Ultra-Stable Protein-Polymer Bioconjugates

Kyle S. Cole
University of Connecticut, kcole912@gmail.com

Recommended Citation
http://digitalcommons.uconn.edu/gs_theses/365
Ultra-Stable Protein-Polymer Bioconjugates

Kyle Stephen Cole

B.S., University of Connecticut, 2011

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
At the
University of Connecticut
2012
Masters of Science Thesis

Ultra-Stable Protein-Polymer Bioconjugates

Presented by
Kyle Stephen Cole, B.S.

Major Advisor________________________________
Dr. Challa V. Kumar

Associate Advisor________________________________
Dr. Steven L. Suib

Associate Advisor________________________________
Dr. Rajeswari M. Kasi

University of Connecticut
2012
Chapter 1: Ultra-Stable Hemoglobin-Poly(Acrylic Acid) Conjugates

1.1 Abstract

1.2 Introduction

1.3 Experimental
   1.3.1 Chemicals and Materials 9
   1.3.2 Polyamines 9
   1.3.3 Protein-Polymer Conjugate Synthesis 9
   1.3.4 Protein-Polymer Cross-Linking with Polyamines 13
   1.3.5 Agarose Gel Electrophoresis 13
   1.3.6 Transmission Electron Microscopy 14
   1.3.7 Dynamic Light Scattering 14
   1.3.8 Spectral Measurements 14
   1.3.9 Circular Dichroism 15
   1.3.10 Enzymatic Activity Assays 15
   1.3.11 Differential Scanning Calorimetry 15
   1.3.12 Steam Sterilization 16

1.4 Results and Discussion
   1.4.1 Synthesis of Hb-PAA Nanoparticles 17
   1.4.2 Agarose Gel Electrophoresis 18
   1.4.3 Elemental Analysis 22
   1.4.4 TEM and DLS 22
   1.4.5 Hemoglobin Structure in Hb-PAA Conjugates 27
   1.4.6 Peroxidase-like Enzymatic Activities of Hb-PAA 31
   1.4.7 Melt Curves and DSC Studies 37
   1.4.8 Reversibility of Thermal Denaturation 42
   1.4.9 Stability Towards Steam Sterilization 44
   1.4.10 Effect of Cross-Linking with Polyamines 46

1.5 Conclusions

51
Chapter 2: A General Approach Towards Ultra-Stable Protein-Polymer Bioconjugates

2.1 Abstract

2.2 Introduction

2.3 Experimental
   2.3.1 Chemicals and Materials
   2.3.2 Protein-Polymer Conjugate Synthesis
   2.3.3 Agarose Gel Electrophoresis
   2.3.4 Spectral Measurements
   2.3.5 Enzymatic Activity Assays

2.4 Results and Discussion
   2.4.1 Protein-Polymer Conjugates Synthesis
   2.4.2 Agarose Gel Electrophoresis
   2.4.3 Enzymatic Activity Assays

2.5 Conclusions

References
List of Tables

Table 1.1. Optimized Reaction Conditions for the synthesis of Hb-PAA(100)-6/7/8 and Hb-PAA(1000)-6/7/8 from Hb (10 μM, 500 μL, 50 nmol), PAA (270/2700 μL, 1 or 10 mM, 5 or 50 μmol, 1:100 or 1:1000 Hb to PAA mole ratio) and EDC (100 mM, 95.8 mg, 500 μmol) in sodium dibasic phosphate buffer.

Table 1.2. Optimized Reaction Conditions for Synthesis of Polyamine Cross-linked Bioconjugates from Hb-PAA(1000)-8 (5 μM, 2500 μL, 25 nmol), polyamine (en [2 M], teta [1 M] or tepa [1 M]) and EDC (100 mM, 95.8 mg, 500 μmol) in 20 mM Na₂HPO₄, pH 8.

Table 1.3. Far UV and Soret CD intensities (mdeg/µM.cm) of Hb-PAA(100) conjugates.

Table 1.4. Specific activities (x10⁻³ µM/mg) of Hb-PAA nanoparticles measured at 25 °C, 20 mM phosphate buffer, pH 7.2.

Table 1.5. Thermodynamic parameters for the denaturation of Hb-PAA nanoparticles.

Table 2.1. Reaction conditions for the synthesis of protein-PAA conjugates from Catalase, Glucose Oxidase or Lysozyme (10 μM, 500 μL, 50 nmol) and EDC (100 mM, 95.8 mg, 500 μmol) in sodium dibasic phosphate buffer, pH 7.

Table 2.2. Summary of extinction coefficients used for various proteins.
List of Figures

Figure 1.1. Agarose gel of Hb (lane 1), Hb/PAA physical mixture (lane 2), Hb/EDC mixture (lane 3) and Hb-PAA(100) nanoparticles synthesized at pH 6, 7 and 8 (lanes 4-6, respectively) spotted at the center of the gel (0.5% agarose, 40 mM Tris acetate, pH 6.5). The Hb, Hb/PAA physical mixture and Hb/EDC mixture migrated towards the negative electrode, while all the three Hb-PAA(100) covalent conjugates moved towards the positive electrode. These lanes did not show any unreacted Hb.

Figure 1.2. Agarose gel of Hb (lane 1), Hb-PAA(100) samples synthesized at pH 6, 7, and 8 (lanes 104, respectively) and Hb-PAA(1000) samples synthesized pH 6, 7, and 8, (lanes 5-7, respectively) spotted at the center of the gel (0.5% agarose, 40 mM Tris acetate, pH 7). Hb, migrated towards the negative electrode, while all the three Hb-PAA(100) covalent conjugates moved towards the positive electrode. These lanes did not show any unreacted Hb.

Figure 1.3. TEM images of Hb-PAA(100) and Hb-PAA(1000) samples synthesized at pH 6, 7 or 8 after ruthenium oxide staining: a) Hb-PAA(100)-6, b) Hb-PAA(100)-7, c) Hb-PAA(100)-8, d) Hb-PAA(1000)-6, e) Hb-PAA(1000)-7 and f) Hb-PAA(1000)-8. In some micrographs, the dark spots at the centers (insets) are clearly surrounded by a very light corona.

Figure 1.4. Dynamic light scattering plots for Hb-PAA(100) and Hb-PAA(1000) conjugates synthesized at pH 6, 7 and 8, respectively. The conjugates synthesized at pH 6.0 show major distribution, around 150 nm, while the conjugates synthesized at pH 7.0 and 8.0 show the major size distribution around 145 and 114 nm, respectively. All samples were in 20 mM Na$_2$HPO$_4$ buffer.

Figure 1.5. (a) Far UV CD of Hb, and Hb-PAA(100) conjugates synthesized at pH 6 (red), 7 (green) and 8 (blue) when compared to that of Hb (black); (b) Soret CD of the Hb-PAA(100) samples with the same color code, as the left panel. All spectra were normalized to obtain molar rotation per unit path length.

Figure 1.6. (a) The peroxidase-like activities of Hb-PAA(100)-6 (green curve), Hb, (black curve) and mixture of Hb and PAA (black dotted curve), and in the absence of hydrogen peroxide (red curve) (1 μM protein, 2.5 mM guaiacol and 1 mM H$_2$O$_2$ in 20 mM Na$_2$HPO$_4$ buffer, pH 7.2). (b) The peroxidase-like activities of Hb-PAA(1000)-7 (green curve), Hb, (black curve) and mixture of Hb and PAA (black dotted curve), and in the absence of hydrogen peroxide (red curve) (1 μM protein, 2.5 mM guaiacol and 1 mM H$_2$O$_2$ in 20 mM Na$_2$HPO$_4$ buffer, pH 7.2). (c) Comparison of the specific activities of Hb-PAA nanoparticles with that of Hb and physical mixtures, all measured at room temperature, 1 μM protein, 2.5 mM guaiacol and 1 mM H$_2$O$_2$ in 20 mM Na$_2$HPO$_4$ buffer, pH 7.2.

Figure 1.7. Lineweaver-Burk plots for the peroxidase-like activities of Hb-PAA(100)-6 (black dots), Hb-PAA(100)-7 (red dots). Lines are best fits to the data using the Lineweaver-Burk equation. All reactions were carried out in the presence of 1 μM Hb-PAA(100)-6 or Hb-PAA(100)-7, increasing guaiacol concentration and 1 mM H$_2$O$_2$ in 20 mM Na$_2$HPO$_4$ buffer, pH 7.2, room temperature.
**Figure 1.8.** (a) Melt curves of Hb, Hb/PAA physical mixture, and Hb-PAA(100)-7 recorded by monitoring heme absorption at 410 nm as a function of temperature. Improved stability of the conjugate is indicated by the shift of its melt curve to higher temperatures; (b) DSC thermograms (molar heat capacity plots) of Hb-PAA(100)-7 (green curve), Hb (black curve), and the Hb/PAA physical mixture (dotted line) as a function of temperature.

**Figure 1.9.** Far UV CD of Hb-PAA(100)-7 (red thick line) and Hb (black thin line) before heating is compared with the corresponding spectra obtained after heating the samples to 90 °C for 15 minutes, followed by cooling to room temperature for 15 min (red dashed, and black dashed lines, respectively).

**Figure 1.10.** Effect of steam sterilization (122 °C, 40 minutes, 17-20 psi) on the relative activities of: (a) Hb-PAA nanoparticles compared with those of Hb, Hb/PAA physical mixtures which are also treated similarly, and (b) polyamine crosslinked Hb-PAA(1000)-8 nanoparticles compared with that of Hb, before and after steam sterilization. All measurements were in phosphate buffer, pH 7.2, at room temperature.

**Figure 1.11.** Agarose gel (0.5%) of Hb (lane 1), Hb-PAA(1000) (lane 2), and Hb-PAA(1000)-teta (lanes 3-6) synthesized in phosphate buffer, pH 8. The samples were spotted at the bottom of the gel (running buffer, 40 mM Tris acetate pH 7.2). While Hb moved as a streak (lane 1, faint band), Hb-PAA(1000)-8 (lane 2) and Hb-PAA(1000)-teta (lanes 3-6, crosslinked with 60, 40, 20 and 10 mM teta, respectively) showed single bands. Hb-PAA(1000)-teta crosslinked nanoparticles moved lesser toward the positive electrode than Hb-PAA(1000)-8 due to neutralization of the negative charge by the reaction with the amine.

**Figure 1.12 (a-c).** Absorbance spectra of Hb-PAA(1000)-8-polyamine conjugates before heat denaturation at room temperature (blue curve), during denaturation at 90 °C (red curve) and after cooling back to room temperature (green curve), panels as labeled. Thermal denaturation clearly shifted the Soret band to higher temperatures and upon cooling most of the Soret band intensity and position are recovered, which suggests a high degree of reversibility of the thermal denaturation of these samples. In contrast, Hb underwent near complete irreversible thermal denaturation, under the same conditions.

