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Toward the Design of Novel Biodegradable Materials by Engineering the Protein-Colloid Interface

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This dissertation focuses on development of protein-derived materials, with the goal of incorporation into biodegradable devices such as enzymatic fuel cells or light emitting diodes (LEDs). Proteins are the key building blocks, for their inherent functions, hierarchical 3D structures, diversity of functional groups, and natural biodegradability. The interfaces between proteins and colloidal materials were exploited in the rational design of these materials.

First, enzyme-poly(acrylic acid) (PAA) conjugates with enhanced stability and catalytic efficiency were explored. Conjugation of cytochrome c (cyt c) to PAA dramatically improved cyt c peroxidase activity, achieving 34-fold enhancement in $k_{\text{cat}}$. Here, polyanionic PAA controlled the pH microenvironment and suppressed wasteful reaction intermediates. Next, we conjugated brush-like poly(norbornene)-graft-poly(acrylic acid) (PNPAA) to cyt c, and found that conjugates retained their structure under denaturing conditions.

We also produced a white emitting hydrogel from crosslinked bovine serum albumin (BSA) with fluorescent dyes incorporated. We explored the applications of this hydrogel in LED coating and pH biosensing. Additionally, we demonstrated enzymatic glucose detection by incorporation of the enzymes glucose oxidase and peroxidase into the gel.

Finally, we demonstrated that cationic silica nanoparticles can regulate the aggregation behavior of poly-L-(glutamate) (PLG). In the early stages, increased PLG concentration at the NP surface increased the nucleation rate. As fiber growth proceeded, NP binding to the growing fiber slowed the growth rate. These effects were controlled by manipulating the PLG-nanoparticle binding affinity, such as by increasing the temperature. Together, these examples provide useful insights into the design of robust, protein-derived materials.
Toward the Design of Novel Biodegradable Materials by Engineering the Protein-Colloid Interface

Kyle Robert Benson

B.A., Wesleyan University, 2006

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut

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2019
Toward the Design of Novel Biodegradable Materials by Engineering the Protein-Colloid Interface

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University of Connecticut
2019
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List of Abbreviations

- (dash) covalent conjugation using EDC
/ (slash) physical mixtures (no EDC)

APTES (3-aminopropyl)-triethoxysilane

Arg arginine
Asp aspartic acid

BSA bovine serum albumin

C460 Coumarin 460

CCT correlated color temperature

CD circular dichroism

COOH carboxylic acid
cpyt c cytochrome c

DLS dynamic light scattering

DP degree of polymerization

EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

F fluorescein

FPA fluorescence polarization anisotropy

FRET Förster resonance energy transfer

FTIR Fourier transform infrared spectroscopy

Glu glutamic acid

GOx glucose oxidase

GPC gel permeation chromatography

Hb hemoglobin

HRP peroxidase from horseradish

$k_{cat}$ enzyme turnover number
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<tr>
<td>$K_M$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>$\lambda_{em}$</td>
<td>emission wavelength</td>
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<tr>
<td>$\lambda_{ex}$</td>
<td>excitation wavelength</td>
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<tr>
<td>LED</td>
<td>light emitting diode</td>
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<tr>
<td>Lys</td>
<td>lysine</td>
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<tr>
<td>$M_w$</td>
<td>molecular weight</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NP</td>
<td>nanoparticle</td>
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<tr>
<td>PAA</td>
<td>poly(acrylic acid)</td>
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<tr>
<td>PDI</td>
<td>polydispersity index</td>
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<td>PLG</td>
<td>poly-L-glutamic acid</td>
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<td>PNPAA</td>
<td>polynorbornene-$graft$-poly(acrylic acid)</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>ROX</td>
<td>5(6)-carboxy-x-rhodamine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>$T_M$</td>
<td>melting/denaturation temperature</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>ThT</td>
<td>thioflavin T</td>
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<tr>
<td>TNBSA</td>
<td>trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
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Chapter 1: Introduction

Proteins are polymers of amino acids, linked together by amide bonds, with defined three-dimensional structures. Naturally occurring proteins can have a variety of functions, from structural to catalytic, and have been refined by nature over thousands of iterations, far more experiments than any one chemist could complete in a lifetime. The protein sequence contains a wide variety of functional groups, including acids, bases, thiols, hydrophobic groups, and aromatic ring systems, among others. With their well-defined structures, diversity of chemical groups, and inherent functionality, they provide attractive building blocks for biological materials. Proteins have the added benefit of being naturally biodegradable, an important material design consideration in today's world, where millions of tons of plastic are dumped into the ocean each year.\textsuperscript{1}

