, 1 lysozyme molecule binds to 1 PAA molecule at low pH, while a large number of lysozymes bind to 1 PAA at high pHs (Scheme 1.1), or the enzyme is densely packed on the polymer.
Figure 2.5. Zeta potentials of lysozyme/PAA complex at (A) pH 7.0 under molar stoichiometric ratio condition. (B) pH 3.0 under charge stoichiometric ratio condition.

Figure 2.6. (A) Change in enthalpy as a function of degree of ionization of PAA and total charge per n monomers. (B) Plot of TΔS as a function of degree of ionization of PAA and total charge per n monomers.
The above thermodynamic data were further correlated with the estimated charges on PAA to establish quantitative relations between charge and the thermodynamic parameters. Plots of $\Delta H$ and $T\Delta S$ as a function of degree of ionization of PAA, or charge per binding site on the polymer occupied by lysozyme, are linear (Figure 2.6). For a particular PAA/lysozyme ratio, an order of magnitude difference in enthalpy between pH 3.0 and 8.0 indicates its charge dependency and dominance of electrostatic nature of the binding interactions.

The change in $K_b$ as function of pH is shown in table 2.2. The binding constant varied from minimum of $10^5$ to a maximum of $10^7$ M$^{-1}$, but it did not follow any specific pattern. The $\Delta G$ values obtained from the above $\Delta H$ and $\Delta S$ values did not show significant variation with pH, and remained fairly the same, within experimental error (Table 2.2). The mechanism of enthalpy-entropy compensation in protein/polymer binding explains the small variations in $\Delta G$ with pH, but this remains to be tested. We further characterized the interactions between lysozyme and PAA by physical and biochemical methods to gain further understanding of particular contributors to the binding.

2.4.3. **Circular dichroism (CD) studies.** Another source of binding free energy could arise from the loss of protein structure due to hydrophobic interactions with the polymer backbone during the binding event. We examined these contributions, if any, by monitoring the CD spectra of each set of lysozyme and lysozyme/PAA complexes at particular pHs (3.0, 5.5 and 7.0), under similar conditions. The CD spectra of unbound lysozyme at pH 7 (red line) followed that of lysozyme/PAA complex (blue curve) at the same pH (Figure 2.7A). The lysozyme/PAA complex at pH 7 indicated some
improvement in the intensities of its 211 and 222 nm UV CD bands and this could be due to tightening of the protein structure in the complex, when compared to the unbound protein. Such small improvements in structure were noted when proteins bind to solid substrates.$^{9,28}$
**Figure 2.7.** (A) Circular dichroism spectra of Lysozyme (red curve) and lysozyme/PAA (blue curve) at pH 7.0. (B) The secondary structure retention of lysozyme/PAA (red) as compared to unbound lysozyme (blue) at pH 3.0, 5.5 and 7.0.
The extent of structure retention was estimated by recording the ratio of the intensities of the 222 nm minimum of lysozyme to that of lysozyme PAA complex, both spectra recorded at the same pH. The percent structure retention at pH 3.0, 5.5 and 7.0 were compared in the bar graph (Figure 2.7B). A small decrease in structure retention (~15%) at pH 3 was noted for the lysozyme/PAA complex, and this could be due to minor structural changes induced by binding at this low pH, promoted by protonation of some of its residues. Such a decrease in the CD signal was previously observed when lysozyme was complexed with poly(sodium (sulfamate-carboxylate) isoprene) at pH 7.0.26 In the context of prior work, current data indicated that PAA has very little impact on the secondary structure of lysozyme at neutral pH but had only minor influence at pH 3. Therefore, these very minor structural changes in lysozyme cannot account for the large changes noted in the thermodynamic parameters (ΔH and ΔS), and the protein/polymer complex retained most of its secondary structure. This outcome could be of practical value for biocatalytic applications.

2.4.4. Dynamic light scattering (DLS) studies. The formation and sizes of the lysozyme/PAA complexes prepared at three different pHs (3.0, 5.5 and 7.0) were determined by DLS. The complex formed at pH 3.0 was around 68 nm, while that at pH 4.5, 5.5 and 7.0 were around 100 nm, 115 nm and 150 nm, respectively. DLS data of lysozyme, PAA and lysozyme/PAA complex are shown in Figure 2.8 and the linear increase of hydrodynamic radius of the lysozyme/PAA complex as a function of pH is shown in Figure 2.9A. Two different factors may contribute to this observed linearity: increase in negative charge on PAA stretching the polymer at high pH and increase in the stoichiometry of lysozyme binding to PAA at high pH.
Figure 2.8. DLS of lysozyme/PAA complex at (A) pH 3.0. (B) pH 4.5 (C) pH 5.5 and (D) pH 7.0

Figure 2.9. Plots of (A) radii and (B) ln(V) of the lysozyme/PAA complex as a function of pH.
The linear increase in the hydrodynamic radius of lysozyme/PAA complex with pH is in support of the ITC data presented above. At pH 8.0, ITC studies show that ~16 lysozyme molecules binds with 1 PAA molecule (16:1) or higher mass at higher pH, but this stoichiometry decreased to ~1:1 at pH 3.0 or reduced the size of the lysozyme/PAA complex. Assuming that density of the complex remained unchanged with pH, ITC studies suggest that the mass as well as the volume of the complex should increase with pH, which is independently derived from Figure 2.4A as well. This agreement between two independent approaches confirms the current findings.

The linear increase in hydrodynamic radius requires further evaluation. The volume (V) of complex is proportional to the cube of its radius (r), which could be considered as its hydrodynamic radius. As shown in Figure 2.9B, a plot of ln V as a function of pH was also linear, but plot of V vs pH was not. Therefore, the increase in the number of lysozyme molecules bound per PAA result in increased radius as a function of pH, but contributions from the changes in the structure of the porotein-polymer complex also needs to be considered. At low pH, the 1:1 complex is expected to be compact, spherical but at alkaline pH, the PAA is stretched out and large number of lysozymes binds. Thus, change in the shape of the complex also contributes to the increased radius. Such volume changes were noted in the pH dependent study of the complexation between chitosan (biopolymer of isoelectric point 6.4) and pepsin (isoelectric point, 1). Increase in pH from 3.0 to 4.0 decreased pepsin/chitosan complex size from 410 to 180 nm. This size contraction was attributed to a decrease in positive charge on the biopolymer, allowing it to collapse, and increase in the negative charge of pepsin. Thus, size measurements could be used to monitor the complex formation.
2.5 Conclusions

Stimuli responsive protein-polymers composites are useful for controlled delivery of biological cargo using pH as a stimulus. However, the molecular nature of the interactions of the protein with the polymer as a function of pH is still being developed. Here, lysozyme and PAA are used as models to investigate the thermodynamics of their interactions and to evaluate and establish rules that control molecular details of enthalpy/entropy changes, stoichiometry, size of the complex and the role of pH.

The binding of lysozyme to PAA was exothermic process from pH 3-8 and driven by enthalpy, with an entropic penalty. The exothermicity increased 3-fold, accompanied by a 5-fold increase in negative charge on PAA and 2-fold decrease in the positive charge on lysozyme, as the pH increased from 3 to 8. If the electrostatic interactions between the protein and the polymer are sole contributors to binding then the exothermicity should have increased by a factor of (5/1)*(1/2) or 2.5-fold. But this is not the case and the discrepancy between the observed and estimated values is due to other interactions that are not included in a simple pH dependent electrostatic model. For example, more H-bonds could be formed at lower pH than at higher pH and contribute to binding, as a function of pH. The ΔΔH value per unit charge, deduced from the above data, turns out to be -0.63 kcal/mol of per unit charge on lysozyme and unit charge on PAA, and suggest the strong role of electrostatic interactions on binding enthalpies but not binding free energies. This scenario is in contrast to the binding of Hb to PAA, published earlier, where both binding partners are negatively charged and the electrostatic interactions are not favorable for binding.
The strong decrease in entropy (ΔS) as a function of pH is due to the loss in various degrees of freedom when the protein/polymer complex is formed as well as proton uptake/release by the complex. At high pHs, compared to low pH, the polymer is expected to be stretched out due to the repulsion between the ionized COOH groups and polymer binding to the protein neutralizes the charge locally, and binding resembles protein beads attached to a polymer string. This is a model proposed to account for the observations here, and needs to be tested in future studies. Protein-loaded polymer could be more rigid than the unbound polymer itself. The ΔΔS value per unit charge, deduced from the above data, turns out to be -0.62 kcal/mol per unit charge on lysozyme and unit charge on PAA. At low pH values, greater number of negative charges were accommodated in the complex, stoichiometric charge ratio being 1.8, which is greater than anticipated value from the known degrees of ionization of the protein and the polymer, charge ratio of 1. Both protein binding and protonation of the COO- groups of the PAA in the protein-polymer complex can account for this discrepancy. Together, charge neutralization due to lysozyme binding and by protonation of the carboxylates proceed, at least under acidic conditions. Thus, binding and protonation proceed until charge neutralization is achieved in the lysozyme/PAA complex. This conclusion needs to be further tested with other proteins and polymers, but current data provides a strong case for continued studies along these lines.

The above scenario of binding enthalpy and entropy dependencies on pH is consistent with the observed increase in the binding stoichiometry from 1 lysozyme molecule binding per PAA at pH 3 to 16 lysozyme molecules binding per PAA at pH 8.0. However, overall binding affinities are nearly independent of pH. The physical
insights gained from this study will be of significant value in the design of novel protein-delivery systems, where the protein can be loaded onto a polymer and unloaded, under pH control for the delivery of therapeutic proteins even when the binding affinities are nearly independent of pH.

2.6 References


Chapter 3

‘Stable-on-theTable’ Biosensors: Hemoglobin-Poly (acrylic acid) Nanogel BioElectrodes with High Thermal Stability and Enhanced Electroactivity

3.1 Abstract

In our efforts toward producing environmentally responsible but highly stable bioelectrodes with high electroactivities, we report here a simple, inexpensive, autoclavable high sensitivity biosensor based on enzyme-polymer nanogels. Met-hemoglobin (Hb) is stabilized by wrapping it in high molecular weight poly(acrylic acid) (PAA, Mw 450k), and the resulting nanogels abbreviated as Hb-PAA-450k, withstood exposure to high temperatures for extended periods under steam sterilization conditions (122 °C, 10 minutes, 17-20 psi) without loss of Hb structure or its peroxidase-like activities. The bioelectrodes prepared by coating Hb-PAA-450k nanogels on glassy carbon showed well-defined quasi-reversible redox peaks at -0.279 and -0.334 V in cyclic voltammetry (CV) and retained >95% electroactivity after storing for 14 days at room temperature. Similarly, the bioelectrode showed ~90% retention in electrochemical properties after autoclaving under steam sterilization conditions. The ultra stable bioelectrode was used to detect hydrogen peroxide and demonstrated an excellent detection limit of 0.5 µM, the best among the Hb-based electrochemical biosensors. This is the first electrochemical demonstration of steam-sterilizable, storable, modular bioelectrode that undergoes reversible-thermal denaturation and retains electroactivity for protein based electrochemical applications.

3.2. Introduction
Redox enzyme based electrodes have attracted increasing interest because of their applications in sensing, biofuel cells and bioelectronic devices\textsuperscript{1}. Among many redox proteins, met-hemoglobin (Hb) is considered as a model to study direct electrochemistry-based biosensors, because of its catalytic activity and commercial availability. Although hemoglobin comprises of four iron containing heme groups, its electron transfer activity is hindered due to the extended three dimensional protein envelope around the heme, which buries the electroactive centers away from the electrode surfaces\textsuperscript{2}. As a result, only a weak redox current appears with Hb-based electrodes upon application of large over voltages. The electron transfer rate may be enhanced by the addition of promoters and mediators such as surfactants\textsuperscript{3}, polymers\textsuperscript{4} and specific nanomaterials\textsuperscript{5}. Using different techniques, Hb has been embedded in these films, which led to enhanced electron transfer rates as well as better adhesion to the electrode surface. This facilitated the use of Hb-based electrodes for biosensing in food, pharmaceutical, clinical and environmental issues. Despite all this progress, a major concern and unmet challenge is the poor stability of Hb and Hb encapsulated derivatives under non-physiological environments such as high temperatures, extreme pHSs and electrode surface\textsuperscript{6}.

Improved thermal stabilities of enzyme electrodes will directly enhance the shelf life for storage at ambient temperatures and facilitate biocatalysis and electrocatalysis at higher temperatures. Particularly, steam-sterilizable bioelectrodes will be useful for biomedical applications. Therefore, there is an urgent need to develop methods to stabilize Hb, as an example, and characterize these new materials for use as bioelectrodes.
A variety of approaches were developed to enhance protein thermal stability, such as (i) intercalation in layered materials\textsuperscript{7-14} and (ii) introducing multiple covalent attachment points to a polymer which may lead to restricted unfolding and conformational stabilization\textsuperscript{15,16}. Protein properties are enhanced by forming hydrophilic matrix around the protein\textsuperscript{17} via the construction of enzyme polymer conjugates\textsuperscript{18-23}.

We are interested in enhancing storage, shelf-life and thermal stabilities of enzymes and proteins by covalent conjugation with water soluble, flexible polymers such as poly(acrylic acid) (PAA)\textsuperscript{24}. The synthesis and characterization of Hb-PAA nanoparticles using low molecular weight PAA (Mw 8k) was reported earlier and these withstood steam sterilized conditions without significant loss of structure or biological activities. However, the particle nature of Hb-PAA-8k nanoparticle platform is not favorable for electrochemical application because Hb is encapsulated in the polymer nanoparticles, potentially hindering the accessibility of Hb active center to the electrode surface. Also, the nanoparticles limit the number of Hb molecules that could be brought close to the electrode surface and some Hb molecules may be far from the electrode surface. Conversely, protein-polymer nanogels could be more amenable to enhance electrical contact between the redox active sites and the electrode surfaces. However, when low molecular weight PAA was used to form Hb-PAA nanogels, Hb was not protected against steam sterilization conditions (122 °C, 17-20 psi, 10 minutes)\textsuperscript{24}.

To amend these above issues, we currently report the use of high molecular weight PAA (Mw 450k) to cross-link Hb molecules and form unique Hb-nanogels such that their thermal stability as well electroactivity at the electrode surface may be improved. This can be due to increased rigidity to the local environment surrounding Hb
by the higher molecular weight polymer. The mole ratio of Hb:PAA was optimized and its molecular weight increased such that low overlap concentrations of the high molecular weight PAA would favor nanogel formation instead of nanoparticles. Also, the mole ratio of the crosslinking reagent (carbodiimide) to the number of COOH groups of PAA that are available for linking to the protein has been optimized systematically to produce water-soluble, highly active and thermally stable nanogels. Furthermore, PAA matrix around Hb was further strengthened by cross-linking carboxylic acid groups of PAA with tetraethylenepentamine (TEPA) or polyenthyleneimine (PEI) using the carbodiimide chemistry.