**Figure 2.1.** Agarose gel of Catalase (lane 6), Cat-PAA(100) (lane 5), Cat-PAA(500) (lane 4), Cat-PAA(1000) (lane 3), Cat-PAA(1500) (lane 2) and Cat-PAA(2000) (lane 1). The conjugates used here were synthesized at pH 8. All samples were spotted in the center of the gel (0.5% agarose, 40 mM Tris-Acetate, pH 7.2) and allowed to run for 30 min at 100V. The unmodified catalase did not migrate far from the center of the gel, towards the positive electrode. The Cat-PAA conjugates all migrated further towards the positive electrode and do not show any unreacted catalase in the lanes.

**Figure 2.2.** Agarose gel of GOx (lanes 1 and 12) and GOx-PAA conjugates (lanes 2-11). The unmodified GOx in lanes 1 and 12 show two distinct bands because the protein sample consists of the monomer and dimer forms of GOx. The conjugates on this gel were synthesized at pH 6 and 7. The lanes consist of GOx-PAA(100) (lane 11, pH 6; lane 10, pH 7), GOx-PAA(500) (lane
9, pH 6; lane 8, pH 7), GOx-PAA(1000) (lane 7, pH 6; lane 6, pH 7), GOx-PAA(1500) (lane 5, pH 6; lane 4, pH 7) and GOx-PAA(2000) (lane 3, pH 6; lane 2, pH 7). All samples were spotted in the center of the gel (0.5% agarose, 40 mM Tris-Acetate, pH 7.2) and allowed to run for 30 min at 100 V. The GOx-PAA samples migrated further toward the negative electrode than the unmodified GOx samples, retaining the two band shape of the unmodified samples, thus there is no unreacted GOx in the lanes.

**Figure 2.3.** Agarose gel of lysozyme (lane 2) and Lys-PAA(100)-7 (lane 1). The unmodified lysozyme migrated towards the negative electrode, which means that the unmodified lysozyme has a net positive charge. The Lys-PAA(100) migrated towards the positive electrode and do not show any unreacted lysozyme in the lane. The samples were spotted in the center of the gel (0.5% agarose, 40 mM Tris-Acetate, pH 7.2) and allowed to run for 30 min at 100 V.

**Figure 2.4.** The decomposition activity of hydrogen peroxide by catalase (blue curve) and Cat-PAA(1000)-8 (green curve). All activity data was measured at room temperature in the presence of 0.5 µM protein and 20 mM H₂O₂ in 20 mM Na₂HPO₄, pH 7.2.

**Figure 2.5.** (a) Kinetics traces for GOx (red curve) and GOx-PAA conjugate samples (glucose (0.2 mM), GOx (0.5 µM), HRP (2 µM) and guaiacol (10 mM) in 20 mM Na₂HPO₄, pH 7.2). (b) Comparison of relative activities of the GOx-PAA conjugates with that of the unmodified GOx at room temperature under the same reaction conditions.

**Figure 2.6.** Kinetic traces for Lysozyme (red curve) and Lys-PAA(100)-6 (blue curve). Activity data was collected in a solution of *M. lysodeikticus* cells (0.36 mg/mL) and lysozyme (1 µM) in 20 mM Na₂HPO₄, pH 7.2 at room temperature.
List of Schemes

**Scheme 1.1.** (Top) Irreversible denaturation, conversion of the native state of the protein (N) to the corresponding denatured state (D), at the thermal denaturation temperature ($T_d$). (Bottom) Resistance to thermal deactivation can be achieved by stabilizing the native state with a suitable polymer (red lines) and/or by facilitating the denatured state to re-fold to the native state (reversible denaturation).

**Scheme 1.2.** The design of protein-polymer nanoparticles by covalent linking of the amino groups of hemoglobin (Hb) with the carboxyl functions of low molecular weight poly(acrylic acid) (PAA, MW 8000). Crosslinking of the polymer layer with ethylenediamine (en), triethylenetetrammine (teta) or tetraethylenpentamine (tepa) to further stiffen the polymer chains, and enhance resistance to thermal deactivation under steam sterilization conditions.

**Scheme 2.1.** The synthesis schematic for the formation of the protein-polymer conjugates by linking the amino groups of the protein to the carboxyl groups of the polymer, PAA. The coupling agent used here is 1-ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC).
Chapter 1

Ultra-Stable Hemoglobin-Poly(Acrylic Acid) Conjugates
1.1 Abstract

Stabilization of proteins against thermal deactivation is a major challenge, and a simple, facile, novel chemical approach is described here to overcome this hurdle. We report here, for the first time, the successful synthesis of ultrastable protein nanoparticles consisting of met-hemoglobin (Hb) conjugated with low molecular weight poly(acrylic acid) (PAA, $M_w$ 8,000) to form discrete nanoparticles. Hb-PAA nanoparticles were not deactivated when subjected to prolonged thermal treatment such as steam sterilization conditions (122 °C, 40 minutes, 17-20 psi), while the unprotected Hb lost most of its activity, when subjected to the same heating conditions. Several Hb-PAA derivatives which resist thermal inactivation, in a similar manner, are produced and characterized. Interestingly, the highest activity retention, after the above thermal treatment, was ~100% of the untreated samples. This resistance to heat is attributed to the enhanced thermodynamic stability of the Hb-PAA conjugate and improved re-folding of the denatured state to the native form, facilitated by PAA conjugation to Hb. This is a unique approach to stabilize Hb against thermal inactivation, and it is a major breakthrough in the production of stable Hb-based nanomaterials that can be safely sterilized in an autoclave for biomedical/in vivo applications.
1.2 Introduction

Improving protein thermal stability is a fundamental problem that is being intensely investigated,\textsuperscript{1,2} and steam sterilization conditions provide a bench-mark to evaluate the success of the strategies used for protein stabilization. Steam sterilization of proteins causes extensive loss of their biological activity. This is a serious drawback for applications that require sterilization,\textsuperscript{3,4,5} and protein stabilization against steam sterilization has been an elusive goal, so far. However, it is imperative to improve proteins’ resistance to steam sterilization since this technique is more commonly available in most laboratories for sterilization using an ordinary autoclave, effective, and inexpensive to perform. Even the highly stable thermophilic proteins are deactivated by steam sterilization and proteins designed by rational approaches are promising, but so far they met with only limited success in this regard.\textsuperscript{6} Enhanced thermal stability is also important for improved storage stability of proteins under different environmental temperatures as well as for biocatalytic applications at high temperatures where the reactions can be further accelerated. To date, there have been no established theoretical or experimental approaches to stabilize proteins against steam sterilization and this important goal of protein thermal stability needs to be achieved.\textsuperscript{7,8}

Protein-polymer nanosystems that are not deactivated by extreme heat, provide unique avenues to mass-produce thermally stable, steam-sterilizable biomaterials and products.\textsuperscript{9,10} Suitably designed protein-polymer nanosystems can address this important issue by exploiting and synergistically combining favorable materials aspects of polymers such as their high thermal stability with the desired biological activities of proteins.\textsuperscript{11} The attachment of strongly hydrophilic polymers such as polyethylene glycol (PEG) to proteins are suggested to stabilize
proteins against denaturation or loss of its three dimensional structure, but only limited progress has been made in terms of improved thermal stability, but none of these examples demonstrated resistance to deactivation against thermal treatments such as steam sterilization. Several conjugates showed significant retention of activity and improved storage stability but these also did not indicate resistance against steam sterilization or high temperature biocatalytic activities. Although constructing a nanoporous wall around the protein was expected to improve protein stability, resistance of the nanoconjugate to heat denaturation has not been evaluated. There are no known examples of general methodologies to stabilize proteins against steam sterilization via conjugation with polymers or otherwise, and overcoming this major hurdle could have a significant impact on a variety of applications of proteins as biomaterials, especially those that require sterilization, high temperature performance or long term stability. Here, we constructed novel protein-polymer nanoparticles and tested them for their thermal stability against steam sterilization conditions, as a bench mark to evaluate their thermal stability.
Scheme 1.1. (Top) Irreversible denaturation, conversion of the native state of the protein (N) to the corresponding denatured state (D), at the thermal denaturation temperature ($T_d$). (Bottom) Resistance to thermal deactivation can be achieved by stabilizing the native state with a suitable polymer (red lines) and/or by facilitating the denatured state to re-fold to the native state (reversible denaturation).
Our hypothesis is that stability can be improved by wrapping the protein in a soft, hydrophilic, water-soluble, flexible, polyelectrolyte where the hydrophilic polymer chains improve thermal stability of the protein. Improvement in stability can be due to two factors, 1) decrease in the conformational entropy of the native state (N) of the protein due to its encapsulation in the polymer matrix, thereby increasing intrinsic thermal stability of the protein; and 2) enhanced solubility of the denatured state (D) due to the covalently-bound hydrophilic polymer chains, which can inhibit aggregation of the denatured peptide and facilitate refolding back to the native state. Therefore, even if the protein undergoes thermal denaturation under steam sterilization conditions (122 °C, 40 min, 17-20 psi), in principle, it could re-fold into the native conformation and regain its biological activity, Scheme 1. This hypothesis has been tested in the current studies.

We successfully show that covalent linking of the carboxyl functions of a water-soluble anionic polymer to a model protein (Scheme 2) resulted in protein-polymer conjugates that resist deactivation by steam sterilization conditions.
Scheme 1.2. The design of protein-polymer nanoparticles by covalent linking of the amino groups of hemoglobin (Hb) with the carboxyl functions of low molecular weight poly(acrylic acid) (PAA, MW 8000). Crosslinking of the polymer layer with ethylenediamine (en), triethylenetetrammine (teta) or tetraethylenpentamine (tepa) to further stiffen the polymer chains, and enhance resistance to thermal deactivation under steam sterilization conditions.
We have chosen poly(acrylic acid) (PAA) as the water-soluble, flexible, anionic polymer and methemoglobin (Hb)\textsuperscript{20,21} as a model protein with multiple amino groups to test the above strategy. Improving Hb stability to sterilization is important because it has the potential to be used as a biocompatible artificial blood substitute or as a biocatalyst with improved thermal stability. Hb has been linked to PEG, or entrapped in liposomes and vesicles to improve its stability and potential as a blood substitute.\textsuperscript{22,23} Use of Hb as an artificial blood substitute, however, would require retention of its biofunction after sterilization and long term storage stability.\textsuperscript{24}

Previously, we have shown that random crosslinking of a high molecular weight PAA (MW 450,000) with Hb results in soluble, Hb-PAA nanogels.\textsuperscript{17} Careful and systematic evaluation of the nanogels showed that Hb encased in the polymer matrix retained its biological structure and activities, and that the crosslinking has been benign. However, the nanogels did not indicate considerable improvement in thermal stability or protection against steam sterilization.

In sharp contrast, the data presented here shows that protein stability can be increased substantially by attaching, low molecular weight PAA to Hb to form discrete Hb-PAA nanoparticles, as opposed to nanogels, via a general, simple, efficient and facile synthetic strategy (Scheme 2). The resulting Hb-PAA nanoparticles resist deactivation by steam sterilization. This simple strategy may be extended to other protein-PAA conjugates and attaining such thermal stability is a major breakthrough for the biomedical/biomaterial applications that require sterilization or long term stability.\textsuperscript{25,26}
1.3 Experimental

1.3.1 Chemicals and Materials

Bovine met-hemoglobin (Hb) was purchased from MP Biomedicals (Solon, OH). Protein solutions were prepared in the respective buffers used for synthesis, as indicated, and the concentrations determined from the Soret absorbance of Hb using extinction co-efficient 303,956 M$^{-1}$cm$^{-1}$ at 407 nm.$^{27}$

1.3.2 Polyamines

Ethylenediamine (en), triethylenetetramine (teta), and tetraethylenepentamine (tepa) were purchased from Sigma (St. Louis, MO). Stock solutions of the polyamines were prepared in deionized water (DI) and the pH was adjusted to 7.2 with concentrated hydrochloric acid before the solutions were made to their final volumes.