One challenge in utilizing proteins to produce biomaterials is the limited range of temperature, pH, and solvent conditions over which they are stable. Clearly, even a biodegradable material must at least survive on a time scale relevant to its application before degrading, and must sustain the working conditions to which it is subjected. Many chemical modification or immobilization approaches have been explored to expand the stability of proteins or modulate their self-assembly behavior, and exploiting the interaction of proteins with colloids, such as polymers, nanoparticles, or hydrogels, is a promising approach. In the past, for example, we have demonstrated that conjugation of enzymes to synthetic polymers such as poly(acrylic acid) (PAA) can improve the thermal and chemical stability of enzymes, and even allow them to function in organic solvents that normally induce denaturation.\textsuperscript{2–5}

The major goal of this study was to demonstrate the ways in which protein-colloid interactions can be used to produce novel, biodegradable materials with diverse functions from sustainable building blocks. In doing so, the following topics were explored; (1) how the physical properties and morphology of PAA influences the thermal stability and catalytic efficiency of conjugated enzymes; (2) the use of proteins to produce multi-functional hydrogels for white light
generation and biosensing; and (3) the influence of nanoparticles (NPs) on the supramolecular assembly of proteins into amyloid fibrils, which could eventually be used to produce nanostructured films.
1.1. Modulating activity and stability of enzyme-polymer conjugates

Enzymes are molecular bioreactors engineered by nature to catalyze diverse reactions with high specificity, enatioselectivity, and catalytic efficiency, and thus have applications in fields such as biomedicine and catalysis.\textsuperscript{6} However, their activity is typically confined to a narrow range of pH and temperature specific to each enzyme. As such, considerable attention has been devoted to stabilize enzymes against thermal or chemical denaturation, and conjugation of enzymes to synthetic polymers is a widely studied and broadly successful strategy.\textsuperscript{7–9} Additionally, the use of specific polymer conjugation techniques to systematically and rationally modulate enzyme kinetics has garnered significant attention recently. Some examples include conjugation of acidic polymers to cytochrome \textit{c} to manipulate the pH microenvironment and expand the enzyme’s pH range,\textsuperscript{10} or the use of charged polymers to control conjugate substrate/inhibitor affinity for chymotrypsin,\textsuperscript{11} horseradish peroxidase,\textsuperscript{12} or catalase.\textsuperscript{2}

We have previously demonstrated that conjugation to PAA significantly improves the thermal and chemical stability of enzymes, with little to no reduction in the catalytic efficiency.\textsuperscript{2–5,13,14} Our hypothesis is that multi-site conjugation to PAA restricts the number of conformations available to the denatured state, reducing the conformational entropy of this state and thus the entropy change upon denaturation. This, in turn, increases the free energy of denaturation, making it less favorable. The hydrophilic polymer shroud also prevents aggregation of denatured proteins, improving the reversibility of denaturation. In the current work, we will explore how the strong negative charge of PAA can be harnessed to modulate the enzymatic activity of conjugated enzymes, and how the PAA morphology (linear versus comb-like) affects the conjugate thermal stability.
1.2. Protein-derived hydrogels and white light generation

Materials capable of efficiently generating white light emission are of current interest in response to demands of the lighting and display industries.\textsuperscript{15} The global yearly energy demand is in excess of 17 TW, and continues to grow.\textsuperscript{16} In light of these factors, energy efficient light emitting diodes (LEDs) are attractive. However, the most common commercially available LEDs contain rare earth metals in the emissive components and phosphor coatings, which are used to convert UV emission to broad white emission.\textsuperscript{17,18} These are problematic long term because of the scarcity of the materials and the difficulty in recovering rare earth metals from spent LEDs.\textsuperscript{19} Alternative materials such as quantum dots, fluorescent polymers, metal-organic frameworks, inorganic-organic hybrids, and organic fluorophores have been explored, but each has its associated challenges, such efficiency, cost, ease of synthesis, biodegradability, and toxicity.\textsuperscript{15,17–20}

Our eventual goal is to develop a white LED (WLED) entirely from sustainable and biodegradable biological materials. In pursuit of this goal, we first developed a biomaterial coating for conventional LEDs to convert UV emission to broad white emission, which we named “biophosphor.” For these purposes, we developed a hydrogel derived from bovine serum albumin (BSA), with red, green, and blue fluorescent dyes incorporated. When excited by a UV LED, a combination of direct dye excitation and Förster Resonance Energy Transfer (FRET) among dyes resulted in the appropriate ratio of blue, green, and red emission to produce white.