In this manuscript, by optimizing the $M_w$ of PAA, reactant mole ratios and the crosslinking conditions, we obtained novel Hb-PAA nanogels that are stable at high temperatures and also show excellent electrochemical behavior over extended periods of time, which has not been realized before to the best of our knowledge. We also show that Hb by itself has poor adhesion to the electrode surface and poor electron transfer rates (Scheme 1, top) while the nanogels are superior. Also, Hb in these nanogels undergoes reversible thermal denaturation, while Hb by itself does not (Scheme 1, bottom).
**Scheme 3.1**: Hemoglobin binding to PAA (middle) and their corresponding cyclic voltammograms (right) and TEM's (left).
More importantly, we exploit this general approach of random conjugation of Hb
to PAA to build, for the first time, excellent bioelectrodes, which not only show better
adhesion to the electrode surface and enhanced electron transfer rates but also better
function at elevated temperatures and these are sterilizable in an ordinary autoclave
without significant loss of electroactivity.

3.3. Experimental Section

Poly(acrylic acid ) (Mw 450k, Mw 8k), polyethyleneimine (PEI, 8k),
tetraethylene pentamine (TEPA), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide
(EDC), o-methoxyphenol, hydrogen peroxide (H₂O₂) were purchased from Sigma-
Aldrich (St. Louis, MO). Bovine hemoglobin was obtained from MP Biomedicals
(Solon, OH).

3.3.1 Synthesis of Hb-PAA nanogel conjugates

PAA stock solution (2 wt% in water) of pH 7 was prepared by dissolving
appropriate amount of PAA in DI water and pH was adjusted by adding concentrated
NaOH. Hb stock solution (26 mg/ml) was prepared by slowly dissolving the enzyme in
10 mM phosphate buffer at pH 7. According to the amount of PAA and ratio of PAA:
EDC, Table 3.1, EDC was mixed with PAA and stirred for 15 minutes to activate the
PAA. Hb in phosphate buffer was then added to EDC activated PAA solution. The
reaction was stirred gently for four hours. The reaction mixture was dialyzed three times
using 25k dialysis membranes against 10 mM phosphate buffer pH 7. Dialyzed
conjugates were used for further experiments. The samples were referred to as Hb-PAA-
450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) based on the molar ratio of Hb:PAA:EDC.
Therefore, Hb-PAA-450k(1:0.8:1.5) has Hb:PAA ratio as 1.3:1 and Hb-PAA-450k(1:0.3:1.5) has 3.4:1.

3.3.2 PAA-Hb nanogel conjugates cross-linking with polyamines

PEI, Mw 8k and TEPA were used to cross link selected Hb-PAA conjugates, for example, Hb-PAA-450k(1:0.8:1.5). Stock solutions of polyamines were prepared in DI water and the pH was adjusted to 7 using concentrated HCl. Only 0.2% of COOH groups were modified using TEPA (denoted as Hb-PAA-450k(1:0.8:1.5)-TEPA) and 0.7% of COOH were modified with PEI (denoted as Hb-PAA-450k(1:0.8:1.5)-PEI). Before adding polyamines to the Hb-PAA conjugate, EDC was added to the conjugate (EDC:COOH ratio is 2:1) and stirred 15 minutes. After addition of TEPA the reaction mixture was stirred another 4 hours and dialyzed using 25k dialysis membranes with 10 mM phosphate buffer pH 7 three times.
Table 3.1. Synthesis conditions of small library of Hb-PAA conjugates.

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<tr>
<th>Molar Ratio = 1 Hb/0.3 PAA</th>
<th>Entry</th>
<th>PAA (mg/mL)</th>
<th>EDC/COOH</th>
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a L = liquid, TL = thickened liquid, G = homogeneous gel, HG = heterogeneous gel

Table 3.2. Synthetic conditions used to make Hb-PAA conjugates.

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<tr>
<th>Sample</th>
<th>Hb:PAA (Mole Ratio)</th>
<th>Hb:PAA (Mass Ratio)</th>
<th>EDC:COOH</th>
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<td>1 Hb:2.1 PAA</td>
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<td>1 Hb:5.5 PAA</td>
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* Hb-PAA450k(1:0.3:0.5) synthesis and characterization was reported previously1.
3.3.3 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was used to determine the morphology of the Hb-PAA conjugates, Hb and crosslinked Hb-PAA conjugates. Tecnai T12 instrument operating at an accelerating voltage of 120 kV was used to obtain TEM images. Hb concentration was 0.026 mg/ml and PAA concentration was 0.007 mg/mL in the samples that were used for TEM experiment. A drop of each sample solution was deposited on a copper grid covered with Fomvar film. Excess solution was blotted away with a piece of filter paper to leave a thin layer of solution on the grid. The sample was left to dry in air, and then stained with Uranyl acetate for 30 minutes prior to taking images.

3.3.4 Circular Dichroism studies

Secondary and tertiary structure retention of Hb after chemical modification in the presence and absence of PAA was determined using circular dichroism studies. Far UV and Soret CD spectra of Hb, and those in the presence of PAA were recorded using Jasco 710 spectropolarimeter. All samples were in PBS pH 6.4 buffer and the buffer spectrum was subtracted during processing. Step resolution was kept at 0.2 nm/data point and bandwidth and sensitivity were 1 nm and 20 milli degrees, respectively. When collecting far UV CD spectra each sample was scanned from 200 nm to 260 nm and scan speed was maintained at 50 nm/min. Average of four accumulations were recorded using 0.05 cm path length cuvettes. Same samples were used to collect Soret CD spectra and each sample was scanned from 350 to 450 nm and the scan speed was 50 nm/min. Path length used was 1.0 cm and eight accumulations were averaged to get each spectrum.

3.3.5 Catalytic activity studies
The catalytic activities of the Hb-PAA nanogel conjugates were evaluated in term of the peroxidase activity of Hb by following reported methods\(^\text{24}\) The substrate, O-methoxyphenol (2.5 mM) and the oxidant, H\(_2\)O\(_2\) (1 mM) were added to the solution containing 1 \(\mu\)M Hb in phosphate buffer pH 7.4.

### 3.3.6 Heated and cooled

Hb, Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5), Hb-PAA-450k(1:0.8:1.5)-TEPA and Hb-PAA-450k(1:0.8:1.5)-PEI samples were heated at 80 °C for 30 minutes in an oil bath followed by cooling for 1 h and 24 h at room temperature. Activity studies were performed using molecular devices; flex station 3, plate reader (Sunnyvale, CA) at room temperature after cooling the samples.

### 3.3.7 Steam sterilization

Hb, Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5), Hb-PAA-450k(1:0.8:1.5)-PEI and Hb-PAA-450k(1:0.8:1.5)-TEPA were steam-sterilized using Amsco Century Scientific (Model SI-120, Steris, Mentor, OH). All liquid samples were steam sterilized for 10 minutes at 122 °C (17-20 psi). Activity studies were performed after samples were cooled for 1 h and 24 h at room temperature, using molecular devices; flex station 3, plate reader (Sunnyvale, CA).

### 3.3.8 Calculating \(K_M\) and \(V_{\text{max}}\) values

\(K_M\) and \(V_{\text{max}}\) values of Hb and Hb-PAA conjugates were determined by performing peroxidase activity at different \(o\)-methoxyphenol concentrations (0.5 mM to 4 mM) using an HP 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). Lineweaver-Burk plots were constructed by plotting inverse initial rate versus inverse \(o\)-methoxyphenol concentrations. Michaelis-Menton parameter (\(K_M\)) and
the maximum velocity ($V_{\text{max}}$) were extracted from the plot. The concentration of Hb part in all cases was kept at 1 $\mu$M and the H$_2$O$_2$ concentration was 1 mM.

3.3.9 Dynamic light scattering (DLS)

Hydrodynamic radius of Hb and Hb-PAA conjugates were measured using CoolBatch+ dynamic light scattering apparatus, where a Precision detector (Varian Inc.) using a 0.5 x 0.5 cm$^2$ cuvette and 658 nm excitation laser source at 90 °C geometry. Samples (0.5 nM Hb or 0.5 nM Hb-PAA in phosphate buffer pH 7.4) were filtered with 0.2-micron filter (PVDF, 13 mm, Fisher Scientific) prior to the measurements. All samples were equilibrated for 300 s at 26 °C and 5 repetitions with 60 accumulations were done at the same temperature. Precision Elucidate Version 1.1.0.9 was used to run the experiment and Deconvolve Version 5.5 was used to process the data.

3.3.10 Elemental analysis

To confirm the covalent conjugation of PAA (Mw 450k) to Hb, elemental analysis was carried out with conjugates samples and compared the results with unmodified Hb. All samples after covalent conjugation were dialyzed in 25k Mw dialysis tubing against deionized water to remove any unreacted EDC. After that water from the sample was removed by freeze-drying using FreeZone 6 Liter Console Freeze Dry System from LABCONCO (catalog no. 7753024). Freeze-drying was carried out for three days under -25 °C temperature and 0.024T pressure using the above instrument. After that samples were sent to Galbraith Laboratories Inc. for elemental analysis.

3.3.11 Electrochemistry
Cyclic Voltammetry and amperometric experiments were carried out using a Model CHI 601C electrochemical workstation (CH Instruments, USA). A conventional three-electrode system was used with glassy carbon (GC) as working electrode, Ag/AgCl as reference electrode and platinum as counter electrode. Before electrochemical measurement, the GC electrodes were polished with alumina powder (1 µm and 50 nm respectively). Then the electrode is rinsed carefully with deionized water followed by sonication. After drying, 10 µL of 5 µM Hb-PAA nanogel conjugates was drop casted onto the electrode surface and dried in vacuum for five hours. EC experiments were carried out in PBS (0.1 M, pH 7.4). Prior to experiment, all the solutions were purged with nitrogen for 30 minutes and the nitrogen environment is maintained through the experiment. The charge Q under the reduction peak is calculated by integrating the area under the peak using CHI software.

3.4 Results and Discussion

3.4.1 Conjugate synthesis

The nanogel conjugates were synthesized by crosslinking Hb and PAA450k using EDC with 1:x mole ratios of Hb to PAA45k, and y:1 mole ratios of EDC to COOH groups in PAA. The conjugates are named as Hb-PAA-450k(1:x:y). We test the hypothesis that the thermal stability and resistance to steam sterilization conditions (122 °C, 40 minutes, 17-20 psi) will improve by conjugation with the polymer in nanogels by systematically increasing the amount of PAA covalently attached to the protein. Mole ratio of Hb:PAA was altered from 1: 0.3 to 1: 0.8 such that increased PAA mole ratio augmented the average number of polymer chains attached per protein molecule. This resulted in a thicker polymer matrix around the protein, which could allow for improved
stability by inhibiting protein denaturation due to the reduced entropy effect. The polar, water-soluble, ionic polymer shell prevents protein aggregation and it could enhance the reversibility of protein denaturation.

Most mammalian hemoglobins have roughly 44 lysine residues\textsuperscript{24} per each molecule and most of these amines could be attached to the carboxyl groups of PAA chains using EDC chemistry. The mole ratio of EDC:COOH was increased from 0.13 to 1.5, by more than an order of magnitude to evaluate the influence of the degree of crosslinking on the chemical, physical and biological properties of the Hb-PAA conjugate. Our hypothesis is that strengthening of the polymer shell around the protein would increase protein stability and also improve the extent of reversibility of denaturation. Using EDC chemistry lysine –NH\textsubscript{2} groups of Hb are conjugated with carboxyl groups of PAA (Mw 450k). Again, the carboxyl groups of PAA are activated using EDC and the polymer chains are further crosslinked using polyamines TEPA and PEI. Thus, the conjugate was rationally designed and engineered to have the best thermal stability while retaining its enzyme-like activities as well as its secondary structure, as presented below.

Increase in PAA wt\% as well as the mole ratio of EDC:COOH led to rapid heterogeneous gelation (Table 3.1). The samples were labeled based on visible observation of the sample viscosity as L= liquid, TL= thickened liquid, G= homogeneous gel, HG= heterogeneous gel, as in case of Hb-PAA-450k(1:3:0.5) increased molar ratio of PAA resulted in the formation of thickened gel. From this library of Hb-PAA conjugates (Table 3.1 and 3.2) two conjugates Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) of low viscosity were selected for further studies to evaluate their
activity, structure and electrochemistry. Hb-PAA-450k(1:0.3:1.5) has nearly three-times excess EDC per COOH of PAA when compared to previously reported Hb-PAA conjugate\textsuperscript{24}. Therefore, comparison of these three samples revealed the influence of increased conjugation and increased PAA content of the conjugates.