1.3.3 Protein-Polymer Conjugate Synthesis

The Hb-PAA conjugates were synthesized by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), purchased from TCI America (Portland, OR), as the coupling agent by reported methods,$^{28}$ with minor modifications. PAA (MW 8,000) solution 45% (w/w) was purchased from Sigma-Aldrich (St. Louis, MO) and diluted four-fold to a concentration of 18.6 mM by the addition of DI. A mixture of 500 μL of 200 mM Na$_2$HPO$_4$ buffer at pH 6, 7 or 8 with 270 μL of PAA (1 mM) and 3730 μL of deionized water were added to a reaction vial, followed by sonication for 5 minutes. Next, 100 mmol of EDC was added to the vial while stirring. The resulting PAA/EDC mixture was stirred for 5 minutes and then 500 µl of 10 µM Hb (50 nmol) was added and stirred for 12 h. Specific reaction conditions for each of all the samples are
summarized in tables 1 & 2. Excess EDC, PAA and urea by-product were dialyzed 3 times with 1000 times the sample volume with 25 kDa molecular weight cut off dialysis membrane to obtain pure protein-polymer conjugates.

The product obtained from the reaction of 5000 nmol of PAA with Hb (50 nmol) at pH 7 (PAA to Hb mole ratio of 100:1) is designated as Hb-PAA(100)-7, while the reaction of 50,000 nmol PAA with 50 nmol of Hb (PAA to Hb mole ratio of 1000:1) at pH 7 is labeled as Hb-PAA(1000)-7. This nomenclature will be used throughout the manuscript for unique identification of specific Hb-PAA nanoparticle samples. Each sample has been centrifuged at 12,000 RPM and filtered using 0.22 µm filter to remove invisible precipitates, if any.
Table 1.1. Optimized Reaction Conditions for the synthesis of Hb-PAA(100)-6/7/8 and Hb-PAA(1000)-6/7/8 from Hb (10 μM, 500 μL, 50 nmol), PAA (270/2700 μL, 1 or 10 mM, 5 or 50 μmol, 1:100 or 1:1000 Hb to PAA mole ratio) and EDC (100 mM, 95.8 mg, 500 μmol) in sodium dibasic phosphate buffer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[PAA] (mM)</th>
<th>PAA Vol (μL)</th>
<th>Moles PAA</th>
<th>Buffer pH</th>
<th>Buffer Vol (μL)</th>
<th>DI Vol (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb-PAA(100)-6</td>
<td>1</td>
<td>270</td>
<td>5 μmol</td>
<td>6</td>
<td>500</td>
<td>3730</td>
</tr>
<tr>
<td>Hb-PAA(100)-7</td>
<td>1</td>
<td>270</td>
<td>5 μmol</td>
<td>7</td>
<td>500</td>
<td>3730</td>
</tr>
<tr>
<td>Hb-PAA(100)-8</td>
<td>1</td>
<td>270</td>
<td>5 μmol</td>
<td>8</td>
<td>500</td>
<td>3730</td>
</tr>
<tr>
<td>Hb-PAA(1000)-6</td>
<td>10</td>
<td>2700</td>
<td>50 μmol</td>
<td>6</td>
<td>500</td>
<td>1300</td>
</tr>
<tr>
<td>Hb-PAA(1000)-7</td>
<td>10</td>
<td>2700</td>
<td>50 μmol</td>
<td>7</td>
<td>500</td>
<td>1300</td>
</tr>
<tr>
<td>Hb-PAA(1000)-8</td>
<td>10</td>
<td>2700</td>
<td>50 μmol</td>
<td>8</td>
<td>500</td>
<td>1300</td>
</tr>
</tbody>
</table>
Table 1.2. Optimized Reaction Conditions for Synthesis of Polyamine Cross-linked Bioconjugates from Hb-PAA(1000)-8 (5 μM, 2500 μL, 25 nmol), polyamine (en [2 M], teta [1 M] or tepa [1 M]) and EDC (100 mM, 95.8 mg, 500 μmol) in 20 mM Na₂HPO₄, pH 8

<table>
<thead>
<tr>
<th>Sample</th>
<th>[en] (mM)</th>
<th>Moles en (μmol)</th>
<th>[teta] (mM)</th>
<th>Moles teta (μmol)</th>
<th>[tepa] (mM)</th>
<th>Moles tepa (μmol)</th>
</tr>
</thead>
</table>
1.3.4 Protein-Polymer Cross-Linking with Polyamines

Polyamines were used to crosslink the PAA chains of the above Hb-PAA nanoparticles, using EDC coupling chemistry. To Hb-PAA(1000)-8 in 20 mM Na2HPO4 buffer at pH 8 (5 µM protein, 5 mL) was added 100 mmol of EDC and the mixture stirred for another 5 minutes before various volumes of polyamines have been added to gain the desired concentrations of polyamine (en, teta, and tepa, reaction conditions for other samples given in the ESI, Table S2). The reaction mixture was further allowed to stir overnight, at room temperature. Excess EDC, PAA and urea byproduct were removed via dialysis with 25 kDa molecular weight cut off dialysis membrane.

1.3.5 Agarose Gel Electrophoresis

Native agarose gels were prepared by dissolving molecular biology grade agarose (0.5% w/v) (U.S. Biological, Swampscott, MA) in a hot solution of Tris-Acetate (40 mM, pH 7.2). The gel was run in a horizontal gel electrophoresis apparatus (Gibco model 200, Life Technologies Inc, MD) using Tris-Acetate (40 mM pH 7.2) as the running buffer. Samples were loaded into the gel with loading buffer (50 % v/v glycerol and 0.01% m/m bromophenol blue) and electrophoresis carried out for 30 minutes at 100 V at room temperature. The gel was stained overnight with 10% v/v acetic acid, 0.02% m/m Coomassie blue and then destained with 10% v/v acetic acid for an additional night. Polyamine cross-linked protein-polymer nanoparticles were stained by Zn(II) reverse staining procedure. The gel was incubated for 25 minutes in ZnSO4-Imidazole solution (15 mM ZnSO4:30 mM Imidazole) followed by incubation in a 10% (w/v) Na2CO3 solution to develop the bands. The gel was photographed using a Molecular Imager Gel Doc XRS System.
1.3.6 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to examine the morphology of Hb-PAA conjugate, Hb and PAA physical mixture (Hb/PAA), Hb and PAA. The images were obtained with a Tecnai T12 instrument operating at an accelerating voltage of 120 kV. Solutions of Hb-PAA, Hb/PAA and PAA were diluted to 0.1 mg/ml based on PAA concentration. The Hb solution was diluted to 0.1 mg/ml Hb. A drop of each 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 ruthenium tetroxide for 30 minutes. Digital images were collected and presented.

1.3.7 Dynamic Light Scattering

Nanoparticle sizes were determined by Precision Detectors (Varian Inc., Santa Clara, CA) CoolBatch+ dynamic light scattering (DLS) apparatus using a 5 x 5 mm square cuvette, 658 nm excitation laser source with a 90° geometry for the excitation and monitoring optics. Samples were routinely filtered with a 0.2-micron filter prior to analysis to remove dust particles and any large aggregates.

1.3.8 Spectral Measurements

The absorption spectra were recorded on HP 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA). The Hb solutions mixed in the respective buffers was determined from the Soret absorbance of Hb using an extinction coefficient of 303,956 M\(^{-1}\)cm\(^{-1}\) at 407 nm. Further, the denaturation temperatures of Hb, Hb/PAA and Hb-PAA were
determined by measuring the thermal transition points from the plots of Soret absorbance at 407 nm as a function of temperature.

1.3.9 Circular Dichroism

A JASCO model J715 spectropolarimeter (Jasco Inc, Easton, MD) was used to record the circular dichroism (CD) spectra. Scan rates were 50 nm/min with a step resolution of 1 nm/data point. Several scans (8-16) were averaged for each sample (0.05 and 1 cm path length) and data plotted using Kaleidagraph 4.1.1. Samples for CD were prepared as mentioned above and signals have been normalized with respect to concentrations of individual samples and the pathlength.

1.3.10 Enzymatic Activity Assays

The peroxidase-like activity of Hb was measured by following the literature method, as reported earlier. Product absorbance at 470 nm was monitored as a function time of after the addition of H₂O₂ (1 mM) to Hb (1 µM) and o-methoxyphenol (2.5 mM). Lineweaver-Burk plots for the protein-polymer conjugates were constructed by obtaining initial reaction rates at various substrate concentrations and by plotting inverse initial rate vs inverse substrate concentration. The corresponding Michaelis-Menten parameters, the Michaelis constant (Kₘ) and the maximum velocity (Vₘₐₓ), were extracted from this plot as reported earlier. All peroxidase-like activities of steam sterilized samples were measured with 1 µM Hb, 2.5 mM guaiacol and 1 mM H₂O₂ in 20 mM Na₂HPO₄ buffer, pH 7.2, or 8, at room temperature using the above method.

1.3.11 Differential Scanning Calorimetry
A Nano II differential scanning calorimeter (DSC) 6100 from Calorimetry Sciences Corporation (CSC, Utah) was used to perform thermal denaturation experiments. The amount of heat required to increase the temperature of a sample with respect to a reference (Cp) by 1 °C was monitored in a series of heating and cooling scans from 20 to 120 °C at a scan rate of 2 °C/min. Thermodynamic parameters (ΔH, and T_m) were obtained from the DSC traces, as described earlier.

1.3.12 Steam Sterilization

An Amsco Century Scientific (Steris, Mentor, OH) model SI-120 isothermal sterilizer was used for steam sterilization. Liquid samples containing Hb-PAA conjugates and unmodified proteins were steam sterilized for 40 min at 122 °C (17-20 psi). Samples were cooled down to room temperature and the peroxidase-like activity of Hb and Hb-PAA conjugates were measured, as described above. The initial rates were calculated using Kaleidagraph from the kinetic traces of absorbance vs time plots, and specific activities have been calculated.
1.4 Results and Discussion

Hb-PAA covalent conjugates are synthesized by covalent linking of the carboxyl functions of PAA with the amino groups of the lysine side chains of Hb, under controlled conditions of stoichiometry, polymer concentration, polymer molecular weight, pH and reaction time. Previously, we have shown that covalent linking of high molecular weight PAA (MW 450,000) to Hb resulted in the formation of protein-polymer nanogels which did not retain activity after steam sterilization. Here, we lowered the PAA molecular weight and its concentration to suppress network formation, and increased the mole ratio of polymer to protein to produce discrete Hb-PAA nanoparticles, which indicated high resistance to thermal deactivation.