Effect of further rigidification of the local environment was evaluated by cross linking Hb-PAA-450k(1:0.8:1.5) with two different polyamines, TEPA and PEI using EDC chemistry (\textbf{Table 3.3}). The influence of polyamine crosslinking was evaluated in steam sterilization studies.
Table 3.3. Synthetic conditions used to make Hb-PAA-450K(1:0.8:1.5)-amine conjugates. Purified Hb-PAA-450K(1:0.8:1.5) sample were subjected to following synthetic condition to achieve Hb-PAA-450K(1:0.8:1.5)-TEPA and Hb-PAA-450K(1:0.8:1.5)-PEI conjugates. Only 0.2% of total COOH on Hb-PAA-450K(1:0.8:1.5) were modified with the amine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>NH$_2$:COOH</th>
<th>EDC:COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb-PAA-450k(1:0.8:1.5)-TEPA</td>
<td>0.002</td>
<td>0.004</td>
</tr>
<tr>
<td>Hb-PAA-450k(1:0.8:1.5)-PEI</td>
<td>0.002</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Table 3.4. Sizes of conjugates from DLS studies.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb-PAA-450k(1:0.8:1.5)</td>
<td>84.8 nm (94%)</td>
</tr>
<tr>
<td>Hb-PAA-450k(1:0.3:1.5)</td>
<td>62.6 nm (92%)</td>
</tr>
<tr>
<td>Hb-PAA-450k(1:0.8:1.5)-PEI</td>
<td>59.4 nm (84%)</td>
</tr>
<tr>
<td>Hb-PAA-450k(1:0.8:1.5)-TEPA</td>
<td>68.2 nm (82%)</td>
</tr>
</tbody>
</table>
3.4.2 *Agarose gel electrophoresis*

Agarose gel electrophoresis confirmed the covalent attachment of Hb to PAA ([Figure 3.1A](#)). The gel was run in 40 mM Tris acetate at pH 6.5. At this pH, Hb is slightly positively charged and migrated toward the negative electrode, while Hb-PAA samples migrated toward the positive electrode due to the excess negative charge on PAA. Hb (lane 1) migrated towards the negative electrode and lanes 2 & 4, contained the physical mixtures of Hb and PAA with compositions same as those of Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5). The physical mixtures indicated unbound Hb and mere presence of PAA did not alter Hb migration. In sharp contrast to these lanes, the conjugates Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) (lanes 3 and 5, respectively) indicated movement in the opposite direction and indicated essentially complete conjugation of Hb to PAA, and there has been no detectable amounts of unbound Hb in these lanes.
Figure 3.1. Covalent conjugation of Hb with PAA shown by agarose gel and TEM’s. A) Agarose gel of Hb and Hb-PAA conjugates (40 mM Tris acetate pH 6.5). Lane 1, Hb; lane 2, Hb/PAA-450k(1:0.8:1.5) physical mixture; lane 3, Hb-PAA-450k(1:0.8:1.5); lane 4, Hb/PAA-450k(1:0.3:1.5) physical mixture, and lane 5, Hb-PAA-450k(1:0.3:1.5). Hb migrated towards negative electrode (lane 1) and Hb-PAA migrated (lanes 3 and 5) towards the positive electrode due to the negatively charged carboxyl groups of PAA conjugated to Hb. B) TEM image of Hb C) TEM image of Hb-PAA-450k(1:0.8:1.5). D) Hb-PAA-450k(1:0.8:1.5)-TEPA, E) Hb-PAA-450k(1:0.8:1.5)-PEI and F) Hb-PAA-450k(1:0.3:1.5). All TEM’s are after staining with uranyl acetate.
3.4.3 Elemental analysis

Conjugates showed decrease in nitrogen content and increase of carbon content when compared to the corresponding values of Hb. The C:N ratio for Hb is 3.35 while those of Hb-PAA-450k(1:0.3:1.5), Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.8:1.5)-TEPA and Hb-PAA-450k(1:0.8:1.5)-PEI were 5.76, 8.26, 7.98 and 5.64, respectively. The increased C:N ratio proves that the carbon-rich PAA has been conjugated to Hb. Hb-PAA-450k(1:0.8:1.5) showed higher C:N ratio than Hb-PAA-450k(1:0.3:1.5), since mole ratio of Hb:PAA used with Hb-PAA-450k(1:0.8:1.5) was 1:0.8 while that of Hb-PAA-450k(1:0.3:1.5) has been only 1:0.3. In case of cross-linked samples, Hb-PAA-450k(1:0.8:1.5)-PEI showed lesser C:N ratio when compared to Hb-PAA-450k(1:0.8:1.5)-TEPA, since PEI is a polyimine with higher nitrogen content when compared to TEPA. These data strongly support conjugate synthesis and further crosslinking.

3.4.4 TEM and DLS

Morphology of Hb-PAA conjugates were determined using TEM (Experimental in Supplementary Information). Figure 1B shows the TEM image of the Hb in which discrete particles were noted. The TEM of Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) (Figure 3.1C and 3.1F) samples clearly show nanogel structures that resulted from cross linking between Hb and PAA. TEM images are consistent with nanogel structure formation with 450k molecular weight PAA as reported earlier. Extensive network structure was observed with Hb-PAA-450k(1:0.8:1.5)-TEPA and Hb-PAA-450k(1:0.8:1.5)-PEI conjugate samples which arise from excess cross-linking between PAA matrixes (Figure 3.2 A and 3.2B). As previously reported lower molecular
weight PAA (8k) resulted in nanoparticles but in the current case higher molecular weight PAA (450k) resulted in nanogels. As expected, higher PAA mole ratio (Hb-PAA-450k(1:0.8:1.5)) gave nanogels of larger size compared to that of lower mole ratio (Hb-PAA-450k(1:0.3:1.5)). Similarly, the overall size of the Hb-PAA nanogel decreased upon cross-linking.

Dynamic light scattering (DLS) data (Table 3.4) showed particle sizes of 85 nm for Hb-PAA-450k(1:0.8:1.5) and 63 nm for Hb-PAA-450k(1:0.3:1.5) conjugates. Particle size decreased when cross linked with polyamines, 70 nm for Hb-PAA-450k(1:0.8:1.5)-TEPA and 60 nm for Hb-PAA-450k(1:0.8:1.5)-PEI. The size and formation of soluble nanogels from DLS corroborated well with the TEM and agarose gel electrophoresis data.
Figure 3.2. A) Absorbance spectra of Hb, Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5). B) Far UV CD spectra of Hb, Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5). C) Far UV CD spectra of Hb, Hb-PAA-450k(1:0.8:1.5)-PEI(green) and Hb-PAA-450k(1:0.8:1.5)-TEPA(orange). D) Kinetic traces of Hb, Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5). All data collected in 10 mM phosphate buffer pH 7.4.
**3.4.5 Protein Structure determination**

The Soret absorbance bands of Hb-PAA(1:0.8:1.5) and Hb-PAA(1:0.3:1.5) matched well with that of Hb (Figure 3.2A). The small shifts in the peak positions are negligible, and similar shift of the Soret band was observed upon PEGylation or methylation of Hb. If heme were to be removed from its binding pocket during our conjugation reaction, the Soret peak would have shifted blue to almost ~385 nm, which did not happen with our Hb-PAA samples.

The protein secondary structure was assessed by circular dichroism (CD) spectra (Figure 3.2B) and the CD spectra of Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.8:1.5)-TEPA and Hb-PAA-450k(1:0.8:1.5)-PEI were compared with that of Hb. All spectra were corrected for Hb concentration, path length and baseline (Figure 3.3C). Peak positions and intensities of conjugates in the 190-250 nm, which is characteristic of the protein secondary structure, were consistent with the peak positions of unmodified Hb, and thus indicating significant retention of protein secondary structure for the conjugates. The far UV CD spectrum of Hb (black line) has a peak maximum at 195 nm and two minima at 210 and 220 nm. Same positions were observed for the conjugates; Hb-PAA-450k(1:0.8:1.5) (blue line), Hb-PAA-450k(1:0.8:1.5)-PEI and Hb-PAA-450k(1:0.8:1.5)-TEPA (Figure 3.3C) with 70-90% retention of the peak intensities.

**3.4.6 Peroxidase-like activity**

Prior to doing the electrochemical work, the conjugates were further characterized to ensure the retention of the redox activities of the heme center present in Hb in the conjugates. Even though Hb does not function as an enzyme in biological systems, its
peroxidase-like activity is well known and it has been used to assess the biochemical competence of hemoglobin\textsuperscript{27}.
Figure 3.3. A) Specific activities (compared to Hb, 100%) of Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5), and Hb-PAA-8k(1:100:1) at room temperature. B) Lineweaver-Burk plots for peroxidase activities of Hb, Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) samples. C) Comparison of specific activities of Hb (compared to Hb, 100%), Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5), and Hb-PAA-8k(1:100:1) before (red) and after (blue) steam sterilization (cooled for 24 h). Each bar is an average of three trials. All reactions were carried out in the presence of 1 µM protein and 1 mM H$_2$O$_2$ in phosphate buffer pH 7.4, at room temperature.

Figure 3.4. Specific activities (compared to Hb, 100%) of Hb-PAA-450k(1:0.8:1.5)-PEI, Hb-PAA-450k(1:0.8:1.5)-TEPA at room temperature. Hb concentration was 1 µM and the H$_2$O$_2$ and guaiacol concentrations were 1 mM and 2.5 mM, respectively. All activity traces were collected at room temperature in phosphate buffer pH 7.4.
Peroxidase-like activities of Hb and its conjugates were monitored by following the oxidation of guaiacol (substrate) by hydrogen peroxide (oxidant). The resulting product absorbs at 470 nm and kinetics were determined from the initial slopes (Figure 3.2D). Specific activities of Hb and Hb-PAA conjugates (Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5)), (Hb-PAA-450k(1:0.8:1.5)-PEI and Hb-PAA-450k(1:0.8:1.5)-TEPA) were estimated from the kinetic data (Figure 3.3A and Figure 3.4). The activities of the conjugates are shown relative to Hb as 100%.

Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) indicated improved activities (150%) when compared to that of Hb and these are better than that of Hb-PAA-8k (4th bar). The gains were less when the conjugates were cross-linked with polyamines (Figure 3.4). Analysis of the initial rates with increasing substrate concentrations but under similar conditions of buffer, ionic strength and pH by Michaelis-Menton plots and Lineweaver-Burk plots (Figure 3.3B) to obtain the Michaelis-Menton constant (K_M) and the maximum velocity (V_max) (Table 3.5) Figure 3.3B.
Table 3.5. $K_M$, $V_{\text{max}}$, $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ values for Hb, Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) samples. $V_{\text{max}}$ values of Hb-PAA conjugates are comparable to Hb and $K_M$ values decreased considerably for Hb-PAA conjugates in comparison to Hb.

<table>
<thead>
<tr>
<th></th>
<th>$K_M$</th>
<th>$V_{\text{max}}$</th>
<th>$k_{\text{cat}}$</th>
<th>$k_{\text{cat}}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>0.76 mM</td>
<td>0.345 $\mu$M s$^{-1}$</td>
<td>0.345 s$^{-1}$</td>
<td>4.54 x 10$^2$ M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>Hb-PAA-450k(1:0.8:1.5)</td>
<td>0.10 mM</td>
<td>0.332 $\mu$M s$^{-1}$</td>
<td>0.332 s$^{-1}$</td>
<td>3.32 x 10$^3$ M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>Hb-PAA-450k(1:0.3:1.5)</td>
<td>0.15 mM</td>
<td>0.383 $\mu$M s$^{-1}$</td>
<td>0.383 s$^{-1}$</td>
<td>2.55 x 10$^3$ M$^{-1}$ s$^{-1}$</td>
</tr>
</tbody>
</table>
The Vmax values of Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) are comparable to Hb, but the $K_M$ values decreased seven fold (Hb-PAA-450k(1:0.8:1.5)) when compared to that of Hb. Decreased $K_M$ indicates increased substrate affinity to the Hb active site. Hb-PAA-450k(1:0.8:1.5) has smaller $K_M$ than Hb-PAA-450k(1:0.3:0.5). This would directly increase the overall rate of the catalytic reaction, which is appreciated for practical applications.

3.4.7 Reversibility of thermal denaturation

Prior to steam sterilization of the samples, we initially tested the thermal stability of the conjugates by heating the samples beyond the denaturation temperature (80 °C). If the denaturation is reversible, then the heat treated samples would have considerable retention of the Soret band and the catalytic activity. Absorbance spectra of Hb, Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) samples before heating, at 80 °C and after cooling down to room temperature (Figure 3.5) showed that both Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) retained ~90% of the Soret band absorbance while Hb lost almost 50% of the Soret band intensity (Figure 3.5-B and C). The conjugates underwent reversible thermal denaturation and these conclusions are tested by CD spectra.

The Soret CD spectra of Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5) and Hb before and after heating to 80 °C (Figure 3.6). Hb-PAA-450k(1:0.8:1.5) and Hb-PAA-450k(1:0.3:1.5) conjugates retained ~ 50% of their initial Soret CD while Hb lost almost 70% of the band intensity. Hence, it is evident that at least ~50% of the sample has refolded after thermal denaturation and cooling to room temperature and the extent of recovery depended on cooling time.
**Figure 3.5.** Absorbance spectra of A) Hb B) Hb-PAA-450k(1:0.8:1.5) and C) Hb-PAA-450k(1:0.3:1.5) at room temperature (red line), heated at 80 °C (black line) and cooled for 24 h (blue dash line) at room temperature. All the spectra were corrected for Hb concentration and samples were in 10 mM phosphate buffer pH 7.4.

**Figure 3.6.** Soret circular dichroism spectra of A) Hb B) Hb-PAA-450k(1:0.8:1.5) and C) Hb-PAA-450k(1:0.3:1.5) at room temperature (red line), heated at 80 °C (black line) and cooled for 24 hrs (blue dashed line) at room temperature. All spectra were normalized with respect to protein concentration and path length (10 mM phosphate buffer pH 7.4).
The influence of heating and cooling on the peroxidase activities were determined to
gauge the extent of recovery of activity, not just the secondary structure observed in the
CD studies. Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.8:1.5)-TEPA and Hb-PAA-
450k(1:0.8:1.5)-PEI heated to 80 °C and cooled only 1 h retained 75-85% of the initial
activities (Figure 3.7) while Hb retained only ~15% of its initial activity. Similarly, Hb-
PAA-450k(1:0.3:1.5) retained 50% of its initial activity upon heat treatment followed by
cooling for 1 hour. The activities of Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5),
Hb-PAA-450k(1:0.8:1.5)-PEI and Hb-PAA-450k(1:0.8:1.5)-TEPA improved after
cooling for 24 h to 70%, 65%, 95% and 90%, respectively. The crosslinking with the
polyamines improved the extent of activity retention, in support of our hypothesis. These
data prompted us to test the thermal stabilities of the conjugates upon exposure to steam
sterilization conditions as a benchmark for thermal stability.
Figure 3.7. Specific activities (compared to Hb as 100%) of Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.3:1.5), Hb-PAA-450K(1:0.8:1.5)-PEI and Hb-PAA-450k(1:0.8:1.5)-TEPA at room temperature, after heating to 80 °C and cooling for 1 h, and 24 h. Hb concentration was 1 µM and the H₂O₂ and guaiacol concentrations were 1 and 2.5 mM, respectively. All activity traces were collected at room temperature in phosphate buffer, pH 7.4.
3.4.8 Stability towards steam sterilization

Peroxidase-like activities of conjugates were monitored after the samples were steam sterilized (10 minutes at 122°C and 17-20 psi, and cooling for 30 minutes) and cooled back to room temperature for 24 h (Figure 3.3C). The samples, Hb-PAA-450k(1:0.8:1.5), Hb-PAA-450k(1:0.8:1.5)-PEI and Hb-PAA-450k(1:0.8:1.5)-TEPA retained almost 80-90% of their initial activities, while Hb retained only 20% of its initial activity. Thus, (1) re-folding of denatured Hb depends on the extent of crosslinking, cooling time and it is a kinetic phenomenon. Thus, in addition to the use of PAA to prepare and stabilize conjugates, polyamine cross-linking enhanced protein re-folding.