1.4.1 Synthesis of Hb-PAA Nanoparticles

Proteins, in general, are polyfunctional molecules with large numbers of amine and carboxyl groups on them. The amino groups of the lysine side chains of proteins have been successfully coupled with a large number of substrates consisting of carboxyl groups, via the standard carbodiimide chemistry. A similar strategy was followed here (Scheme 2). Covalent attachment of a large excess of PAA (MW 8000, 100 or 1000-fold molar excess) to the lysine side chains of Hb was carried out by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at pH 6, 7 or 8, in 20 mM Na2HPO4 buffer (ESI, Table S1). All the conjugates reported here were completely soluble at all times, and there have been no precipitates or gel formation. Most mammalian Hb molecules have ~44 lysine residues, and a large number of them can potentially react with the activated carboxyl groups of PAA. The Hb/PAA/EDC reaction mixture was allowed to react for 12 h and dialyzed to remove the unreacted PAA, EDC or urea by-products.
All samples reported here are completely water-soluble and there have been no precipitates under our reaction conditions. Progress of the crosslinking reaction was followed by agarose gel electrophoresis, described below.

1.4.2 Agarose Gel Electrophoresis

The covalent attachment of anionic PAA to Hb is expected to give rise to negatively charged Hb-PAA conjugates, and this conversion of positively charged Hb to negatively charged conjugate is expected to increase its electrophoretic mobility toward the positive electrode. In contrast, if the synthesis resulted in the formation of macroscopic protein-polymer macrogels, the gels would not migrate through the narrow pores due to their large size, when compared to the gel pores (~450 nm). As anticipated, the Hb-PAA(100) conjugates (100-fold excess PAA per Hb, lanes 4-6, Fig. 1) moved towards positive electrode, while Hb, Hb/PAA physical mixture, or Hb/EDC mixture (lanes 1-3, respectively) migrated towards the negative electrode. Migration reversal is not due to physical binding of PAA to Hb, as the mixture (lane 2) migrated toward the negative electrode but not the positive electrode. The presence of a new band in lanes 4-6 and the absence of the Hb band, indicated complete conversion of Hb to the corresponding Hb-PAA conjugate. In contrast, the Hb-PAA nanogels produced from the conjugation of high molecular weight PAA (MW 450,000) with Hb, moved as streaks, under the same conditions of electrophoresis.\textsuperscript{17a} Thus, the covalent linking of the anionic polymer to Hb to form discrete particles is clearly demonstrated.
Figure 1.1. Agarose gel of Hb (lane 1), Hb/PAA physical mixture (lane 2), Hb/EDC mixture (lane 3) and Hb-PAA(100) nanoparticles synthesized at pH 6, 7 and 8 (lanes 4-6, respectively) spotted at the center of the gel (0.5% agarose, 40 mM Tris acetate, pH 6.5). The Hb, Hb/PAA physical mixture and Hb/EDC mixture migrated towards the negative electrode, while all the three Hb-PAA(100) covalent conjugates moved towards the positive electrode. These lanes did not show any unreacted Hb.
We also prepared Hb-PAA(1000) samples at pH 6, 7, and 8, where there has been 1000-fold molar excess of the polymer, and compared the mobilities of these samples with those of Hb-PAA(100) samples (Figure 2). While the Hb-PAA(100) samples indicated tight bands (lanes 2-4), the Hb-PAA(1000) samples (lanes 5-7, respectively) had more diffuse bands due to higher concentrations of PAA. These lanes also did not indicate free Hb (lane 1) and both reaction conditions resulted in the complete conjugation of Hb with the polymer, and there has been no visible precipitation under any of the reaction conditions reported here.

Electrophoretic mobilities of all the Hb-PAA conjugates (both 1:100 and 1:1000 mole ratio samples, prepared under three different pH conditions) indicated a measure of the charge and size. The average pore size of agarose gel used in Figure 1 & S1 (0.5% gel) is around 450 nm, \(^{34}\) and all the Hb-PAA conjugates moved readily through these pores. Therefore, the conjugates are likely to be much smaller than 450 nm. Additionally, there is no formation of macroscopically crosslinked gels, which would have indicated streaks of bands or precipitation in the wells.\(^{17a}\) This conclusion is consistent with the observed high solubility of the conjugates in the reaction mixture and the low overlap concentration of low molecular weight of PAA (MW 8,000) used in the current studies. The agarose gel data confirm the clean ligation of Hb to PAA with particle sizes well below 450 nm.\(^{35}\) All conjugates reported here are completely water soluble and there have been no precipitations under any of these conditions.
Figure 1.2. Agarose gel of Hb (lane 1), Hb-PAA(100) samples synthesized at pH 6, 7, and 8 (lanes 4, respectively) and Hb-PAA(1000) samples synthesized pH 6, 7, and 8, (lanes 5-7, respectively) spotted at the center of the gel (0.5% agarose, 40 mM Tris acetate, pH 7). Hb, migrated towards the negative electrode, while all the three Hb-PAA(100) covalent conjugates moved towards the positive electrode. These lanes did not show any unreacted Hb.
1.4.3 Elemental Analysis

Covalent conjugation of carbon rich PAA polymer to Hb is expected to increase the C:N ratio, and from this ratio, one can assess the amount of polymer bound per protein molecule. For example, the C:N ratio of 6.34 was measured for the Hb-PAA(1000)-8 which increased from 3.51 for the untreated Hb. Since the nanoparticles were extensively dialyzed to remove unreacted PAA, the increase in C:N ratio is indicative of increase in carbon content (PAA) of the conjugate. From this ratio we deduce that on average nearly 7 PAA polymer chains were attached for every Hb in Hb-PAA(1000)-8, giving a Hb:PAA stoichiometry of 1:7. Since molecular weight of PAA is 8000, that of Hb is 64,500, and seven of these chains are attached to one Hb molecule, the average mass ratio of protein to polymer is nearly (64,500):(7x8,000) or ~1:0.9, or approximately equimass ratio. Thus, each protein molecule is surrounded by several low molecular weight polymer chains, and even at 1000-fold higher concentration of PAA only a few chains are attached to the protein.

1.4.4 TEM and DLS

The Hb-PAA samples were visualized by TEM to determine their morphology, size, shape and phase separation characteristics. In general, TEM micrographs indicated spherical particles and we did not see macrogels or highly crosslinked products (Figure 2). Hb-PAA(100) synthesized at pH 7, for example, (Figure 2b) indicated spherical spots with a dark core and very light corona (inset).
Figure 1.3. TEM images of Hb-PAA(100) and Hb-PAA(1000) samples synthesized at pH 6, 7 or 8 after ruthenium oxide staining: a) Hb-PAA(100)-6, b) Hb-PAA(100)-7, c) Hb-PAA(100)-8, d) Hb-PAA(1000)-6, e) Hb-PAA(1000)-7 and f) Hb-PAA(1000)-8. In some micrographs, the dark spots at the centers (insets) are clearly surrounded by a very light corona.
The TEMs of the Hb-PAA(100) samples synthesized at 6, 7 and 8, are compared in Figure 2(a-c) and none of these indicated nanogels or macroscopically crosslinked gels. The insets in these panels are expanded views of randomly chosen particles to illustrate their contours better, and the average sizes of the Hb-PAA(100) particles synthesized at pHs 6, 7, and 8 were ~250, ~125 and ~100 nm (Figure 2a-c), respectively. The particle size decreased, perceptibly, with increase in pH, at Hb:PAA concentration ratio of 1:100. This decrease in size could be due to increased charge repulsion between the PAA and Hb chains, as the pH is increased and fewer molecules are attached at higher pH.

Next, we examined the influence of PAA concentration on the particle formation and the TEM micrographs of Hb-PAA(1000) samples prepared with a protein to polymer ratio of 1:1000 also indicated nanoparticles (Figure 2e). These are also nearly spherical, with a dark core and a very light corona, in some cases, and they also did not indicate the formation of any macroscopic gels. These are compared with those synthesized at pH 6 and 8 (Figure 2d-f) which also showed spherical nanoscopic species, and none of them indicated nano- or macrogels. The Hb-PAA(1000) samples synthesized at pH 6, 7, and 8 had average sizes ~80, ~90 and ~100 nm, respectively, and these are smaller than those obtained at lower stoichiometries of Hb to PAA. Higher polymer concentration (higher stochiometry) decreased the particle size, and this could be due to the greater distribution of Hb molecules among a larger number of PAA chains, resulting in smaller particle formation.

The sizes of the 1000:1 samples increased slightly with increase in pH which is opposite to what was noted with the 100:1 samples, where the size decreased with increase in pH. Thus, not only the pH but also the polymer concentration influenced the particle size to a measurable extent, while the shape and morphology appears to be nearly independent of these factors.
Lower pH and higher PAA concentration resulted in the smallest particles. Thus, the particle size can be tuned from about 250 nm to around 80 nm, in a predictable manner, depending on the reaction conditions.

The formation and size of the Hb-PAA nanoparticles determined from TEM are also corroborated by dynamic light scattering (DLS) data (Figure 3), where the trends in size with pH and polymer concentration are similar to those obtained from TEM micrographs. The conjugates synthesized at pH 6.0 show major distribution, around 150 nm, while the conjugates synthesized at pH 7.0 and 8.0 show the major size distribution around 145 and 114 nm, respectively.
Figure 1.4. Dynamic light scattering plots for Hb-PAA(100) and Hb-PAA(1000) conjugates synthesized at pH 6, 7 and 8, respectively. The conjugates synthesized at pH 6.0 show major distribution, around 150 nm, while the conjugates synthesized at pH 7.0 and 8.0 show the major size distribution around 145 and 114 nm, respectively. All samples were in 20 mM Na$_2$HPO$_4$ buffer.
1.4.5 Hemoglobin Structure in Hb-PAA Conjugates

Native structure of the encapsulated protein is critical for the retention of its biological activity, and we used circular dichroism (CD) spectroscopy to assess Hb structure before and after conjugation to PAA. The CD spectra of the Hb-PAA nanoparticles in the far-UV region (Figure 4a) are similar to that of Hb,\textsuperscript{36} and these indicated significant retention of its native-like secondary structure upon conjugation with PAA. These peak positions, intensities and ratio of the intensities of 222/209 nm peaks are compared in Table 1. In conclusion, the UV CD data confirm that the protein secondary structure is not adversely effected by the conjugation reaction.
Figure 1.5. (a) Far UV CD of Hb, and Hb-PAA(100) conjugates synthesized at pH 6 (red), 7 (green) and 8 (blue) when compared to that of Hb (black); (b) Soret CD of the Hb-PAA(100) samples with the same color code, as the left panel. All spectra were normalized to obtain molar rotation per unit path length.
The Soret absorption band of Hb also gives rise to the corresponding CD and it is a sensitive signature of the structural changes at the heme binding pocket. Soret CD served as another means to interrogate the influence of conjugation on protein structure. The Soret CD of the Hb-PAA(100) samples synthesized at pH 6, 7 and 8 are compared with that of Hb (Figure 4b), and these indicated some loss in intensity after conjugation, but the peak positions of the nanoparticles matched with that of Hb. The Soret CD spectra suggest that the heme binding pocket has undergone some distortion, even though the secondary structure of Hb remained mostly intact. These CD data are summarized in Table 3, for convenience, and these clearly show significant retention of the secondary structure, but the loss in Soret intensity of some derivatives was of concern, and hence, we tested if these changes adversely influenced the peroxidase-like activity of Hb-PAA nanoparticles, as discussed below.
Table 1.3. Far UV and Soret CD intensities (mdeg/µM.cm) of Hb-PAA(100) conjugates

<table>
<thead>
<tr>
<th></th>
<th>222 nm</th>
<th>209 nm</th>
<th>222/209 nm</th>
<th>409 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>-80</td>
<td>-74</td>
<td>1.07</td>
<td>2.17</td>
</tr>
<tr>
<td>Hb/PAA</td>
<td>-92</td>
<td>-89</td>
<td>1.03</td>
<td>2.05</td>
</tr>
<tr>
<td>Hb-PAA(100)-6</td>
<td>-93</td>
<td>-88</td>
<td>1.06</td>
<td>1.13</td>
</tr>
<tr>
<td>Hb-PAA(100)-7</td>
<td>-91</td>
<td>-88</td>
<td>1.03</td>
<td>1.93</td>
</tr>
<tr>
<td>Hb-PAA(100)-8</td>
<td>-86</td>
<td>-91</td>
<td>1.00</td>
<td>1.56</td>
</tr>
</tbody>
</table>
1.4.6 Peroxidase-like Enzymatic Activities of Hb-PAA

Hb does not function as an enzyme in biological systems but its peroxidase-like activity has long been known.\textsuperscript{37,38} Catalytic activity of Hb-PAA nanoparticles was monitored by the reaction of guaiacol (substrate) with hydrogen peroxide (oxidant), and formation of the colored product has been monitored by its absorption at 470 nm, as a function of time (Figure 4). For example, the product formation catalyzed by Hb-PAA(100)-6 (Figure 4a, green curve) is nearly comparable to that of Hb (black curve), there has been no reaction in the absence of hydrogen peroxide (red curve), and PAA did not catalyze the reaction (data not shown). A mixture of Hb and PAA (black dashed curve) indicated activities that are lower than that of the corresponding Hb-PAA conjugate.

Similarly, the peroxidase activity of Hb-PAA(1000)-7 is shown in Supplementary Materials S4, and the activities are comparable to that of Hb or the physical mixture of Hb/PAA. These data are consistent with the conclusion that PAA did not adversely influence peroxidase activity of Hb-PAA nanoparticles.
Figure 1.6. (a) The peroxidase-like activities of Hb-PAA(100)-6 (green curve), Hb, (black curve) and mixture of Hb and PAA (black dotted curve), and in the absence of hydrogen peroxide (red curve) (1 μM protein, 2.5 mM guaiacol and 1 mM H₂O₂ in 20 mM Na₂HPO₄ buffer, pH 7.2). (b) The peroxidase-like activities of Hb-PAA(1000)-7 (green curve), Hb, (black curve) and mixture of Hb and PAA (black dotted curve), and in the absence of hydrogen peroxide (red curve) (1 μM protein, 2.5 mM guaiacol and 1 mM H₂O₂ in 20 mM Na₂HPO₄ buffer, pH 7.2). (c) Comparison of the specific activities of Hb-PAA nanoparticles with that of Hb and physical mixtures, all measured at room temperature, 1 μM protein, 2.5 mM guaiacol and 1 mM H₂O₂ in 20 mM Na₂HPO₄ buffer, pH 7.2.
The initial rates from the kinetic traces of all the six Hb-PAA nanoparticle samples were measured and compared with those of Hb and Hb/PAA physical mixtures (Figure 5c). The conjugates showed significant activity, and only in the case of Hb-PAA(100)-8, substantial reduction has been noted (~40% loss), the remaining five samples had specific activities close to that of Hb (Table 5). Some improvements may have been noted in some cases but these are well within the experimental errors indicated. While the Hb/PAA(100) physical mixture was nearly as active as Hb, the Hb/PAA(1000) physical mixture was measurably less active and this could be due to increased viscosity of the solution due to the higher PAA concentration in the mixture. The majority of Hb-PAA nanoparticles, however, retained most of their peroxidase-like activities.

Initial reaction rates from the kinetic data were obtained at a series of substrate concentrations and they have been used to construct Michaelis-Menton plots for the extraction of the corresponding enzymatic parameters, Michaelis constant (Km) and the maximum velocity (Vmax). The plot of inverse of the initial rate as a function of the inverse of the substrate concentration indicated linear plots for Hb-PAA(100)-6 and Hb-PAA(100)-7 (Figure 6).
Figure 1.7. Lineweaver-Burk plots for the peroxidase-like activities of Hb-PAA(100)-6 (black dots), Hb-PAA(100)-7 (red dots). Lines are best fits to the data using the Lineweaver-Burk equation. All reactions were carried out in the presence of 1 µM Hb-PAA(100)-6 or Hb-PAA(100)-7, increasing guaiacol concentration and 1 mM H$_2$O$_2$ in 20 mM Na$_2$HPO$_4$ buffer, pH 7.2, room temperature.
**Table 1.4.** Specific activities (x10^{-3} \mu M/mg) of Hb-PAA nanoparticles measured at 25 °C, 20 mM phosphate buffer, pH 7.2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity (x10^{-3} \mu M/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Hb/PAA(100)</td>
<td>2.3 ± 0.07</td>
</tr>
<tr>
<td>Hb-PAA(100)- 6</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Hb-PAA(100)- 7</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Hb-PAA(100)- 8</td>
<td>1.4 ± 0.07</td>
</tr>
<tr>
<td>Hb/PAA(1000)</td>
<td>2.0 ± 0.07</td>
</tr>
<tr>
<td>Hb-PAA(1000)- 6</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Hb-PAA(1000)- 7</td>
<td>2.6 ± 0.07</td>
</tr>
<tr>
<td>Hb-PAA(1000)- 8</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>
Linear fits to these data (Figure 6) using the Michaelis-Menton model indicated $V_{\text{max}}$ of 0.0028, 0.0025 and 0.0026 mM/s and $K_m$ of 1.25, 0.375 and 0.50 mM, for Hb-PAA(100)-6, Hb-PAA(100)-7 and Hb,\textsuperscript{39} respectively. under similar conditions of buffer, ionic strength, temperature and pH. The maximum velocities observed with all the three samples, for example, are similar, and polymer conjugation did not alter this parameter to a significant extent. While the $K_m$ of Hb-PAA(100)-6 is three times larger than that of Hb, the $K_m$ of Hb-PAA(100)-7 matched well with that of Hb. Enhanced $K_m$ of Hb-PAA(100)-6 is indicative of a decrease in its affinity for the substrate, and this observation is intriguing but it also coincides with the substantial loss of its Soret CD band. Therefore, in this case, the heme-pocket in the globin is distorted enough to cause substantial reduction of the binding of the substrate at the active site but not its catalytic efficiency. Overall, the catalytic parameters found for Hb-PAA nanoparticles are consistent with the notion that formation of the covalently conjugated nanoparticles did not substantially alter Hb native-like structure or its peroxidase-like activities.
1.4.7 Melt Curves and DSC Studies

Encouraged by the retention of secondary structure and biocatalytic activities of the Hb-PAA nanoparticles, we next assessed their thermal stability by recording Soret absorbance as a function of temperature (melt curves) as well as by differential scanning calorimetry (DSC). While the melt curves provide a quick measure of thermal stability, reversibility and precipitation of the sample during or after denaturation, the DSC\textsuperscript{40} provides a direct quantitative measure of the thermodynamic parameters of thermal denaturation.\textsuperscript{41}
Figure 1.8. (a) Melt curves of Hb, Hb/PAA physical mixture, and Hb-PAA(100)-7 recorded by monitoring heme absorption at 410 nm as a function of temperature. Improved stability of the conjugate is indicated by the shift of its melt curve to higher temperatures; (b) DSC thermograms (molar heat capacity plots) of Hb-PAA(100)-7 (green curve), Hb (black curve), and the Hb/PAA physical mixture (dotted line) as a function of temperature.
On one hand, a plot of the ratio of the Soret absorbance of the sample at 407 nm (A) to initial absorbance of the sample (Ao) as a function of temperature (Figure 7a), indicated a sharp increase followed by decrease with a peak around 65 °C for Hb (black curve), and this is consistent with the denaturation of Hb followed by its precipitation from solution, around this temperature. On the other hand, the melt curve for Hb-PAA(100)-7 (Figure 7a, green line), under the same conditions of buffer, pH and ionic strength, indicated the beginning of a change around 60 °C, which continued upto 100 °C but no precipitation has been noted. The physical mixture of Hb and PAA (black dotted line) indicated some stabilization followed by denaturation but eventually precipitated. Thus, Hb-PAA(100)-7 indicated improved thermal stability when compared to that of Hb or the corresponding Hb/PAA physical mixture. The melt curves of other Hb-PAA conjugates also indicated improved stability when compared to that of Hb, no precipitation, and the Soret absorption band recovered upon cooling the samples to room temperature. These details are discussed latter.
Table 1.5. Thermodynamic parameters for the denaturation of Hb-PAA nanoparticles

<table>
<thead>
<tr>
<th></th>
<th>Hb</th>
<th>Hb/PAA</th>
<th>Hb-PAA(100)-6</th>
<th>Hb-PAA(100)-7</th>
<th>Hb-PAA(100)-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_d/ ^\circ \text{C}$</td>
<td>$68\pm0.2$</td>
<td>$68\pm0.5$</td>
<td>$61\pm3.0$</td>
<td>$77\pm3.0$</td>
<td>$65\pm1.2$</td>
</tr>
<tr>
<td>$\Delta H/\text{kcal/mol}$</td>
<td>$135\pm5$</td>
<td>$145\pm10$</td>
<td>$310\pm40$</td>
<td>$540\pm35$</td>
<td>$130\pm20$</td>
</tr>
</tbody>
</table>
To quantify the thermodynamic parameters of protein denaturation, the DSC curves of Hb-PAA(100)-7, Hb and Hb/PAA physical mixture were recorded and the denaturation temperatures (T\text{d}) have been determined (Figure 6b). The DSC profile of Hb-PAA(100)-7 (green curve), for example, indicated a small peak around 77 °C, and then a much larger transition near 100 °C. The first, smaller peak is slightly shifted to higher temperature when compared to that of Hb (black curve, T\text{d} of 68 °C). The DSC profile of the Hb/PAA physical mixture (balck dotted curve) indicated an endothermic transition larger than that of Hb, with a T\text{d} of 63 °C, below that of Hb. PAA itself (thin black line) did not indicate any detectable transition in this temperature range, and hence, the nanoparticles indicated much greater stability (two separate transitions) when compared to that of Hb or the corresponding physical mixture. Such high thermal stability is unusual for most proteins.