3.4.9 Direct electrochemistry of Hb-PAA nanogels

Encouraged by the above improvements in the thermal stabilities of the conjugates and due to their nanogel morphology, we hypothesized that these could serve as good model systems to evaluate their function in bioelectrodes. Direct electron transfer of Hb-derivatives was already demonstrated by other researchers but the stability and activity of these systems have not been systematically investigated. We used Cyclic Voltammetry to study the direct electron transfer of Hb-PAA modified electrodes.
Figure 3.8. Improved stability: Cyclic voltammograms of Hb-PAA electrodes, (A) in pH 7.4 PBS (0.1 M) before heat treatment; B) Hb-PAA-450k(1:0.8:1.5) electrode after steam sterilization (SS) with 2 h cooling (black) and 24 h cooling (red) in pH 7.4 PBS (0.1 M). C) Stability of Hb-PAA-450k(1:0.8:1.5) electrodes at room temperature over 14 days.

Figure 3.9. Cyclic voltammograms of Hb-PAA-450k(1:0.8:1.5) with and without nafion immersed in 0.1M PBS pH 7.4 after 30 minutes.
Cyclic voltammograms (CVs) of the Hb-electrodes in 0.1 M phosphate buffered (PB, pH 7.4) are shown in Figure 3.8A. The CV of the Hb-electrode (Scheme 1, right top panel and black curve in Figure 3.8A showed very poor redox peaks while the electrode modified with Hb-PAA showed a pair of well-defined quasi-reversible redox peaks (red, green and blue). Thus, PAA not only favorably impacts the catalytic efficiency of Hb but also provide the suitable microenvironment for electron transfer between Hb and the underlying electrode. An increase in catalytic activity and electron transfer rate was seen when Myoglobin (Mb) is covalently linked with PAA functionalized iron nanoparticles\textsuperscript{29,30}. Among different Hb-PAA conjugates, Hb-PAA-450k(1:0.8:1.5) displayed more enhanced and sensitive redox peaks than the others. Based on the excellent peroxidase like activity (Figure 3.3A) and thermal stabilities (Figure 3.3C), Hb-PAA-450k(1:0.8:1.5) conjugate is chosen to be the best for further electrochemical studies.

The enhanced response of Hb-PAA-450k(1:0.8:1.5) clearly indicated that the direct electron transfer is greatly promoted by increased polymer concentration and increased covalent conjugation. The polymer dependent electron transfer is further verified by comparing the CVs of Hb-PAA-8k Hb-PAA-450k. Due to the highly water-soluble nature of Hb-PAA nanogels, the CVs were collected and compared in presence and absence of nafion after 30 minutes in 0.1 M PBS with continuous nitrogen bubbling. In both the cases, it displayed the same redox peak intensity (Figure 3.9), which indicates Hb-PAA nanogels are well adsorbed on the electrode surface.

The anodic and cathodic peak of Hb in Hb-PAA (Hb-PAA-450k(1:0.8:1.5)) are located at 0.279 v and 0.334 v with peak to peak separation of 55 mV at the scan rate of
100 mV/s. This peak to peak separation is smaller than 70 mV for Nafion/Hb-MWCNT system in ionic liquid modified carbon paste electrode\textsuperscript{31}, 56 mV for Hb microbelts\textsuperscript{32}, 70 mV for Hb immobilized in sodium alginate-MWCNT composite film\textsuperscript{33} but is higher than Hb at mesoporous carbon modified GC electrode (53 mV)\textsuperscript{34} at the same scan rate. This indicates quasi-reversible electron transfer of Hb in the Hb-PAA conjugate and is consistent with the characteristics redox of the heme proteins [Fe(III)/Fe(II)]\textsuperscript{33}. The concentration (mol/cm\textsuperscript{2}) of electroactive Hb on the electrode surface was determined by integrating the reduction peak according the equation, $\Gamma = Q/nFA$, where $\Gamma$ is the concentration of electroactive Hb, Q is charge involved in reaction calculated by integrating the reduction peak using software (5.665x10\textsuperscript{-7} C), n is number of electrons transferred, A is area of the electrode (7.06x10\textsuperscript{-2} cm\textsuperscript{2}) and F is Faraday constant (96485 C/mol). Out of 7.08x10\textsuperscript{-7} mol/cm\textsuperscript{2} deposited on electrode surface, the concentration of electroactive Hb was estimated to be 8.31x10\textsuperscript{-11} mol/cm\textsuperscript{2} (0.01%), assuming one electron transfer. This is 4.4 times higher than the theoretical monolayer coverage of Hb, 1.89x10\textsuperscript{-11} mol/cm\textsuperscript{2}, which is higher than 1.6 times for Hb entrapped in agarose hydrogel in RT ionic liquid\textsuperscript{36}, 4.0 times for Hb immobilized in polyacrylamide-P123\textsuperscript{37} but smaller than most of the system with Hb immobilized in CNTs. CNTs are known to bridge between the layers thus making the electron hopping possible to larger distance from electrode surface thus resulting 114 times more electroactive species for Hb immobilized in graphene/CNTs\textsuperscript{31}, 21 times more electroactive species for Hb immobilized in sodium alginate-MWCNT composite film\textsuperscript{33}. Our data, in comparison to published literature, suggests multi layers of Hb-PAA are present on the electrode and enough concentration
of electroactive Hb species is present in these layers which allows this material to be electroactive even in the absence of conducting carbon inclusions.

3.4.10 Stability of Hb-PAA-450k(1:0.8:1.5) modified GC electrodes

3.4.10.1) Cyclic voltammetry of steam sterilized samples

The cyclic voltammetry of Hb-PAA-450k(1:0.8:1.5) was recorded before and after steam-sterilization (SS). Figure 4B shows ~65% retention in peak current within 2 h (black) and ~ 90% after 24 h (red) of cooling at room temperature. This observation further supports the idea that Hb re-folding in Hb-PAA conjugate is a kinetic phenomenon and substantial re-folding occurs within 24 h. The peak intensity of Hb intercalated in zirconium phosphate decreased beyond 85°C indicating the denaturation of the protein.

3.4.10.2) Stability with time at room temperature

The extended stability of Hb-PAA modified electrode at room temperature was tested by monitoring the electroactivities of three Hb-PAA (Hb-PAA-450k(1:0.8:1.5)) electrodes maintained at ambient conditions. The peak current decreased by only 5% after two weeks, indicating the high stability of the electrodes (Figure 3.8C) over this time period at room temperature. Most enzymes are usually unstable and the enzyme-electrodes are generally stored at 4°C. Increased stability of the Hb-PAA electrodes (95% retention in peak current) is comparable to 96% retention reported using Hb loaded onto ZnO nanoparticle/ionic liquid system, 95% retention in graphene/Fe₃O₄ nanocomposite at 4°C, over the same time period. The increased stability observed with Hb-PAA offer opportunities to build better biosensors with enhanced room temperature storage or wide
temperature range applications in contrast to denaturation and subsequent loss in the electroactivity of unprotected enzymes.

3.4.11 Hb-PAA-450k(1:0.8:1.5) modified GC electrode for H2O2 detection

The peroxidase like activities of Hb-PAA conjugates synthesized using 450k PAA in solutions was better than Hb itself. Now, the electrocatalytic activity of the same was investigated using cyclic voltammetry (CV).
Figure 3.10. A) Cyclic Voltammograms of Hb-PAA-450k(1:0.8:1.5) modified GC electrode at different concentration of H$_2$O$_2$ Inset: corresponding calibration plot B) Amperometric response of Hb-PAA modified electrode with successive additions of H$_2$O$_2$ in PB pH 7.4 at an applied potential of −0.335 V at 25°C. C) Corresponding calibration plot of amperometric response towards H$_2$O$_2$. 
Figure 5 A shows the CV’s of Hb-PAA (Hb-PAA-450k(1:0.8:1.5)) in absence and presence of different concentration of H$_2$O$_2$. The peak current increases linearly with H$_2$O$_2$ concentration and saturates at about 370 µM. The corresponding calibration plot is shown in inset of figure 5A. Amperometry is more than CV and was used to determine the response of the Hb-PAA modified electrode with different concentration of H$_2$O$_2$ (Figure 3.10B) The amperometry showed linear response up to 120 µM H$_2$O$_2$ (Figure 3.10C) at an applied potential of -0.335 v with a detection limit of 0.5 µM. This detection limit is compared with the recently reported Hb based H$_2$O$_2$ biosensors, Table 2, and proved that the detection limit of our system is comparable to other Hb/polymer systems albeit with enhanced storage stability and ability to reversibly denature.
**Table 3.6.** Relevant data from recently reported Hb based H$_2$O$_2$ biosensors.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Applied Potential</th>
<th>Electrolyte, pH</th>
<th>Detection Limit (µM)</th>
<th>Reference</th>
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</thead>
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<tr>
<td>Hb/PAN-co-PAA</td>
<td>-</td>
<td>0.1 M phosphate, pH 7.0</td>
<td>4.5</td>
<td>41</td>
</tr>
<tr>
<td>Hb/sodium alginate-MWCNTs</td>
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<td>0.1 M phosphate, pH 7.0</td>
<td>16.41</td>
<td>33</td>
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<tr>
<td>Hb microbelts</td>
<td>−0.377 V (vs. Ag/AgCl)</td>
<td>0.1 M phosphate, pH 7.0</td>
<td>0.61</td>
<td>32</td>
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<tr>
<td><em>Hb/flexi</em></td>
<td>-</td>
<td>0.1 M phosphate, pH 7.0</td>
<td>6.7</td>
<td>42</td>
</tr>
<tr>
<td>Polystyrene-Block-PAA/Hb</td>
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<td>0.1 M phosphate, pH 6.5</td>
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<td>43</td>
</tr>
<tr>
<td>Hb-PAA-450k</td>
<td>−0.335 V (vs. Ag/AgCl)</td>
<td>0.1 M phosphate, pH 7.4</td>
<td>0.5</td>
<td>This study</td>
</tr>
</tbody>
</table>
3.5. Conclusions

We systematically investigated the influence of polymer molecular weight, Hb to polymer mole ratio and the amount of crosslinking on the thermal and electrochemical properties of Hb-PAA nanogels. Nanogels produced with high molecular weight PAA (Mw 450k) at high Hb:PAA ratio, at high EDC:COOH mole ratios indicated reversible thermal denaturation of conjugated Hb. Interestingly, this Hb-PAA nanogel in the absence of a mediator presented excellent electrochemical response towards H$_2$O$_2$ with the detection limit of 0.5 µM. Thus, chemical modification of Hb with PAA enhanced direct communication of the Hb redox center with the underlying electrode. These Hb-PAA modified electrodes are stable at room temperature for two weeks with 95% retention in their electrochemical properties, which removes the need for refrigeration of the bioelectrodes. The approach for bioelectrode stabilization is modular, and current approach can be extended to other proteins or enzymes that have appropriate ligation sites on their surfaces.

There are several features of the Hb-PAA nanogels that are likely responsible for the observed retention in biocatalytic and electrocatalytic activity after exposure to denaturing conditions. First, a major factor is the presence of a substantial polymer shroud around the protein and in the nanogel network that effectively prevented the agglomeration and precipitation of the denatured protein. Further crosslinking by amines robust the polymeric shell around the protein and helps in refolding. This polymeric layer also prevented the denaturation of protein at the electrode surface. Second, physical confinement of Hb molecules within nanogel networks shield the protein from the external microenvironment and thus enhanced its stability. By increasing the polymer
molecular weight, polymer to Hb mole ratio, and with increased EDC:COOH mole ratio, the polymer shroud around Hb is strengthened, thickened or rigidified to enhanced its ability to protect Hb against denaturation and promoted refolding of the denatured protein. This aspect is similar to the effect of networks in shape memory polymers. Finally, crosslinking between the protein and hydrophilic PAA maintained a hydrophilic environment at the electrode surface and protected protein secondary structure and heme retention in the active site. Hydrophilic environment around the protein has been shown to increase electron transfer rates\textsuperscript{44} and this could be an important parameter in the current studies.

When all of the above discussed factors act in concert and the synergistic result is the increased resistance of the Hb-electrode to thermal deactivation and promoted efficient electron transfer. Overall, higher resistance to deactivation by heat is a welcome change for applications in high temperature biocatalysis, sterilization and in the production of novel, sterile biomaterials and robust biocatalysts for sensing and fuel cell applications. Since the improvements in the bioelectrode properties are mostly due to the polymer matrix surrounding the protein, our modular approach is likely to be useful in stabilizing bioelectrodes of other enzymes as well, but this needs to be tested in future studies. This hypothesis will be valuable in directing research to enhance bioelectrode stabilities and their electroactivities.

3.6 References


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40. Wang, Y.; Zhang, H.; Yao, D.; Pu, J.; Zhang, Y.; Gao, X.; Sun, Y. Direct electrochemistry of hemoglobin on graphene/Fe3O4 nanocomposite-modified glass


Chapter 4

Three-Dimensional, Enzyme Biohydrogel Electrode for Improved Bioelectrocatalysis

4.1 Abstract

Higher loading of enzymes on electrode and efficient electron transfer from the enzyme to the electrode are urgently needed to enhance the current density of biofuel cells. The two-dimensional nature of electrode surface limits the enzyme loading on the surface and unfavorable interaction with electrode surfaces cause inactivation of the enzyme. Benign biohydrogels are designed here to address enzyme degradation and the three-dimensional nature of the biohydrogel enhanced the enzyme density per unit area. A general strategy is demonstrated here using a redox active enzyme glucose oxidase (GOx) embedded in bovine serum albumin (BSA) biohydrogel on flexible carbon cloth (CC) electrodes. In presence of ferricyanide as mediator, this bioelectrode generated a maximum current density ($j_{\text{max}}$) of 13.2 mA.cm$^{-2}$ at 0.45 V in the presence of glucose with a sensitivity of 67 µA.mol$^{-1}$.cm$^{-2}$ and a half-life of >2 weeks at room temperature. Strong correlation of current density with water uptake by the biohydrogel was observed. Moreover, a soluble mediator (sodium ferricyanide) in the biohydrogel enhanced the current density by ~1000 folds and citrate-phosphate buffer has been found to be the best to achieve the maximum current density. A record 2.2% of the loaded enzyme was electroactive, which is greater than the highest value reported (2 folds)$^{1,2,3,4,5}$. Stabilization of the enzyme in the biohydrogel resulted in retention of the enzymatic activity over a wide range of pH (4.0-8.0). We showed here that biohydrogels are an excellent media for enzymatic electron transfer reactions required for bioelectronics and biofuel cell applications.
4.2 Introduction

A novel method to interlock bovine serum albumin (BSA) and glucose oxidase (GOx) with water-soluble amino acids (Xaa) using carbodiimide (EDC) chemistry to form a highly electroactive enzyme network around the fibers of carbon cloth is reported here. This method helps overcome three major challenges (i) increasing the loading of electroactive GOx species (ii) increasing the percent GOx electroactive and (iii) increasing the stability of GOx. This BSA-GOx-Arg (Arginine, Arg) coated on carbon cloth anode presents enhanced current densities and increase in percentage of electroactive GOx species when compared to other state-of-the-art bio-electrodes.\(^1\)\(^-\)\(^5\) This glucose oxidase modified carbon cloth may serve as an anode for sugar-to-power conversion in biofuel cells (BFC).