The denaturation enthalpies (\Delta H) were estimated from the DSC curves, and these are compared in Table 2. The \Delta H for the denaturation of the nanoparticles ranged from 130 to 540 kcal/mol, and enthalpy changes of Hb-PAA(100)-6 and Hb-PAA(100)-7 are significantly higher than those of Hb (135 kcal/mol) as well as Hb/PAA physical mixture (145 kcal/mol). Hb-PAA(100)-8 had denaturation enthalpy and peak transition temperatures similar to that of Hb. The improved thermal stability of Hb-PAA(100)-7 (T\text{d} shifted to higher temperature, another transition at higher temperature as well as higher enthalpy of denaturation) is clearly demonstrated. Thus, the thermal stabilities are either comparable to that of Hb or higher, and hence, we decided to examine the extent of reversibility of the thermal denaturation of the Hb-PAA conjugates. If the denaturation is fairly reversible, then the nanoparticles are of high promise for steam sterilization studies, and we used steam sterilization as a benchmark of thermal stability.
1.4.8 Reversibility of Thermal Denaturation

To test the reversibility of the thermal denaturation of the Hb-PAA nanoparticles, these were heated to 90 °C followed by cooling to room temperature for 15 minutes, and the CD spectra of the samples have been recorded before and after heat treatment. A comparison of the CD spectra of Hb-PAA(100)-7 before and after 90 °C treatment are compared to those of the Hb treated in a similar manner (Figure 7). The Hb-PAA(100)-7 sample, which showed the best thermal stability among the three samples described in the DSC studies, indicated extensive retention of UV CD after the 90 °C heat treatment (Figure 8). The CD spectrum of the sample after heating (red dotted curve) indicated the same minima and maxima peak positions as that of the sample before heating (red curve). However, small decreases in the intensities of the heat treated sample is clearly evident, and these suggest that the thermal denaturation is mostly reversible, but not 100%.
**Figure 1.9.** Far UV CD of Hb-PAA(100)-7 (red thick line) and Hb (black thin line) before heating is compared with the corresponding spectra obtained after heating the samples to 90 °C for 15 minutes, followed by cooling to room temperature for 15 min (red dashed, and black dashed lines, respectively).
In contrast, the CD spectra of Hb before (black solid curve) and after 90 °C heat treatment (black dashed curve) show that the peak intensities decreased substantially, and its thermal denaturation is essentially irreversible, under the same conditions of pH, buffer and ionic strength. Near-complete reversibility of thermal denaturation of the Hb-PAA(100)-7, however, is clearly demonstrated above, and the soft, hydrophilic polymer might facilitate refolding, upon cooling to room temperature. Improved resistance to heat deactivation and facilitated recovery of the native-like structure after heating, are in support of the initial hypothesis, and next, we examined the resistance to steam sterilization as a benchmark of protein thermal stability.

1.4.9 Stability towards steam sterilization

Improved resistance of Hb-PAA(100)-7 toward heat and near-complete reversibility of denaturation indicated that the Hb-PAA nanoparticles are potentially promising candidates to test their ability to resist deactivation by steam sterilization. We examined the peroxidase-like activities of Hb-PAA samples after they have been subjected to steam sterilization conditions (122 °C, 40 minutes, 17-20 psi) and cooled back to room temperature. If the samples retained their activities after steam sterilization, it would be a major breakthrough in improving Hb stability.
Figure 1.10. Effect of steam sterilization (122 °C, 40 minutes, 17-20 psi) on the relative activities of: (a) Hb-PAA nanoparticles compared with those of Hb, Hb/PAA physical mixtures which are also treated similarly, and (b) polyamine crosslinked Hb-PAA(1000)-8 nanoparticles compared with that of Hb, before and after steam sterilization. All measurements were in phosphate buffer, pH 7.2, at room temperature.
The peroxidase-like activities of the steam sterilized samples are measured at room temperature, and the extent of recovery of activity or the ratio of the initial rate before heating to initial rate after heating of each sample is shown in Figure 9a. If the activity is not affected by heating, this ratio will be 1, while the actual value shows the per cent activity retention, so that all samples can be compared with a single measure.

Essentially all Hb-PAA(100) samples, retained their activities by 75-85% and this is remarkable. Hb-PAA(100)-6, for example, retained nearly 80% of its activity after steam sterilization. In contrast, Hb lost almost 80% of its activity after sterilization, retained only ~20% of its original activity, and the Hb-PAA(100) nanoparticles showed extensive resistance to deactivation by steam sterilization. Hb-PAA(1000)-7 and Hb-PAA(1000)-8 also retained appreciable activities, but much less than their Hb-PAA(100) counterparts. Extensive activity retention is a surprise, as the Hb-PAA nanogels prepared from high molecular weight PAA (450,000) did not retain their activities after steam sterilization. Thus, the polymer molecular weight played a major role in the resistance to thermal deactivation. Note that Hb/PAA physical mixtures also resisted thermal deactivation to a substantial extent and this clearly shows the role of the polymer in assisting the refolding of the denatured Hb or the prevention of the aggregation of denatured Hb molecules. The advantage of the covalent linking approach of the current Hb-PAA nanoparticles is that these samples are discrete and they will not be subjected to leaching of the polymer from the protein or when diluted for practical applications.

1.4.10 Effect of Cross-Linking with Polyamines

Since Hb-PAA(1000)-8 retained only about half of its activity, we decided to examine if crosslinking of the COOH groups of the PAA chains with polyamines would improve their
resistance to thermal deactivation. The crosslinking could stiffen the polymer chain and might enhance protein stability or improve the reversibility of denaturation. The Hb-PAA(1000)-8 samples, accordingly, were crosslinked with polyamines, en, teta or tepa using EDC chemistry (Scheme 2), purified by dialysis, and their purity confirmed by agarose gel electrophoressis (Figure 10). Due to the high PAA content of these samples, their bands do not stain properly with Coomassie Blue, and we used Zn(II)-imidazole reverse staining to locate the bands in the gel. These amine-crosslinked samples also indicated discrete, negatively charged bands where the extent of negative charge inversely varied with the extent of amine-adduction. All samples were completely soluble under these conditions, and no precipitation or macroscopic networks are formed. Reaction with the polydentate amines reduced the net charge on the particles, as seen in the agarose gels, but the reaction conditions were adjusted such that amines did not crosslink different particles, that is intraparticle crosslinking is achieved over interparticle crosslinking.
Figure 1.1 Agarose gel (0.5%) of Hb (lane 1), Hb-PAA(1000) (lane 2), and Hb-PAA(1000)-teta (lanes 3-6) synthesized in phosphate buffer, pH 8. The samples were spotted at the bottom of the gel (running buffer, 40 mM Tris acetate pH 7.2). While Hb moved as a streak (lane 1, faint band), Hb-PAA(1000)-8 (lane 2) and Hb-PAA(1000)-teta (lanes 3-6, crosslinked with 60, 40, 20 and 10 mM teta, respectively) showed single bands. Hb-PAA(1000)-teta crosslinked nanoparticles moved lesser toward the positive electrode than Hb-PAA(1000)-8 due to neutralization of the negative charge by the reaction with the amine.
We tested stabilities and reversibility of the thermal denaturation of these amine-conjugated Hb-PAA nanoparticles (Figure 11). Absorbance spectra of Hb-PAA(1000)-8-polyamine conjugates before heat denaturation at room temperature (blue curve), during denaturation at 90 °C (red curve) and after cooling back to room temperature (green curve), are recorded and analyzed. For example, the Hb-PAAA(1000)-20teta sample indicated that its Soret band shifted from 410 to 395 nm on heating to 90 °C, and the Soret band shifted back to 410 nm upon cooling the sample back to room temperature. The blue shift of the Soret band at 90˚C clearly indicates the denaturation of the protein structure around the heme binding pocket, and its recovery back to 410 nm upon cooling supports the notion that the protein structure is recovered substantially. All three crosslinked samples (Hb-PAA(1000)-teta, Hb-PAA(1000)-tepa and Hb-PAA(1000)-en) indicated similar, reversible thermal denaturation, under these conditions. The thermal denaturation of the entrapped Hb in the conjugates, is therefore, mostly reversible. This conclusion is supported by the fact that these samples also recovered their peroxidase-like activity to a significant extent, after steam sterilization, described earlier. In contrast to the reversible denaturation of these amine conjugates, Hb underwent near-complete irreversible thermal denaturation, under the same conditions.
Figure 1.12 (a-c). Absorbance spectra of Hb-PAA(1000)-8-polyamine conjugates before heat denaturation at room temperature (blue curve), during denaturation at 90 °C (red curve) and after cooling back to room temperature (green curve), panels as labeled. Thermal denaturation clearly shifted the Soret band to higher temperatures and upon cooling most of the Soret band intensity and position are recovered, which suggests a high degree of reversibility of the thermal denaturation of these samples. In contrast, Hb underwent near complete irreversible thermal denaturation, under the same conditions.
These amine-modified Hb-PAA(1000)-8 samples were then evaluated for the retention of their peroxidase-like activity after steam sterilization. Percent activities of the samples after steam sterilization with respect to that of Hb before steam sterilization are shown in Figure 8b as a bar graph. All the crosslinked samples retained greater activities than Hb, and the activity retention with respect to Hb-PAA(1000)-8 improved initially, with the concentration of amine used for crosslinking, and as the amine concentration used for crosslinking increased the activities after steam sterilization decreased. Some improvement over Hb-PAA(1000)-8 was noted in several cases and overall, the crosslinking had substantial improvement in thermal stability or reversibility of the denaturation. In total, we tested 30 distinct samples or mixtures, and most of them except Hb, indicated substantial retention of activity after steam sterilization.

1.5 Conclusions

Covalent conjugation of suitable polymers with appropriate proteins provides exciting new opportunities in materials chemistry, but only a limited progress has been made due to the widespread use of very few polymers such as PEG carrying a very limited number of functional groups. Here, we demonstrate a simple, modular approach, where the covalent conjugation of a water-soluble, multifunctional polymer, PAA, to lysine residues of a generic protein such as Hb can be controlled to produce Hb-PAA nanoparticles with controlled size, high solubility, improved thermal stability and near-reversible thermal denaturation. The conjugates are endowed with the biological activities of the protein with unique, novel attributes.

Based on the TEM/DLS data, and gel electrophoresis, we suggest that discrete nanoparticles are formed, and covalent linking allows the co-habitation of the protein and the polymer phases within the same particles. Surprisingly, the Hb-PAA nanoparticles indicated
considerable retention of the elegant hierarchical 3D structure of Hb, its peroxidase-like activity and fairly narrow size distribution for a major population of the particles. TEM data strongly suggest that the conjugates are primarily made of protein-PAA nanoparticles, not gels. The physical characteristics of the nanoparticles such as size, structure retention and stability are influenced by the composition of the conjugate as well as the reaction conditions such as pH and stoichiometries.

One exciting observation is that all the Hb-PAA nanoparticles tested here, retained their biological activities after steam sterilization, and this is highly unusual. Most of them retained substantial activities while Hb lost most of its activity under these conditions. Steam sterilization has been known to deactivate proteins, and it is a stumbling block in the development of protein therapeutics and protein-based materials for biomedical applications. Our earlier study on Hb-PAA conjugates which used higher molecular weight PAA (MW 450,000) did not indicate resistance to thermal deactivation. Therefore, the current results demonstrate dependence on the molecular weight of PAA as well as the high mole ratio of the protein to polymer of the nanoparticles. Excess PAA reduced the extent of resistance to thermal deactivation while crosslinking with polyamines improved the resistance only marginally. Overall, several examples of Hb-PAA are shown here, which resist thermal deactivation well and set new standards for the thermal stabilities of proteins. Our approach may provide new opportunities for the design of novel protein-polymer conjugates, carefully tailored, for a variety of applications where sterilized protein-based products are essential.

We speculate that several unique features of these nanoassemblies are responsible for the observed retention of activity after steam sterilization. One major factor, among them, is that the polymer assists the solubilization of the denatured protein and the individual nanoparticles are
kept apart from agglomeration and precipitation by the anionic polymer, so that they refold to the active form on cooling. Shielding of the protein from the external microenvironment by the polymer could enhance the intrinsic stability of the protein. There could be a kinetic factor for the slow denaturation of the protein or partial retention of the structure after denaturation, and when these factors are combined in a synergistic manner, the overall result is increased resistance to thermal deactivation. These novel Hb-PAA nanoparticles provide a major breakthrough in the preparation of bioactive hybrid nanomaterials, which can be sterilized, without deactivation, for biomedical and bionsensing applications.

Overall, Hb-PAA nanoparticles reported here are highly resistant to deactivation by steam sterilization, and these are welcome changes for applications in biocatalysis and biomaterials. These findings are significant because most proteins lose their biological activities when they are subjected to steam sterilization, while Hb-PAA nanomaterials retained most of their activity. Additionally, the protein hierarchical structure and activity in nanoparticles is retained to a large extent. High thermal stability of the hybrid materials, unlike the poor thermal stability of most proteins, is unique, novel and extremely useful for applications that require sterile products. These pioneering results might provide a new paradigm for the use of protein-polymer materials in biosensors, protein therapeutics, tissue engineering scaffolds and implants.
References


Chapter 2

A General Approach Towards Ultra-Stable Protein-Polymer Bioconjugates
2.1 Abstract

Proteins and enzymes are naturally occurring polymeric biological macromolecules with well-defined hierarchial structures and highly specific biological activities, however, they are very sensitive to their environment. Environmental stimuli such as pH, temperature, bacterial degradation and organic solvents can readily deanture an enzyme\textsuperscript{1}. The limitations of the enzymes can be overcome when conjugated to suitable polymers via synthetic approaches\textsuperscript{2}. Here we report the synthesis of a small library of protein-polymer conjugates using a facile, self-assembled chemical method we have previously published\textsuperscript{3}. Here we have produced protein-polymer conjugates from covalent linking of low molecular weight poly(acrylic acid) (PAA, MW 8,000) to various proteins and enzyme under different experimental conditions to form discrete nanoparticles. The bioconjugates retained significant biological activities when compared to their unmodified counterparts and showed no nanogel network formation.
2.2 Introduction

Proteins and enzymes are naturally occurring polymeric biological macromolecules with well-defined hierarchical structures and highly specific biological activities. Enzymes can accelerate chemical reactions by orders of magnitude with very high selectivity, under ideal conditions. However, proteins are generally not suited to function as biomaterials due to their limitations and sensitivity to environmental stimuli such as pH, temperature, bacterial degradation and organic solvents. The limitations of the enzymes can be overcome when conjugated to suitable polymers via synthetic approaches. The combination of proteins and polymers can lead to new structures and the design of unique biomaterials that can enhance the properties of both the protein and the polymer for specific applications. Protein-polymer conjugates can be synthesized by site-specific conjugation or random conjugation. Usually this conjugation is through a covalent link which the functional groups of the polymer are bound to the surface groups of the protein. Hybrid bioconjugates can potentially be used for applications in biosensing, biocatalysis or biomedical applications if engineered appropriately.

By wrapping the proteins herein with a soft, hydrophilic, flexible polymer such as the low molecular weight poly(acrylic acid) (PAA, MW 8000), the stabilities of the proteins can be improved. Here, the synthesis of a small library of protein-polymer bioconjugates using a previously developed simple, facile, self-assembled approach is reported. The data shows that the method previously developed can be used as a general approach to successfully synthesize protein-polymer conjugates by covalently linking the carboxyl functional groups of the anionic polymer to the amine containing lysine amino acids on the protein surface via amide bond formation through standard carbodiimide chemistry (Scheme 1).
Scheme 2.1. The synthesis schematic for the formation of the protein-polymer conjugates by linking the amino groups of the protein to the carboxyl groups of the polymer, PAA. The coupling agent used here is 1-ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC).
Previously it has been shown that conjugation of the proteins with this low molecular weight PAA (MW 8000) yields highly soluble, discrete nanoparticle formation which is resistant to steam sterilization\textsuperscript{7}. It has also been shown that random cross-linking of high molecular weight PAA (MW 450,000) resulted in the formation of soluble nanogel networks. These nanogels showed streaking and particle precipitation in the wells of the agarose gels\textsuperscript{8}. No streaking or precipitation was seen previously or shown presently with conjugation to the low molecular weight PAA. Also, these protein-polymer conjugates have shown significant activity retention after conjugation when compared to their unmodified counterparts.
2.3 Experimental

2.3.1 Chemicals and Materials

Glucose Oxidase (GOx) and Lysozyme (Lys) were purchased from Sigma (St.Louis, MO). Catalase (Cat) was purchased from Worthington Biochemicals (Lakewood, NJ). Protein solutions were prepared in the respective buffer solutions used for synthesis, as indicated, and the concentrations were determined from the absorbance of each protein using the appropriate extinction coefficient at 280 nm. The coupling agent used for synthesis, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), was purchased from TCI America (Portland, OR). Poly(acrylic acid) (PAA, 45 wt%, MW 8,000) was purchased from Sigma (St. Louis, MO).

2.3.2 Protein-Polymer Conjugate Synthesis

The protein-polymer conjugates were synthesized by using EDC as the coupling agent with minor modifications to reported methods\(^9\). A mixture of 500 µL of 200 mM Na\(_2\)HPO\(_4\) buffer at pH 6, 7 or 8 with 270 µL of PAA (1 mmol) and 3730 µL of deionized water were added to a reaction vial, followed by sonication for 5 minutes. Next 100 mmol of EDC was added to the vial while stirring. The resulting PAA/EDC mixture was stirred for 5 minutes, followed by the addition of 500 µL of 100 µM protein (50 nmol) and stirred for 12 hours. Specific reaction conditions for each of the samples are summarized in Table 1. Excess EDC, PAA, and urea by-product were dialyzed three times with 1000 times the sample volume with 25 kDa molecular weight cut off dialysis membrane to ensure pure protein-polymer conjugates. The product obtained from the reaction of PAA (5000 nmol) with GOx (50 nmol) at pH 7 (PAA to GOx mole ratio of 100:1) is designated as GOx-PAA(10)-7, while the reaction of 50,000 nmol PAA with GOx (50 nmol) at pH 7 (PAA to Hb mole ratio of 1000:1) is designated
as GOx-PAA(1000)-7. This nomenclature will be used throughout the manuscript for unique identification of specific protein-polymer conjugate samples. Each sample has been centrifuged at 12,500 rpm and filtered using a 0.22 µm filter to remove invisible precipitates, if any.
Table 2.1. Reaction conditions for the synthesis of protein-PAA conjugates from Catalase, Glucose Oxidase or Lysozyme (10 μM, 500 μL, 50 nmol) and EDC (100 mM, 95.8 mg, 500 μmol) in sodium dibasic phosphate buffer, pH 7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[PAA] (mM)</th>
<th>PAA Vol (μL)</th>
<th>Moles PAA</th>
<th>Buffer Vol (μL)</th>
<th>DI Vol (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-PAA(100)</td>
<td>1</td>
<td>135</td>
<td>5 μmol</td>
<td>500</td>
<td>3865</td>
</tr>
<tr>
<td>Protein-PAA(500)</td>
<td>5</td>
<td>675</td>
<td>25 μmol</td>
<td>500</td>
<td>3325</td>
</tr>
<tr>
<td>Protein-PAA(1000)</td>
<td>10</td>
<td>1,350</td>
<td>50 μmol</td>
<td>500</td>
<td>2650</td>
</tr>
<tr>
<td>Protein-PAA(1500)</td>
<td>15</td>
<td>2,025</td>
<td>75 μmol</td>
<td>500</td>
<td>1975</td>
</tr>
<tr>
<td>Protein-PAA(2000)</td>
<td>20</td>
<td>2700</td>
<td>100 μmol</td>
<td>500</td>
<td>1300</td>
</tr>
</tbody>
</table>
2.3.3 Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving molecular biology grade agarose (0.5% w/v) (Hoefer Inc., Holliston, MA) in a hot solution of Tris-Acetate (40 mM, pH 7.2). The gels were run in a horizontal gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc., Grand Island, NY) using Tris-Acetate (40 mM, pH 7.2) as the running buffer. Samples were loaded into the gel with loading buffer (50 % v/v glycerol and 0.01% m/m bromophenol blue) and electrophoresis carried out for 30 minutes at 100 V at room temperature. The gel was stained overnight with 10% v/v acetic acid, 0.02% m/m Coomassie blue and then destained with 10% v/v acetic acid for an additional night. The gels were photographed using a BioRad Chemidoc XRS+ (Hercules, CA).

2.3.4 Spectral Measurements

Absorption spectra were recorded on an HP8453 uv-visible spectrophotometer (Agilent Technologies, Santa Clara, CA). The concentration of protein in solution was determined from the absorbance using the appropriate extinction coefficient at 280 nm (Table 2). All data was plotted using Kaleidagraph 4.1.1.
Table 2.2. Summary of extinction coefficients used for various proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Extinction Coefficient (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>420,000</td>
</tr>
<tr>
<td>Glucose Oxidase</td>
<td>267,200</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>38,940</td>
</tr>
</tbody>
</table>
2.3.5 **Enzymatic Activity Assays**

For catalase, the decomposition of hydrogen peroxide ($\text{H}_2\text{O}_2$) was followed from reported methods\(^{10}\). Catalase (0.5 µM) was added to a solution of $\text{H}_2\text{O}_2$ (20 mM) and the disappearance of the $\text{H}_2\text{O}_2$ was monitored at 240 nm with respect to time at room temperature.

For glucose oxidase, the activity was determined by a previously reported method\(^{11,12}\). A solution of glucose (0.2 mM) was added to a mixture of GOx (0.5 µM), HRP (2 µM) and guaiacol ($o$-methoxyphenol) (10 mM) in DI water. Oxidation of the substrate, guaiacol, to a dimeric product ($3,3'$-dimethoxy-4,4'$'$-biphenoquinone) which has absorption maxima at 470 nm was monitored as a function of time at room temperature.

For lysozyme, the rate of lysis of *Micrococcus lysodeikticus* cells is monitored as reported in the literature\(^{13}\). A suspension of *M. Lysodeikticus* cells (0.36 mg/mL) was placed in the spectrophotometer and a blank rate was established by observing the absorbance at 450 nm with respect to time. Next, a solution of lysozyme (1 µM) was added to the cuvette and the rate of lysis of the cell walls was established. All enzymatic activity data was plotted using Kaleidagraph v4.1.1.