The power output of bioelectrodes depends on the fraction of redox active enzyme on the electrode, its activity, stability and its electrical contact with the electrode. Maximizing the loading of electroactive enzyme on the electrode is challenging because of spontaneous enzyme denaturation on the electrode surface. Several different methods have been developed to improve loading of electroactive enzymes and prevent leaching of the enzyme from the electrode surface. This includes the use of (i) entrapping enzymes within polymer like nafion\(^2\)\(^,\)\(^5\) and cellulose,\(^1\) (ii) covalent immobilization of enzyme onto the functionalized carbon electrode\(^6\) and (iii) formation of 3D network of enzymes on the electrode surface\(^7\)\(^-\)\(^9\). The first two methods have been extensively used in sensing while the third method enhanced loading of the enzyme\(^10\) for BFC applications, which is the focus of this work.

Recently, 3D networks with high water content have gained much attention as electrode materials.\(^11\)\(^,\)\(^12\) Compared to other enzyme immobilization techniques, formation
of 3D network on the electrode surface not only improves enzyme loading per unit area of the electrode surface, but also protects the enzyme from denaturation by maintaining a crosslinked hydrophilic environment. These crosslinked enzyme networks are often derived from synthetic polymers, carbohydrates\textsuperscript{13}, proteins\textsuperscript{14}, DNA\textsuperscript{13,15} and other water soluble or dispersible systems. These 3D networks on modified electrodes are generally synthesized by (i) drop casting or physical adsorption of the network onto the electrode surface\textsuperscript{13,15,17} (ii) chemical crosslinking with functionalized electrode surface\textsuperscript{18} and (iii) entrapping the network using polymers, like chitosan\textsuperscript{17}. These methods enhanced enzyme loading but electro-active enzymes is still very low (<0.1\%)\textsuperscript{3}.

Enzyme redox sites are often buried deep in the enzyme and efficient electron transfer between the redox center and the electrode is difficult to achieve. This is often addressed by using mediators\textsuperscript{2,19} and conducting networks\textsuperscript{1,20,21}. For example, GOx is the most extensively used enzyme for glucose based BFC’s.\textsuperscript{22} The redox center of GOx, flavin adenine dinucleotide (FAD), is buried 13 Å deep in the protein structure, limiting the efficiency of direct electron transfer with the electrode\textsuperscript{23}. Integration of enzyme with carbon based materials like CNTs, where the high conductivity and small diameter of CNTs have been useful in wiring the redox centers to the electrode surface.\textsuperscript{1,4} By using external mediators which help wire the active center of enzyme to the electrode surface, making electron transfer independent of the enzyme position or enzyme orientation at the electrode surface\textsuperscript{24}. The most commonly used mediators for GOx include quinone and its derivatives\textsuperscript{3}, ferrocene and its derivatives\textsuperscript{13,25}, osmium\textsuperscript{4} and ruthenium complexes\textsuperscript{26}. Most of these mediators are covalently linked or physically entrapped along with the enzyme. Some metal complexes and small molecules have been used as mobile mediators to
enhance electron transport to the electrode\textsuperscript{27,28}. Although significant work has been done to improve wiring of enzymes on the electrode surface, the current densities of BFCs are still limited.

A rational approach to address the above issues is presented here. Previously, enzymes with hydrophilic poly (acrylic acid) improved enzyme stability even under steam sterilization conditions (122 °C, 10 min, 17-20 psi).\textsuperscript{29,30} A method was recently reported for the interlocking enzymes within the fibers of cellulose, without any surface activation, with enhanced enzyme stability and eliminated enzyme leaching.\textsuperscript{31} The interlocking concept was extended in this paper to enhance enzyme loading onto the fibers of carbon cloth electrodes with improved electrical contact and high operational stability.

Here, we report a biohydrogel network interlocked within the pores of carbon cloth electrode by crosslinking the proteins with water-soluble amino acids as linkers, under amide coupling conditions (Scheme 1). Using this general synthetic method, the percentage of electroactive GOx species on the anode is enhanced significantly while leaching of the electroactive species is significantly reduced. The current density was established using mobile (ferricyanide and ferrocene dicarboxylic acid), immobilized or entrapped mediators (1,4-Naphthoquinone (NQ) and 2-methyl 1,4-Naphthoquinone (VK\textsubscript{3})). Under optimized condition and ferricyanide as a mediator, the bioelectrode was able to produce $j_{\text{max}}$ of 13.2±0.27 mA.cm\textsuperscript{2} at 0.45 V with a sensitivity of 67 µA.mol\textsuperscript{-1}.cm\textsuperscript{-2}. These high performance bioelectrodes, in the presence of mobile mediators, are more suitable for biofuel cells with higher power densities and longer lifetimes.
Scheme 4.1. Synthesis of BSA-GOx-Arg/CC electrode using EDC chemistry. **Top right:** Corresponding cyclic voltammograms. **Middle right:** Multi-channel fluorescence microscopy image of BSA-GOx-Arg modified CC electrode at 20x magnification. BSA was labeled with fluorescein-5-isothiocyanate (FITC) (channel 2, green) and GOx was labeled with 5(6)carboxy-x-rhodamine N succinimidyl ester (ROX) (channel 3, red), then merged together (channel 1, overlay of red and green) and transmitted light (channel 4). **Bottom right:** Amperometric response for glucose in the presence of 10 mM ferricyanide. **Bottom middle:** Catalytic activity.
4.3. Experimental Section

4.3.1 Materials

Bovine Serum Albumin (BSA) was purchased from Equitech Bio (Kerville TX). Glucose oxidase (GOx), amino acids, o-methoxyphenol, hydrogen peroxide (H₂O₂), D-(+)-glucose, fluorescein-5-isothiocyanate (FITC), 5(6)carboxy-x-rhodamine N succinimidyl ester (ROX) and 2-methyl 1,4-Naphthoquinone (VK₃) were purchased from Sigma-Aldrich (St. Louis, MO). 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from TCL America (Portland, OR). Carbon cloth was purchased from Fuel Cell Earth LLC (Woburn, MA).

4.3.2 Synthesis of BSA-GOx-Xaa biohydrogel networks

BSA (150 mg/mL), GOx (150 mg/mL) and arginine (300 mM) stock solutions were prepared by dissolving the appropriate amount in deionized water (DI) and by further adding hydrochloric acid (HCl) to attain pH of 4.5. To synthesize BSA-GOx-Arg network, first, a network precursor containing 50 mg/mL BSA, 50 mg/mL GOx, and 100 mM Arg was prepared using the stock solutions. Second, solid EDC (100 mM final concentration) was added to the precursor mixture and was allowed to react for 30 min. Finally, the formation of the solid network was confirmed by a simple inversion test. Prior to further experiments, the network was washed with DI to remove the excess of unreacted reagents and the reaction byproducts. Similar methods were used to prepare BSA-GOx-Gly (Glycine, Gly) and BSA-GOx-Phe (Phenylalanine, Phe) networks.

BSA-GOx-Arg networks with different GOx and BSA mass fractions were synthesized by varying GOx (10-60 mg/mL) and BSA (90-40 mg/mL) concentrations.
Here, total protein (BSA and GOX = 100 mg/mL), arginine (100 mM) and EDC (100 mM) concentrations were maintained as explained.

### 4.3.3 Synthesis of BSA-Xaa biohydrogel network

The BSA-Xaa networks (Xaa = water-soluble amino acids) were prepared as shown below. BSA (200 mg/mL) and Xaa (200 mM stock solution, pH 4.5) were prepared as previously described. To this network precursor of 100 mg/mL BSA and 100 mM Xaa, solid EDC was added. The reaction mixture was left to react for 30 minutes and the formation of the solid network was confirmed by an inversion test. Prior to further experiments, the network was washed with DI to remove the excess of unreacted reagents and the reaction byproducts.

### 4.3.4 Preparation of BSA-GOx-Xaa modified electrodes

The network precursor with 50 mg/mL BSA, 50 mg/mL GOx, and 100 mM Xaa at pH 4.5 was prepared and mixed with EDC (100 mM final concentration). Immediately, 50 µL this solution was drop cast over the 1cm X 1cm area of carbon cloth electrode and allowed to react for 30 min. The BSA-GOx-Xaa networks were formed within the pores as well as on the surface of carbon cloth. The remaining untreated area of the carbon cloth was treated and covered with water repellent epoxy glue. This BSA-GOx-Xaa/CC electrode is immersed in DI water for an hour to wash out the unreacted enzymes, carbodiimde and other reaction byproducts.

BSA-GOx-Arg/CC electrodes with either NQ or VK₃ mediators were prepared by two different methods. First method: Different volumes (10 µL, 25 µL, 50 µL and 100 µL) of the mediator solution (0.25 M in acetone) were drop cast on carbon cloth and allowed to dry for 20
mins at room temperature. This was further treated with precursor network solution containing BSA, GOx, Arg and EDC and allowed to react for 30 minutes, washed/dried as described above leading to the synthesis of BSA-GOX-Arg/CC containing mediator.

Second Method: Different amounts of mediator (0.5 mg, 1 mg, 2.5 mg, 5 mg) were mixed with the network precursor containing BSA-GOx-Arg followed by addition of EDC. This precursor solution was immediately drop cast onto carbon cloth and allowed to react for 30 mins, followed by washing and drying as previously described leading to the synthesis of BSA-GOx-Arg/CC containing mediator.

4.3.5 Synthesis of FITC labelled BSA and ROX labelled GOx

Dyes (FITC or ROX) in DMSO (10 mg/mL, 40 µL) was slowly added to protein solution (BSA or GOx, 20 mg/mL, 1 mL) in bicarbonate buffer (0.1 M, pH 9.5). The solution was then stirred for 8 hours in the dark at room temperature. The unreacted dyes were then removed by dialyzing against PB (pH 7.0, 10 mM).

4.3.6 Laser Confocal Fluorescence Microscopy

The distribution of BSA and GOx on the surface of biohydrogel modified CC electrode was studied using Laser Confocal Fluorescence Microscopy. BSA was labeled with fluorescein-5-isothiocyanate (BSA-FITC) and GOx was labeled with 5(6)carboxy-x-rhodamine N succinimidyl ester (GOx-ROX), prior to network formation, following the procedure described in ESI. BSA-GOx-Arg modified CC was placed directly on the microscope stage. FITC was exited at 492 nm and emission monitored at 525 nm. ROX was excited at 575 nm and emission monitored at 600 nm. Images were collected with a Nikon A1R Microscope (20x), Argon laser (power level 2.6) for excitation, PMT HV 120 to record emission and the instrument pinhole has been set at 1.2 µm.
4.3.7 Preparation of dyes labeled BSA-GOx-Arg modified Carbon cloth

The network precursor with 50 mg/ml BSA (containing 10 % FITC labeled BSA), 50 mg/ml GOx (containing 10 % ROX labeled GOx), and 100 mM Xaa at pH 4.5 was prepared and mixed with EDC (100 mM final concentration). Immediately, 50 µl this solution was drop cast over the 1cm X 1cm area of carbon cloth electrode and allowed to react for 30 min.

4.3.8 Leaching experiments

The stability of interlocked protein networks within the pores of carbon cloth was tested by leaching experiments. BSA-GOx-Arg/CC and BSA-Arg/CC were synthesized without mediators as described in section 2.4. These electrodes were immersed in 10 mL of deionized water (DI). The absorbance of supernatant was recorded every 15 minutes for 2 hours and once after 12, 24 and 48 hours at 280 nm (aromatic residues) and 450 nm (FAD) using a HP8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA).

4.3.9 Scanning Electron microscope (SEM):

The SEM image of BSA-GOx-Arg biohydrogel was obtained under freeze dried condition. To prepare freeze dried samples, swollen BSA-GOx-Arg biohydrogel was flash frozen in liquid nitrogen and then dried under vacuum at room temperature. Freeze dried samples were sectioned with a razor blade and then coated with Au-Pd in Edwards E306A Coating System, prior to imaging. FEI Teneo LVSEM using 10 kV accelerating voltage was used for analysis.

4.3.10 Water uptake (Hydration) Studies:
The hydration of BSA-GOx-Xaa network and its variants were studied using a conventional gravimetric technique. The network was vacuum dried at room temperature for 48 hours, when constant weight was obtained ($m_{\text{dry}}$). The network was immersed in DI water and weighed every 15 minutes until the equilibrium was attained ($m_{\text{swollen}}$). The hydration percentage was calculated using the equation,

$$\text{Hydration percent (HP)} = \frac{m_{\text{swollen}} - m_{\text{dry}}}{m_{\text{dry}}} \times 100$$

4.3.11 Circular Dichroism studies

Secondary and tertiary structure retention of BSA and GOx in biohydrogel network was determined using circular dichroism studies. Far UV (200 nm to 260 nm) and near UV (300 nm to 450 nm) CD spectra of BSA and GOx before and after formation of matrix were recorded using a JASCO 710 spectropolarimeter (Easton, MD) at a scan rate of 50 nm/min. All samples were synthesized at pH 4.5 and the water spectrum at the same pH was subtracted during processing. Step resolution was maintained at 0.2 nm/data point and bandwidth and sensitivity were 1 nm and 20 millidegrees, respectively. An average of 20 accumulations were recorded using 0.01 cm path length cuvettes.

4.3.12 Electrochemistry

The electrochemical properties of BSA-GOx-Arg/CC were established using a Model CHI 600E electrochemical workstation (CH Instruments, Austin, TX). A conventional three-electrode system was used with BSA-GOx-Arg/CC (with or without mediators) as the working electrode, Ag/AgCl as the reference electrode and platinum wire as the counter electrode. The electrochemical cell was purged with nitrogen for 30 minutes prior to EC experiments and the nitrogen environment is maintained through the
EC experiment. For amperometric experiments, appropriate potentials were applied to the working electrode and the current was recorded with time. EC experiments were carried out in PBS or citrate/phosphate buffer (0.2 M, pH 7.0).

4.4. Results and Discussion

4.4.1 Synthesis of BSA and BSA-Xaa 3D networks

BSA and BSA-Xaa networks were prepared by carbodiimide (EDC) cross-linking of carboxyl and amine groups in the protein and/or in the amino acids. Bovine serum albumin, BSA, has 59 lysine residues and 99 acidic residues (Asp and Glu). Under EDC (100 mM) coupling condition and high BSA concentration (>75 mg/ml, table 4.1), the extensive crosslinking generated a solid 3D network of BSA. The formation of the solid network was confirmed by simple tube inversion test.

BSA-Xaa networks were synthesized using BSA, water-soluble amino acids and EDC (table 4.1). Here, the concentrations of BSA (100 mg/ml), Xaa (100 mM) and EDC (100 mM) were kept constant for all experiments. Solid network formation, confirmed by inversion test, was observed in most of the cases except when aspartic acid, glutamic acid and cysteine were used as amino acids. Amino acids with different side chain (R) (hydrophilic vs hydrophobic, charged vs neutral) were expected to impart different hydration properties to the network formed. After general screening, networks with arginine (Arg, positive side chain, hydrophilic), phenylalanine (Phe, neutral side chain, hydrophobic) and glycine (Gly, neutral, no side chain) were chosen for further experiments and compared with just protein networks.

4.4.2 Design and Synthesis of BSA-GOx-Xaa 3D network
A network of BSA-GOX-Arg was formed directly on carbon cloth without chemical activation of the electrode surface. The enzyme network was made by carbodiimide (EDC) cross-linking of the carboxyl and amine groups on BSA, GOx. BSA has 59 lysine residues and 99 acidic residues (Asp and Glu) and GOx has 16 lysine residues and 68 acidic residues. A solution of BSA (50 mg/mL), GOx (50 mg/mL), and Arg (100 mM) were mixed to give a total protein concentration of 100 mg/mL. These were coupled using EDC (100 mM) to result in an extensive 3D network of BSA-GOx-Arg. The biohydrogel was formed on CC due to extensive crosslinking of BSA, GOx and Xaa on the pores and around the CC fibers and the network has been mechanically interlocked around the fibers.

The reagent stoichiometry was adjusted by varying each component systematically and 3D networks with most desirable properties are identified. For example, we examined the effects of (1) concentration of BSA, (2) concentration of GOx, (3) type and quantity of amino acid spacer used (Arg vs. other water-soluble amino acids, (hydrophilic vs hydrophobic amino acids) and (4) concentration of the coupling agent (EDC, tables 4.1, 4.2 and 4.3). The effect of BSA and GOx concentration on network formation was established for total protein concentration of 100 mg/mL (BSA (40-90 mg/mL), GOx (10-60 mg/mL)), 100 mM Arg and 100 mM EDC (table 4.1).
Table 4.1. Synthesis conditions and formation of BSA-Arg networks

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<tr>
<th></th>
<th>BSA (mg/ml)</th>
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<th>EDC (mM)</th>
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Table 4.2. Preparation of BSA-GOx-Arg network from BSA, GOx and Arg

<table>
<thead>
<tr>
<th></th>
<th>BSA (mg/ml)</th>
<th>GOx (mg/ml)</th>
<th>Arg (mM)</th>
<th>EDC (mM)</th>
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Table 4.3. Synthesis conditions and formation of BSA-Xaa networks

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4.4.3 Leaching Experiments

The stability of BSA-GOx-Arg and BSA-GOx-Arg/CC networks were tested by a leaching experiment. Here, the BSA-GOx-Arg (50 µL) or BSA-GOx-Arg/CC (50 µL in 1 cm X 1 cm carbon cloth) network was immersed in DI water (10 mL), the absorbance of supernatant (280 nm for the aromatic residues and 450 nm for the FAD) was recorded every 15 minutes for 2 hours, and once after 12, 24 and 48 hours. Both BSA-GOx-Arg and BSA-GOx-Arg/CC networks containing BSA (>50 mg/mL) were stable and showed <0.5 % leaching of total protein over 48 h (Figure 1A). These results suggest BSA-GOx-Arg forms stable crosslinked network within the pores and surface of the carbon cloth. The BSA and GOx concentration 50 mg/mL each (total protein = 100 mg/mL) was used as standard for synthesis of networks with other amino acids.

4.4.4 Hydration Studies

Hydration percentage (HP) of the protein network in water gives insight to the extent of crosslinks present in the network. When the network is highly crosslinked, water penetration through the network is limited, and the extent of hydration is reduced. The protein network with most hydration percentage is desirable as electrode material as it could improve the diffusion of the substrate and the mediator. The hydrophilic nature of the network determines its ability to absorb water. Therefore, a network with hydrophilic amino acid side chain is expected to swell more, have higher hydration and provide better diffusion of the reagents through the network. The hydration behavior of specific BSA networks formed with arginine (Arg, hydrophilic side chain), phenylalanine (Phe, hydrophobic side chain) and glycine (Gly, no side chain) were compared to BSA
network in the absence of added amino acid (Figure 4.1). The hydration ratio is calculated as \( \frac{m_{\text{hydrated}} - m_{\text{dry}}}{m_{\text{dry}}} \).

The BSA-Phe network hydrated the least (200%) while BSA-Arg network hydrated the most (1400%). At constant [EDC] and [Arg], an increase in [BSA] from 75 mg/mL to 100 mg/mL resulted in an increase in swelling from 600% to 1400% but further increase in [BSA] caused % hydration to decrease to 200%. The hydration percentage of BSA-GOx-Xaa network is tested for the total protein concentration of 100 mg/mL (50 mg/mL BSA and 50 mg/mL GOx). With 100 mM EDC and 100 mM Arg, BSA-GOx-Arg network showed maximum high hydration of 1000% (Figure 4.2B). Thus, 3D networks with tunable hydration capacity can be designed by manipulating the concentrations of protein, crosslinker and coupling agent. Similar hydration behavior was observed for networks while varying the degree of hydrophobicity or hydrophilicity of the network.32
Figure 4.1. Tunable hydration percentage of different BSA-GOx-Xaa and BSA networks. Error bars shows standard deviation (triplicates).
Figure 4.2. Synthesis of BSA-GOx-Arg network. (A) GOx leaching from BSA-GOx-Arg network. (B) Water uptake studies showing tunable percent water gain by BSA-GOx-Xaa network. (C) Scanning electron microscopy image of BSA-GOx-Arg network. (D) UV-vis absorption spectra of BSA/Arg (blue), GOx/Arg (black) and BSA-GOx-Arg (red). (E) Near UV CD spectra of GOx (black), BSA-GOx-Arg (red), FAD (green) and BSA/FAD (blue). Error bars shows standard deviation (triplicates).
4.4.5 Scanning Electron Microscopy (SEM) Studies

Morphology of BSA-GOx-Arg matrix was examined by SEM. The swollen biohydrogel was flash frozen in liquid nitrogen and freeze-dried at room temperature. Prior to the SEM imaging, the matrix was ground using mortar and pestle. The SEM image showed highly porous honeycomb like structure with a pore size of 5-10 µm (Figure 4.2C).

4.4.6 Effect of Crosslinking in the Microenvironment of FAD Moiety

The changes in the microenvironment of FAD moiety of GOx were studied with UV-vis absorption spectroscopy and circular dichroism spectroscopy. The absorption spectrum of GOx shows three distinct peaks at 277, 382 and 450 nm (Figure 4.3). The 277 nm peak is characteristic of the aromatic rings of amino acid residues whereas 382 and 450 nm peaks are characteristics of the FAD moiety. Upon crosslinking, the 450 nm peak was unaffected, but the 382 nm peak showed increased intensity and was blue shifted to 378 nm (Figure 4.2D). The increased intensity and blue shifts could be due to greater exposure of FAD to the solvent. The change in the microenvironment around FAD moiety was also observed in the CD spectra in the region of 300-450 nm. GOx exhibits characteristic FAD absorption band at 375 nm (Figure 4.2E). Free FAD in solution showed negative peak at 375 nm and positive peak at 340 nm. Upon crosslinking, (i) the 375 nm absorption band was red shifted to 380 nm and showed slight decrease in intensity and (ii) the 340 nm peak, which corresponds to free FAD, increased. However, the leaching experiment showed <0.5 % loss in total protein concentration over 48 h. These results suggest that although the FAD is more exposed to solvent, it is still intact within BSA-GOx-Arg network. The effect of crosslinking on GOx and BSA...
secondary structure was compared by CD spectroscopy in the far UV region of 195-260 nm. GOx, BSA and BSA/GOx-Arg showed two negative bands in CD spectra at ~208 nm and ~222 nm, which is characteristics of α-helix structure. Upon crosslinking, the intensity at 208 and 222 nm decreases, with ~ 90% retention in secondary structure (Figure 4.4).
Figure 4.3. UV-vis absorption spectra of BSA-GOx-Arg (red) compared to GOx/Arg (black, physical mixture) and BSA/Arg (blue, physical mixture).

Figure 4.4. Secondary structure retention: Far UV CD of GOx (black), BSA (blue), BSA/GOx/Arg (green, physical mixture) and BSA-GOx-Arg (red).
4.4.7 Laser Confocal Fluorescence Microscopy

The BSA-GOx-Arg network interlocked CC electrode was examined by laser confocal fluorescence microscopy. BSA was labeled with fluorescein-5-isothiocyanate (BSA-FITC) and GOx was labeled with 5(6)carboxy-x-rhodamine N succinimidyl ester (ROX-GOx). Then, (BSA-FITC)-(GOx-ROX)-Arg/CC was synthesized and imaged by confocal microscopy. Analysis of the images showed uniform presence of ROX and FITC dyes which establishes uniform distribution of BSA and GOX (Scheme 1, middle right).

4.4.8 Electrochemical studies of BSA-GOx-Arg/CC anode

The electrochemical behavior of BSA-GOx-Arg/CC electrode in the absence and the presence of mediators was studied by cyclic voltammetry (CV) and amperometric (i-t) method. In the absence of a mediator, BSA-GOx-Arg/CC electrode showed a pair of well-defined redox peaks by cyclic voltammetry located at -0.34 V and -0.48 V, corresponding to the FAD redox activity\(^2\), with the peak-to-peak separation of 140 mV (Figure 4.5). The anodic and cathodic peak intensity remained the same after 50 complete cycles (Figure 4.6), confirming immobilization of the BSA-GOx-Arg network on the pores of carbon cloth. The effect of GOx concentration is shown in figure 2. CV response for series of BSA-GOx-Arg/CC electrode with different GOx concentration from 10 mg/mL to 60 mg/mL (~ GOx mass fraction of 0.1-0.6) was recorded and its impact on the anodic peak current (\(I_{pa}\)) and cathodic peak current (\(I_{pc}\)) determined. The peak currents increased with GOx concentration. However, for BSA concentration <50 mg/mL, the interlocking of BSA-GOx-Arg network on carbon cloth (CC) was not stable as it leached out 10% of enzyme in 24 h. So, CC electrode modified with network containing BSA (50 mg/mL) and GOx (50 mg/mL) were used for further experiments.
**Figure 4.5** Cyclic voltammograms of BSA-GOx-Arg/CC electrodes with different GOx and BSA concentration (GOx, 10 – 60 mg/mL and BSA, 90 – 40 mg/mL) in 0.2 M PBS (pH 7.0, scan rate: 10 mV/s).

**Figure 4.6.** Cyclic voltammograms of BSA-GOx-Arg/CC electrodes cycle 1 (red) and cycle 50 (black) in 0.2 M PBS (pH 7.0).
The performance of BSA-GOx-Arg/CC electrode was further tested in the presence of 10 mM ferricyanide and compared with that of bare CC electrode (Figure 4.7). Bare CC electrode showed a pair of well defined peaks at 0.11 V and 0.31 V, corresponding to ferricyanide redox activity. Interestingly, the presence of BSA-GOx-Arg network (<2.5 mg GOx) on CC electrode surface substantially improved ferricyanide electro-oxidation. However, further increase of the BSA-GOx-Arg loading per cm² of CC electrode decreased the peak current density. This may be due to increased thickness of the BSA-GOx-Arg layer on CC, which will decrease diffusion of ferricyanide to and from the electrode surface. The linear dependence of $I_{pa}$ and $I_{pc}$ as a function of square root of scan rate ($\nu^{1/2}$) further showed the diffusion-controlled process on the electrode surface (Figure 4.8).
Figure 4.7. Cyclic voltammograms of BSA-GOx-Arg/CC electrodes with different BSA-GOx-Arg loading in 0.2 M PBS (pH 7.0) containing 10 mM ferricyanide solution (scan rate: 10 mV/s).

Figure 4.8. A) Cyclic Voltammetry BSA-GOx-Arg/CC electrode at various scan rates 5mV/s to 100 mV/s in 0.2 M PBS (pH 7.0) containing 10 mM ferricyanide solution. B) The plot of anodic and cathodic peak currents as a function of square root of scan rate.
4.4.9 Mediated Glucose Bioelectrocatalysis

The GOx electrocatalytic activity in BSA-GOx-Arg network towards glucose in the presence of ferricyanide as mediator in 0.2 M PBS, represented by equations 2 and 3, was electrochemically characterized by CV and amperometry. The performance of BSA-GOx-Arg/CC electrode over the range of pH (3.0-8.0) was evaluated and citrate/phosphate buffer (0.2 M) is convenient for this wide range of pH. Figure 4.9A and 4.9C shows CVs of BSA-GOx-Arg/CC electrode in 0.2 M PBS (pH 7.0) and 0.2 M citrate/phosphate buffer (pH 7.0) respectively, containing 10 mM potassium ferricyanide with (red) and without (black) 50 mM glucose. After addition of glucose, a distinct increase in $I_{pa}$ and decrease in $I_{pc}$ is observed in both buffers. This indicates typical electro-oxidation of glucose on electrode surface and effective electron transfer among GOx and electrode surface in the presence of the mediator.
**Figure 4.9.** Electrocatalytic activity (A) Cyclic voltammograms of BSA-GOx-Arg/CC electrodes in the presence of 50 mM glucose (red) and absence of glucose (black) in phosphate buffer. (B) Amperometric response of bioanode with different concentrations of glucose, modified with BSA-GOx-Arg (red), BSA-Arg/FAD (black) and BSA-Arg (blue). (C) Cyclic voltammograms of BSA-GOx-Arg/CC electrodes in the presence of 50 mM glucose (red) and absence of glucose (black) in citrate/phosphate buffer. (D) Michaelis-Menten kinetics of BSA-GOx-Arg/CC electrodes in phosphate buffer and citrate/phosphate buffer. All experiments done in pH 7.0 phosphate or citrate/phosphate buffer (0.2 M) containing 10 mM ferricyanide solution (scan rate: 10 mV/s). Error bars shows standard deviation (triplicates).
Glucose + 2[Fe(CN)₆]³⁻ + H₂O → Gluconolactone + 2[Fe(CN)₆]⁴⁻ (1)

2[Fe(CN)₆]⁴⁻ electrode → 2[Fe(CN)₆]⁴⁻ + 2e⁻ (2)

The electrocatalytic activity of BSA-GOx-Arg/CC electrode is further investigated by amperometric response with increasing concentrations of glucose at a constant potential of 0.45 V. A typical Michaelis-Menten trend (Figure 4.9D) with the maximum current density ($j_{\text{max}}$) 13.2±0.27 mA.cm⁻² (citrate/phosphate buffer) and 4.07±0.13 mA.cm⁻² (phosphate buffer) were obtained. Three-fold increase in current density was achieved by using citrate/phosphate buffer over phosphate buffer. However the apparent $K_M$ obtained in citrate/phosphate buffer (98 mM) was higher than in phosphate buffer (80 mM). These $K_M$ values in both buffers are well within the known range (33-110 mM).³⁵,³⁶ This electrode is among the highest current density generating bioelectrode per U¹⁻² of GOx and has the highest percent electroactive GOX compared to other state-of-the-art bio-electrodes.¹⁻⁵ In contrast, BSA-Arg/CC control electrode didn’t show any response to glucose addition as expected (Figure 4.9B).