2.4 **Results and Discussion**

Here, the successful synthesis of various protein-polymer conjugates using various proteins with low molecular weight PAA (MW 8,000) is reported. Previously it has been shown that hemoglobin (Hb) shows activity retention after steam sterilization, using this technology\(^7\). It has also been previously shown that covalent conjugation of Hb to high molecular weight PAA (MW 450,000) resulted in the formation of nanogels. These nanogels did not show the same
activity retention after steam sterilization\textsuperscript{8}. Herein the successful synthesis and initial enzymatic activity studies for the small library produced are reported.

2.4.1 Protein-Polymer Conjugate Synthesis

Proteins are large, polyfunctional, biological macromolecules with a large variety of surface functional groups, including amines and carboxyl moieties. Previously, the amine groups of the surface lysine side chains have been modified with a large variety of carboxyl group containing substrates via standard carbodiimide chemistry\textsuperscript{14}. Using a similar strategy here, a large excess of PAA (MW 8000, 100 or 1000 molar excess) was covalently attached to the surface lysine side chains of the proteins using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as the coupling agent. After synthesis, all of the conjugates produced remained completely soluble, with no precipitation or nanogel formation. The protein/EDC/PAA reaction mixture was allowed to react for 12 hours, followed by dialysis with a 25 kDa molecular weight cut off dialysis membrane to remove any excess PAA, EDC and urea by product.

2.4.2 Agarose Gel Electrophoresis

Covalent attachment of PAA to the proteins is expected to give rise to negatively charged protein-polymer conjugates like we have shown before\textsuperscript{7}. At pH 7.2, each of these proteins have different net charges. Amylase, Catalase, and Glucose Oxidase all have net negative charges, while Lysozyme has a net positive charge. Thusly, conjugation of the PAA to the various proteins is expected to greatly increase the electrophoretic mobilities of Lysozyme much more than that of Amylase, Catalase, or Glucose Oxidase.
As predicted, the Cat-PAA conjugates (100-2,000 fold excess PAA per Catalase) moved further towards the positive electrode than did the unmodified Catalase sample (Figure 1). This increase in migration is not due to the physical binding of the PAA to the protein, as no change in migration between the unmodified Catalase sample and the physical mixture (not shown). The presence of new bands and the absence of the catalase band in lanes 1-5 indicate the complete conversion of catalase to the Cat-PAA conjugates. In comparison to previously published low molecular weight Hb-PAA conjugates\(^7\), a similar result has been observed with the conjugation of catalase with PAA to form Cat-PAA conjugates. In contrast, it has been previously shown that the nanogel networks formed between the Hb and high molecular weight PAA (MW 450,000) moved as streaks on the agarose gels under the same electrophoresis conditions\(^8\). Thusly, it has been shown that the technology previously developed with the Hb-PAA conjugates can be applied to other proteins and enzymes to form the corresponding protein/enzyme-PAA conjugates.
Figure 2.1. Agarose gel of Catalase (lane 6), Cat-PAA(100) (lane 5), Cat-PAA(500) (lane 4), Cat-PAA(1000) (lane 3), Cat-PAA(1500) (lane 2) and Cat-PAA(2000) (lane 1). The conjugates used here were synthesized at pH 8. All samples were spotted in the center of the gel (0.5% agarose, 40 mM Tris-Acetate, pH 7.2) and allowed to run for 30 min at 100V. The unmodified catalase did not migrate far from the center of the gel, towards the positive electrode. The Cat-PAA conjugates all migrated further towards the positive electrode and do not show any unreacted catalase in the lanes.
Conjugates were also prepared with glucose oxidase (100-2,000 fold molar excess PAA) and saw similar results to those of the catalase conjugates mentioned before. The increase in electrophoretic mobility is again due to the covalent attachment of the PAA. The loss of the band for unmodified glucose oxidase and the presence of a new band (Figure 2, lanes 2-11) indicate the complete conversion to the GOx-PAA conjugates. This again agrees with our general approach to synthesize protein-polymer conjugates through a covalent, self-assembled method.
Figure 2.2. Agarose gel of GOx (lanes 1 and 12) and GOx-PAA conjugates (lanes 2-11). The unmodified GOx in lanes 1 and 12 show two distinct bands because the protein sample consists of the monomer and dimer forms of GOx. The conjugates on this gel were synthesized at pH 6 and 7. The lanes consist of GOx-PAA(100) (lane 11, pH 6; lane 10, pH 7), GOx-PAA(500) (lane 9, pH 6; lane 8, pH 7), GOx-PAA(1000) (lane 7, pH 6; lane 6, pH 7), GOx-PAA(1500) (lane 5, pH 6; lane 4, pH 7) and GOx-PAA(2000) (lane 3, pH 6; lane 2, pH 7). All samples were spotted in the center of the gel (0.5% agarose, 40 mM Tris-Acetate, pH 7.2) and allowed to run for 30 min at 100 V. The GOx-PAA samples migrated further toward the negative electrode than the unmodified GOx samples, retaining the two band shape of the unmodified samples, thus there is no unreacted GOx in the lanes.
Conjugates prepared from lysozyme and the low molecular weight PAA (100-2,000 molar excess) behaved similarly to the two sets of conjugates previously mentioned. Again, using our general approach to prepare the protein-polymer conjugates, it is confirmed that the increase seen in the electrophoretic mobility is due to the covalent conjugation of the PAA to the lysozyme (Figure 3). Further, there is no indication of free lysozyme in the lane with the conjugate, indicating the complete conversion of the enzyme to the conjugate form.
Figure 2.3. Agarose gel of lysozyme (lane 2) and Lys-PAA(100)-7 (lane 1). The unmodified lysozyme migrated towards the negative electrode, which means that the unmodified lysozyme has a net positive charge. The Lys-PAA(100) migrated towards the positive electrode and do not show any unreacted lysozyme in the lane. The samples were spotted in the center of the gel (0.5% agarose, 40 mM Tris-Acetate, pH 7.2) and allowed to run for 30 min at 100 V.
The average pore size of the agarose gels used in Figures 1-3 (0.5% gel) is around 450 nm, and all of the protein-polymer conjugates moved through the gel readily. Thus, it can be assumed that the conjugates are all much smaller than 450 nm in diameter. It can also be assumed that there is no formation of any nanogel networks, which would have been indicated by streaks on the gels instead of clearly defined bands. This is consistent with the observations of highly soluble conjugates and the low overlap concentration of the PAA. The agarose gels confirm the clean and consistent linking of the PAA to the proteins.

2.4.3 Enzymatic Activity Assays

The catalytic activity of catalase was monitored by the decomposition of hydrogen peroxide (substrate) by its decreasing absorption at 240 nm as a function of time (Figure 4). The decomposition of H$_2$O$_2$ by catalase (blue curve) and Cat-PAA(1000)-8 (green curve) are almost identical and PAA by itself did not catalyze the reaction (data not shown). The initial rates of the conjugate sample were compared to the initial rates of the unmodified catalase and about 12% increase in activity of Cat-PAA was observed when compared to the unmodified catalase, but this increase is within the experimental errors.
Figure 2.4. The decomposition activity of hydrogen peroxide by catalase (blue curve) and Cat-PAA(1000)-8 (green curve). All activity data was measured at room temperature in the presence of 0.5 µM protein and 20 mM H₂O₂ in 20 mM Na₂HPO₄, pH 7.2.
The enzymatic activities of glucose oxidase were also measured by following procedures reported in literature\textsuperscript{11,12}. The oxidation of a peroxidase substrate to a dimeric product was monitored at 470 nm as a function of time at room temperature. The activities of the GOx-PAA conjugates were very similar to that of the unmodified GOx. When comparing the relative activities, the conjugates showed significant activities, most being close to that of GOx, with the lowest having 86\% activity retention.
Figure 2.5. (a) Kinetics traces for GOx (red curve) and GOx-PAA conjugate samples (glucose (0.2 mM), GOx (0.5 µM), HRP (2 µM) and guaiacol (10 mM) in 20 mM Na$_2$HPO$_4$, pH 7.2). (b) Comparison of relative activities of the GOx-PAA conjugates with that of the unmodified GOx at room temperature under the same reaction conditions.
In order to determine the activities of lysozyme, the rate of lysis of the cell walls of *M. lysodeikticus* cells was used. A solution of lysozyme (1 µM) was added to a suspension of *M. lysodeikticus* cells (0.36 mg/mL) and the change in absorbance at 450 nm was monitored as a function of time. The lysozyme was injected after 100 seconds of monitoring the absorbance to establish a blank rate, if any, and the kinetic traces are compared below in Figure 6. Here a major difference between the two curves. After the point of lysozyme/Lys-PAA(100)-6 injection (100 sec) the red curve begins to decrease immediately at a steady rate (-6.84 x 10^{-4} µM^{-1} s^{-1}), while the blue curve does not decrease. This is because the cells are too large to gain access to the lysozyme protein inside of the conjugates. We hypothesize that the pore size of the polymer shell around the protein is much smaller than the diameter of the *M. lysodeikticus* cells, thus the cell wall will not be lysed and no change in absorbance will be observed.
Figure 2.6. Kinetic traces for Lysozyme (red curve) and Lys-PAA(100)-6 (blue curve). Activity data was collected in a solution of *M. lysodeikticus* cells (0.36 mg/mL) and lysozyme (1 µM) in 20 mM Na$_2$HPO$_4$, pH 7.2 at room temperature.
2.5 Conclusions

Covalent conjugation of polymers with proteins is providing new and exciting opportunities in biomaterials. However, limited progress has been made in this field due to very few polymers being used, such as polyethylene glycol, which has a limited number of functional groups. Previously, a simple, modular, self-assembled approach of covalent conjugation of low molecular weight PAA (MW 8,000), a multifunctional, hydrophilic polymer to the surface lysine amino acids of Hb has been reported. These bioconjugates were produced with controlled size, solubility, improved thermal stabilities and near-reversible denaturation. Here, the use of the previously optimized technology to synthesize new, discrete nanoparticles with other various proteins is reported.

Based on the agarose gel electrophoresis, it can be suggested that discrete nanoparticles are formed through the covalent conjugation of the low molecular weight PAA (MW 8,000) to the protein surface lysine groups. Nanogel network formation is not observed because they are no streaks observed in the agarose gel, nor is there any precipitation in the wells.

Based on the activity data for the conjugates made with catalase and glucose oxidase, the newly formed conjugates are endowed with the biological activities of their respective unmodified proteins, showing significant activity retention after conjugation. However, in the case of the conjugates made with lysozyme the biological activities of the proteins post-modification cannot be determined because the Micrococcus lysodeikticus cells (substrate) are too large to pass through the pores of the polymer shell.

Overall, previously developed methods were used to create a small library of protein-polymer conjugates. These are welcome for applications in biocatalysis and biomaterials due to
the discrete particle formation and significant activity retention. These results currently add to the idea that one day, protein-polymer biomaterials will be used in biosensors, protein therapeutics if tailored to the proper conditions.
References