The current density per U of GOx loaded on electrode surface is calculated using equation 3 and the specific enzymatic activity of GOx in BSA-GOx-Arg/CC electrode was calculated using equation 4¹:

Current density (U⁻¹GOx) = $\frac{j_{\text{max}}}{m.U}$ (3)

Specific activity (U.mg⁻¹) = $\frac{j_{\text{max}} \cdot 60 \cdot A}{F \cdot n \cdot m}$ (4)
U is the native enzyme activity in μmol.min\(^{-1}\), \(j_{max}\) is the maximum current density (13.2 mA.cm\(^{-2}\)) and m is the mass of GOx (2.5 mg). A is the electrode surface area (1 cm\(^2\)), F is faraday constant and n is the number of electrons involved in electrode reaction (for ferricyanide n=1).

Taking into account that specific activity of GOx in solution is 150 U.mg\(^{-1}\), here, current density per U of GOx loaded on electrode surface was calculated as 10.8 µA.cm\(^{-2}\).U\(^{-1}\) in phosphate buffer and 35.2 µA.cm\(^{-2}\).U\(^{-1}\) in citrate/phosphate buffer. Compared to other best bioanode, which uses GOx based bioanode, BSA-GOX-Arg/CC electrode showed ~ 20-fold increase in activity of GOx. Similarly, GOx specific activity was calculated to be 0.99 U.mg\(^{-1}\) (in phosphate buffer) and 3.28 U.mg\(^{-1}\) (in citrate/phosphate buffer) via one-electron oxidation of ferricyanide ion (n=1 for Fe(CN)\(_6\)\(^{3-}\) to Fe(CN)\(_6\)\(^{4-}\)). These specific activities correspond to 0.67% (in phosphate buffer) and 2.2% (in citrate/phosphate buffer) of the activity of the free GOx in solution (Table 4.4). Compared to other best bioanode, which uses glucose as fuel (GOx or glucose dehydrogenase (GDH) based bioanodes), BSA-GOX-Arg/CC electrode showed ~ 2-fold increase in activity of GOx (Figure 4.10A).\(^{1-5}\)
Table 4.4. Current density ($j_{max}$), $K_M$, activity (U.mg$^{-1}$), % enzyme electroactive for BSA-GOx-Arg/CC electrode in phosphate buffer and phosphate/citrate buffer.

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<th>Current density ($j_{max}$) (mA.cm$^{-2}$)</th>
<th>$K_M$ (mM)</th>
<th>Activity (U.mg$^{-1}$)</th>
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<tr>
<td>Citrate/Phosphate</td>
<td>13.2±0.27</td>
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The performance of BSA-GOx-Arg/CC electrode was optimized by investigating the influence of various components of the system such as amino acids, GOx loading on electrode surface, mediators and pH.

The current density obtained from BSA-GOx-Xaa/CC electrode (Xaa = Arg, Phe, Gly) was compared with GOx/CC electrodes in the presence of ferricyanide as the mediator (Figure 5B). BSA-GOx-Arg/CC electrode showed excellent response towards glucose with a \( j_{\text{max}} \) of 4.07±0.13 mA.cm\(^{-2}\) and open circuit potential (OCP) of 0.25 V followed by BSA-GOx-Gly/CC electrode (\( j_{\text{max}} = 0.8±0.2 \) mA.cm\(^{-2}\), OCP = 0.17 V). GOx/CC electrode showed lowest current density of 0.1±0.01 mA.cm\(^{-2}\). These OCP values are in good agreement with the literature values\(^{10}\).

To obtain the optimal concentration of GOx on the electrode surface, a series of electrodes with different BSA-GOx-Arg loadings (1, 2, 3, 4, 5, 7.5 mg total protein per cm\(^2\) of CC electrode) were prepared. With increase in GOx loading, the current density increased, as expected, shown in figure 5C. However, the current density decreased above 2.5 mg.cm\(^{-2}\) (~ 15 nmol of GOx.cm\(^{-2}\)). This is likely due to increase in the thickness of the enzyme network, which decreases the diffusion of substrate and the mediator. This result was consistent with the CV of BSA-GOx-Arg/CC electrodes in 10 mM ferricyanide solution (Figure 4.7).
Figure 4.10. (A) Comparison of percent enzyme electroactive with other systems.\textsuperscript{1-5} (B) Comparison of maximum current density of BSA-GOx-Arg/CC electrodes with different amino acids. (C) The dependence of current density of BSA-GOx-Arg/CC electrode on different loading of network. (D) Comparison of maximum current density of BSA-GOx-Arg/CC electrode with different mediators. All experiments done in pH 7.0 PBS (0.2 M) containing 10 mM ferricyanide solution. Error bars shows standard deviation (triplicates).
The maximum current density of BSA-GOx-Arg/CC electrodes was investigated in order to compare the performances of four different mediators in PBS (0.2 M, pH 7.0). These are classified into two categories, diffusional/mobile and immobilized. Ferricyanide and ferrocene dicarboxylic acid being water-soluble, are diffusional mediators. 1,4-Naphthoquinone (NQ) and 2-methyl 1,4-Naphthoquinone (VK₃) being water insoluble are trapped within the network and classified as entrapped mediators. The bioanode with the ferricyanide showed maximum current density of 4.07±0.13 mA.cm⁻² and had highest open circuit potential 0.25 V, followed by ferrocene dicarboxylic acid (2.6 mA.cm⁻², OCP = 0.23)) (Figure 4.10D). These values of current densities are two orders of magnitude higher than previous bioanodes using ferricyanide³⁷ and ferrocene carboxylic acid³⁸ mediators.

The optimal concentration of ferricyanide needed for best performance was investigated by comparing the current density at 50 mM glucose by increasing the ferricyanide concentration from 0 to 20 mM. The current density increased drastically and then leveled off above 10 mM ferricyanide (Figure 4.11). These data suggest that mobile phase mediator is suitable for protein-enzyme network modified electrodes, as the 3D porous structure facilitates the mediator/substrate diffusion and provides better electrical communication with the electrode.

The BSA-GOx-Arg/CC electrodes were evaluated over a wide pH range, from 3.0 to 8.0 in citrate/phosphate buffer (0.2 M), in the presence of 50 mM glucose. The electrode showed optimum current density between pH 4.0-8.0 (Figure 4.12). This wide range of working pH of BSA-GOx-Arg/CC electrode in combination with laccase, as biocathode, which has optimum activity around pH 4-5³⁹ could be useful for BFC design.
4.4.10 Stability of BSA-GOx-Arg/CC Electrode at Room Temperature

The stability and performance reproducibility (triplicates) of BSA-GOx-Arg/CC bioanode stored at room temperature and under dry conditions were evaluated by comparing its OCP and current density for a period of 25 days. The bio electrodes showed 50% initial drop in current density and 22% drop in OCP over 20 days (Figure 4.13). The observed stability can be attributed to the confinement of GOx in BSA-GOx-Arg network that prevented the denaturation of enzyme. This observation is in keeping with our previous studies in which we showed that proteins covalently bonded with polyacrylic acids formed nanogel networks within which the protein was confined and stabilized for room temperature storage and steam sterilization applications (122 °C, 10 min, 17-20 psi).29,30
Figure 4.11. The dependence of current density of BSA-GOx-Arg/CC electrode with ferricyanide concentration. Error bars shows standard deviation (triplicates).

Figure 4.12. Performance of BSA-GOx-Arg/CC electrodes at different pH. Error bars shows standard deviation (triplicates).
Figure 4.13. Evolution of open circuit potential (OCP) and current density of BSA-GOx-Arg/CC bioelectrode in the presence of 5 mM glucose. All experiments were done in pH 7.0 PBS (0.2 M) containing 10 mM ferricyanide solution. Error bars show standard deviation (triplicates).
Taking all the results together, BSA-GOx-Arg/CC electrode showed three orders (~1000) of magnitude increment in the current density when ferricyanide mediators are used. This is because FAD in GOx is deeply buried within the protein and is electrochemically less accessible for direct electron transfer with the electrode surface. In the presence of mediators, electrons are transferred from redox center of GOx to the mediator and then on to the electrode surface.\textsuperscript{10,40} When mobile mediators are used, the mediator diffuses to electrode surface through the 3D network. Interestingly, BSA-GOx-Arg/CC bioelectrode showed ~750-fold increment in current density with mobile mediators (ferricyanide and ferrocene dicarboxylic acid) when compared to the immobilized mediators (VK\textsubscript{3} and NQ). The protein network with high levels of hydration is desirable because of its enhanced capabilities to diffuse mediators/substrate/byproducts to and from the electrode surface. Higher diffusion lowers the accumulation of reaction products like gluconolactone, which are known to inhibit the GOx activity.\textsuperscript{41}

4.5 Conclusions

In this article, we report the synthesis and interlocking of protein networks on the pores of the carbon cloth electrode. By combining spectroscopic and electrochemical techniques, we established optimal conditions to design and characterize BSA-GOx-Arg biohydrogel network in BSA-GOx-Arg/CC electrodes. For example, the hydration or water uptake of BSA-GOx-Arg networks can be tuned by using different amino acids or different compositions, as desired. The protein network with Arg has high water uptake capacity, which probably enhanced the substrate/mediator/product diffusion to and from the GOx active site even when present on the electrode. Also, CD spectra showed 90% retention in the secondary structure of the GOx in the network, even after the
crosslinking. Furthermore, the stability of the BSA-GOx-Arg network within the pores of carbon cloth electrode is confirmed by leaching experiments. The BSA-GOx-Arg modified CC electrodes are capable of producing high current density \( j_{\text{max}} \) 13.2±0.27 mA.cm\(^{-2}\) using glucose in the presence of mobile ferricyanide mediator, which is ~1000-fold higher than in the absence of mediator.

Several features of BSA-GOx-Arg biohydrogel network are responsible for the observed high current density and electroactivity. The amino acid, arginine, makes this network hydrophilic, which in turn increases the hydration percentage to 1000%. We believe that increased hydration allows more mobile mediators to come in close proximity with FAD of GOx through high diffusion, enhancing the electron transfer rate. The enhanced hydrophilicity, which provides a shield of hydration, also may prevent denaturation of GOx on carbon cloth surface. The chemical crosslinking of GOx with BSA and/or Arg provides better dispersion and physical confinement of GOx within the 3D network, which prevents the leaching of enzyme from the network.

Compared to other examples in the literature, our bioelectrode showed higher percent of electroactive enzymes and produce higher current density per U of enzyme loaded. This means more efficient mediated electron transfer is obtained between the GOx within the 3D network and the electrode surface. This approach of 3D protein network synthesis and bioelectrode preparation can be extended to other single or multi-enzyme system for the biofuel cell application.

4.6 References


Chapter 5
Multifunctional Macroporous Bovine Serum Albumin Sponge for Efficient Emulsion Separations

5.1 Abstract

Here we present a highly porous, biodegradable and biorenewable protein sponge platform for efficient separation of oil-in-water emulsions. BSA-Sponge was synthesized by covalent crosslinking of BSA molecules using carbodiimide (EDC) chemistry. The as prepared BSA-Sponge showed excellent hydrophilicity and oleophobicity in both dry and wet conditions. These properties were then used to study the surfactant-free and surfactant-stabilized oil-in-water emulsion separation solely driven by gravity. The BSA-Sponge was able to separate oil-in-water emulsion with 99.1% efficiency. In addition, BSA-Sponge showed robust mechanical properties and underwater thickness recovery. When submerged in water, the sponge recovered 100% to its original shape without significant plastic deformation after compression for 50 cycles. This BSA-sponge platform is a low cost, biodegradable-filtering material for efficient separation of oil/water emulsions.

5.2 Introduction

Porous materials such as films and sponges with larger surface area are often used for filtration, adsorption, separation and cleanups.\textsuperscript{1-4} Biocompatible and biodegradable porous materials with shape memory properties are of great importance for biomedical applications such as drug delivery and tissue regeneration.\textsuperscript{5} These porous materials are synthesized using templates, lyophilization and cryogelation techniques.\textsuperscript{6-8} In this present study, a simple, general and modular method to prepare novel and biodegradable Bovine
Serum Albumin (BSA) Sponge with robust shape memory characteristics is presented. This new method can be used to synthesize 3D porous protein based materials for biomedical, biocatalysis and environmental applications. Furthermore, oil-in-water emulsion separation properties of BSA-Sponge will be explored.

Increased exploration of crude oil has lead to several accidental oil spills and release of oil containing wastewater has created enormous environmental damage.\\(^9-11\\)\textsuperscript{9-11} Nominally, phase separated oil and water should be easily separated due to density difference. However, oil-in-water emulsions are difficult to separate because of their stability in the presence of naturally occurring surfactants as well as its dimensions and morphology.\\(^12\\)\textsuperscript{12} Various cleanup techniques such as in situ burning, skimming, centrifugation were traditionally used to deal with oil spills.\\(^13-16\\)\textsuperscript{13-16} However, these techniques come with their own disadvantages like air pollution, and mechanical separations are inefficient, expensive and time consuming. Therefore, there is an urgent need to develop an eco-friendly materials platform with high efficiency to separate the bulk oil in water as well as stable oil-in-water emulsions.

Recently, two different wettable materials for oil/water separations have been studied: 1) Hydrophilic and oleophobic\\(^17-19,11\\)\textsuperscript{17-19,11} 2) Hydrophobic and oleophillic\\(^20-23,19\\)\textsuperscript{20-23,19}. On one hand, hydrophobic/oleophillic materials by absorption methods are not reusable. The poor reusability is due the accumulation of the organic matter on the surface or inside the separation materials, called fouling, which lowers the efficiency of the system.\\(^24\\)\textsuperscript{24} On the other hand hydrophilic materials can be used efficiently for emulsion separation via gravity driven filtration method and has better antifouling properties.
Polymer based membranes are most commonly used materials for filtration process. However, these conventional 2D membranes used for oil/water separations are more likely to be contaminated easily during its long use, have weak mechanical strength and have poor cycling performance.\textsuperscript{25} Therefore, porous 3D biomaterials with tunable pore size are desirable for efficient separation.\textsuperscript{11} Two different methods are commonly used to synthesize the 3D materials: 1) Use of bottom-up self-assembling techniques to construct bulk porous materials, such as carbon nanotube sponges and graphene aerogels.\textsuperscript{23, 26, 27} However, these methods are expensive and limited to lab use only. 2) By post modification of commercially available polyurethane (PU) sponges with CNT’s, graphene and polymers.\textsuperscript{28, 29} Most of these PU sponges are modified to improve their water repellency or oil absorbency, which ignores the potential application of these sponges for filtration technique.\textsuperscript{11} The use of PU as starting material also limits the ability to tune the internal properties of the sponge.\textsuperscript{24} However, availability of low cost, biodegradable, bioderived porous membrane polymer or polymer/hybrid materials platform is currently an unmet challenge.

Here, we designed covalently crosslinked bovine serum albumin (\textbf{Scheme 5.1}) derived sponge for efficient oil/water separation. The natural hydrophilicity of BSA ensures the oleophobicity of BSA-Sponge under hydrated condition and allows continuous oil-in-water emulsion separation simply driven by gravity with 99.1\% efficiency.
Scheme 5.1. Synthesis of BSA-Sponge using EDC chemistry. Top right: Showing Compressibility. Middle right: Solvent uptake (Water and Paraffin oil) by BSA-Sponge showing hydrophilicity. Bottom right: Optical image of BSA-Sponge showing pores.
5.3 Experimental section

5.3.1 Materials

Bovine Serum Albumin (BSA, reagent grade) was purchased from Equitech Bio (Kerville TX). 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from TCL America (Portland, OR). Solvents were purchased from J.T. Baker chemicals (Phillipsburg, NJ). Tween-80 was purchased from Sigma-Aldrich (St. Louis, MO).

5.3.2 Synthesis of BSA-Sponge

BSA (250 mg/mL solutions were prepared by dissolving the appropriate amount in deionized water (DI). The solution was then blended to obtain the foam for 5 min using a microblender as shown in scheme 5.1. Solid EDC (200 mM final concentration) was added to the foam and was allowed to react for 30 min. The formation of the sponge was confirmed by a simple touch test. Prior to further experiments, the sponge was washed with DI to remove the excess of unreacted reagents and the reaction byproducts.

5.3.3 Solvent uptake Studies:

The hydration of BSA-Sponge network was studied using a conventional gravimetric technique. The sponge was vacuum dried at room temperature for 48 hours, when constant weight was obtained ($m_{dry}$). The sponge was immersed in solvent and weighed every 15 minutes until the equilibrium was attained ($m_{swollen}$). The solvent uptake percentage was calculated using the equation,

$$\text{Solvent uptake} \% = \frac{m_{swollen} - m_{dry}}{m_{dry}} \times 100$$

5.3.4 FT-IR Spectroscopy

The FT-IR was used to determine the effect of crosslinking on the secondary structure of protein. The FT-IR spectra were recorded using Nicolet Manga-IR 560 ESP
Spectrophotometer (Nicolet Instrument, Madison WI) from 4000 to 650 nm. The total number of scan was 1232 to improve the signal to noise ratio.

5.3.5 Dynamic Mechanical Analysis

A dynamic mechanical analyzer (DMA 2980, TA instrument, Q800) was used to evaluate the mechanical performance of BSA-Sponge. A submersion compression mode with an initial load force of 0.01N was employed. The dimensions of the tested sample (cylindrical BSA-Sponge) were averagely 10 mm (length) x 22 mm (diameter). Cyclic strain controlled loading (1.0 N/min or 2.0 N/min) was used to evaluate the fatigue behavior of the sample.

5.3.6 Preparation of oil-in-water emulsions

To prepare the oil in water emulsion, 2 mL of toluene was added into 98 mL of DI with 0.25 mL of tween-80 as surfactant. The mixture was then stirred for 3 h. The surfactant stabilized emulsions were stable for more than week.

5.3.7 Emulsion separation experiments

The as prepared BSA-Sponge (d=2.2 cm and h=0.5-1.0 cm) was compressed and inserted in a glass cylinder (d=2.0 cm). The sponge was then hydrated to expand and fit the tube. The oil in water emulsion was poured into the filtration system and the separation process was driven by gravity. The separation efficiency was determined by measuring the oil content in the filtrate and the feed using a HP8453 UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA). The flux was determined by measuring the volume of water filtered through the sponge in 5 min.

5.4. Results and Discussion

5.4.1 Synthesis of BSA-Sponge
BSA has 59 lysine residues and 99 acidic residues (Asp and Glu). The BSA-sponge was synthesized by carbodiimide crosslinking of the carboxyl and amine groups on BSA. A solution containing 250 mg/mL BSA was blended for 5 min using the blender as shown in scheme 5.1. Solid EDC (200 mM final concentration) was then added to the foam produced by the agitation of BSA solution. The extensive crosslinking between the BSA molecules stabilizes the foam resulting in the sponge formation. The reagent stoichiometry was adjusted by varying each component systematically and the conditions needed for the most desirable properties are identified. For example, we examined the effects of concentration of BSA and concentration of the coupling agent (EDC, Table 5.1) on sponge formation. The BSA-Sponges formed with BSA concentration <200 mg/mL and EDC concentration <100 mM were fragile and do not show shape retention after compression.
<table>
<thead>
<tr>
<th>BSA</th>
<th>EDC (mM)</th>
<th>Sponge</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
<td>N</td>
</tr>
<tr>
<td>100</td>
<td>150</td>
<td>N</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>N</td>
</tr>
<tr>
<td>150</td>
<td>200</td>
<td>N</td>
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<tr>
<td>150</td>
<td>100</td>
<td>N</td>
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<tr>
<td>150</td>
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<td>N</td>
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<tr>
<td>150</td>
<td>200</td>
<td>N</td>
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<tr>
<td>200</td>
<td>50</td>
<td>N</td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>Y</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>Y</td>
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<td>200</td>
<td>250</td>
<td>Y</td>
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<tr>
<td>250</td>
<td>50</td>
<td>N</td>
</tr>
<tr>
<td>250</td>
<td>100</td>
<td>Y</td>
</tr>
<tr>
<td>250</td>
<td>200</td>
<td>Y</td>
</tr>
</tbody>
</table>
5.4.2 Solvent uptake Studies:

The sponge when dry is porous and brittle while when hydrated is elastomeric and have shape recovery properties. Thus, all experiments were done under the hydrated condition of sponge. The water uptake by BSA-Sponge was studied using a conventional gravimetric technique. The sponge was vacuum dried at room temperature for 48 hours, when constant weight was obtained (m_{dry}). The network was immersed in water and weighed every 15 minutes until the equilibrium was attained (m_{swollen}). The water uptake percentage was calculated using the equation 5.1.

As shown in the Figure 5.1A, BSA-Sponge showed ~ 850% uptake of water. When dry BSA-Sponge was immersed in organic solvents (toluene or ethanol or hexane or acetone), shows ~200 % solvent absorption (Figure 5.1B). The BSA-Sponge in organic solvent, however remains brittle and doesn’t possess elastomeric and shape memory properties. The hydrophilicity of hydrated BSA-Sponge was further demonstrated by filtration method. The as prepared BSA-Sponge (d=2.2 cm and h=0.75 cm) was compressed and inserted in a glass cylinder (d=2.0 cm). The sponge was then hydrated to expand and fit the tube. 15 ml of solvent (water or toluene or hexane or ethanol or paraffin or acetone) was poured on top of it. Water quickly passes through the sponge while the sponge rejected other solvents.
Figure 1. A) Rate of Swelling of BSA-Sponge. B) Solvent (Water, hexane, toluene, ethanol, acetone) gain (w/w %) by the dry BSA-sponge. Error bars shows standard deviation (n=3).
5.4.3 FT-IR Spectroscopy

FTIR spectroscopy is an important technique to analyze the changes in the conformation of BSA. The amide I band due to C=O stretch (1600-1700 cm\(^{-1}\)) and amide II band due to N-H stretch (~1550 cm\(^{-1}\)) are sensitive to the protein secondary structure.\(^{30, 31}\) FT-IR spectrum of BSA powder (blue) and BSA-Sponge (red) (Figure 5.2) doesn’t show any significant change in protein fingerprint region (amide I and amide II), indicating BSA secondary structure remains intact upon EDC crosslinking.
Figure 5.2. FTIR spectra of BSA (blue) and BSA-Sponge (red)
5.4.4 Dynamic Mechanical Analysis

The long-term mechanical stability of BSA-Sponge is very important for oil-in-water emulsion separation. The as prepared BSA-Sponge showed good compressibility and robustness, in air, against the deformation force without any fracture (Scheme 5.1, Top right). Underwater, beside compressibility BSA-Sponge showed excellent thickness recovery (Figure 5.3). Thus the mechanical properties of BSA-Sponge were studied under submerged condition. Here, the submersion compression stress strain experiments were performed to evaluate the robustness of the BSA-Sponge. The stress-stress curves (figure 5.4A) showed elastomeric nature of BSA-Sponge with two distinct regions for the compression process: The initial elastic region (strain < 50%) and abrupt stress rising region (strain > 50%). The sponge recovered 100% to its original shape (Figure 5.4B) without plastic deformation after compression for 50 cycles.
Figure 5.3. Underwater thickness recovery of BSA-Sponge.

Figure 5.4. (A) Compressive stress-strain curves of BSA-sponges (B) Thickness recovery after 50 cycles.
5.4.5 Separation of oil-in-water emulsion

Due to its hydrophobicity, extensive porosity and mechanical stability, it is an excellent candidate for separation of oil/water mixture. To test the oil in water separation capability of BSA-Sponge, surfactant-free and surfactant-stabilized emulsions were prepared. To prepare the oil in water emulsion, 2 mL of toluene was added into 98 mL of DI with or without 0.25 mL of tween-80 as the surfactant. The mixture was then stirred for 3 h. The as prepared oil in water emulsion was poured into the BSA-Sponge filtration system (prepared as described in section 5.3.2) and the separation process was gravity driven. The water was quickly permeated through the sponge while toluene was retained above the sponge (Figure 5.5). The oil (toluene) was colored with oil red O dye for better visualization. The separation efficiency was determined by measuring the oil content in the filtrate and the feed using a UV-visible spectrophotometer. The flux was determined by measuring the volume of water filtered through the sponge in 5 min (d=2.0 cm and h=0.5-1.0 cm). Since the separation is driven by gravity, this method of oil-in-water emulsions separation is desirable and energy efficient.
Figure 5.5. Oil-in-water emulsions separation (A) The filtration system used for tween-80 stabilized oil/water emulsion. The oil (toluene) is colored red with oil red O dye (B) UV-vis absorption spectra of tween-80 stabilized oil/water emulsion (red), filtrate (blue) and water (black).
The flux rate and oil in water separation efficiency were calculated for three different thicknesses of BSA-Sponge (0.5 cm, 0.75 cm and 1 cm) and are summarized in Table 5.2. The BSA-Sponge showed excellent emulsion separation efficiency of 99.1%. The separation efficiency was comparable to other filtration membranes available in literature (Table 5.3). The reusability BSA-Sponge (0.75 cm) was tested for 10 cycles using 50 mL of the oil-in-water emulsion. Between the filtrations, BSA-Sponge was simply washed with water and the reusability was compared in terms of flux and separation efficiency. After 10 cycles, the BSA-Sponge retained its separation efficiency of 99.0% with the flux of 475 L.m\(^{-2}\).h\(^{-1}\). This demonstrates the stability and antifouling property of BSA-Sponge, which is an important parameter for practical applications.
**Table 5.2.** Flux rate and oil in water emulsion separation efficiency for various thickness of BSA-Sponge

<table>
<thead>
<tr>
<th>Sponge thickness (cm)</th>
<th>Flux rate (L.m².h⁻¹)</th>
<th>Separation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1144.8</td>
<td>95.2</td>
</tr>
<tr>
<td>0.75</td>
<td>477.0</td>
<td>99.1</td>
</tr>
<tr>
<td>1.0</td>
<td>286.2</td>
<td>99.1</td>
</tr>
</tbody>
</table>

**Table 5.3.** Comparison of various materials used for oil in water emulsion separation

<table>
<thead>
<tr>
<th>Materials platform</th>
<th>Separation efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-Sponge</td>
<td>99.1</td>
<td>This study</td>
</tr>
<tr>
<td>5Si-PMF/LDH</td>
<td>99.46</td>
<td>11</td>
</tr>
<tr>
<td>PAA-g-PVDF membrane</td>
<td>99</td>
<td>32</td>
</tr>
<tr>
<td>Cellulose sponge</td>
<td>99.94</td>
<td>17</td>
</tr>
</tbody>
</table>
5.5 Conclusions

In conclusion, we successfully synthesized a chemically crosslinked structured BSA-Sponge via carbodiimide coupling chemistry. The as prepared BSA-Sponge showed underwater oleophobicity with oil/water separation efficiency of 99.1% solely by gravity driven filtration. Since the separation is driven by gravity, this method of oil-in-water emulsions separation is desirable and energy efficient. The BSA-Sponge showed excellent mechanical properties with shape memory properties.

Several features of BSA-Sponge are responsible for observed oleophobicity and oil/water separation. The wetting behavior of materials is determined by the surface chemistry and can be tuned by surface modifications. We believe that the hydrophilic nature of BSA results in hydrophilic nature of the sponge. Furthermore, chemical crosslinking of the BSA in the network makes the pores more stable interconnected and sponge networks more flexible. These combined effects could boost water flux via liquid bridge within the connected channels. With good mechanical properties, antifouling nature hydrophilicity, the BSA-Sponge showed a potential candidate for oil/water separation in practical applications.

5.6 References