The white emitting gel was also explored as a biosensor. First, the emission of incorporated dyes and their binding to BSA was sensitive to the protein microenvironment.\textsuperscript{21–24} Because of the delicate balance required to produce white, any changes to one emissive component would result in off-white emission, which could be readily detected visually and by fluorescence spectroscopy.\textsuperscript{20,25,26} Second, the variety of functional groups on BSA allowed for swelling in response to stimuli such as pH. Finally, enzymes were incorporated into the hydrogel for detection of diverse analytes.
1.3. Regulation of polypeptide aggregation by nanoparticles

Amyloid fibrils are formed by supramolecular assembly of misfolded proteins in non-native conformations that are rich in β-sheet structure. The resulting fibrils can be several hundreds of nanometers in length, and can form into higher order structures with larger dimensions. The beta-sheet structure of amyloid fibrils is highly stable, difficult to reverse, and the fibrils eventually precipitate from solution when their dimensions are large enough. Such amyloid plaques are implicated in neurodegenerative diseases such as Alzheimer's and Parkinson's. Recently, researchers have since demonstrated that the ability to form amyloid-like structures is a generic feature of the peptide backbone, and the protein sequence simply determines the conditions required to form fibers.

With the growth of nanomedicine and the increased use of nanomaterials in biomedical applications, understanding the role of nanoparticles (NPs) in protein amyloidogenesis becomes important. Current studies focused on naturally amyloidogenic proteins reveal that the influence of nanoparticles on amyloidosis can be highly sequence dependent, and thus drawing broad conclusions is difficult. For example, N-isopropylacrylamide:N-tert-butylacrylamide copolymeric NPs enhance fibrillation of β2-microglobulin but inhibit fibrillation of amyloid-β and islet amyloid polypeptide.

The proposed mechanism of NP enhanced aggregation indicates that binding of proteins to NPs increases the local concentration of protein monomers, thus increasing the rate of formation of the high energy nucleus, the slowest step is nucleated supramolecular polymerization. We selected poly(glutamic acid) (PLG), which is known to form amyloid fibrils in acidic pH, to study this phenomenon systematically. We expected that introduction of cationic NPs, with a complementary charge to anionic PLG, will increase the rate of PLG fibrillation. Using this model system, we found that cationic NPs act in a regulatory capacity, and can either inhibit or promote aggregation of PLG depending on the experimental conditions.
Chapter 2. 34-fold enhancement of peroxidase turnover number for cytochrome c-poly(acrylic acid) conjugates

2.1. Abstract

Cytochrome c-poly(acrylic acid) (cyt c-PAA) conjugates with 34-fold enhancement in peroxidase turnover number ($k_{\text{cat}}$) are reported. Cyt c-PAA conjugates were prepared by carbodiimide coupling. PAA with molecular weight ($M_w$) ranging from 1.8k to 250k g mol$^{-1}$ were employed, and the effect of PAA $M_w$ on peroxidase kinetics was assessed. The $k_{\text{cat}}$ value increased with increased $M_w$ of PAA, ranging from 0.077(±0.002) s$^{-1}$ in the absence of PAA to 2.66(±0.08) for the conjugate of cyt c with 250k PAA. Enzymatic activity studies over pH 6-8 indicated improved activity for cyt c-PAA conjugates at neutral or slightly alkaline pH. Examination of the cyt c heme spectroscopy in the presence of H2O2 revealed that formation of Compound III, a reactive intermediate that leads to enzyme inactivation, was suppressed in cyt c-PAA conjugates. Thus, we suggest the $k_{\text{cat}}$ enhancement can be attributed to acidification of the pH microenvironment and inhibition of the formation of a reactive intermediate that deactivates cyt c during the catalytic cycle.
2.2. Introduction

Conjugation of synthetic or natural polymers to enzymes is a proven strategy to improve the thermal and chemical stability of enzymes. The use of polymeric materials, particularly polyelectrolytes, to engineer the protein microenvironment is emerging as an effective means to manipulate the catalytic activity of enzyme-polymer conjugates, and can be viewed as a type of synthetic post-translational modification. For example, the ability of polyelectrolytes to sequester ionic species can influence the local pH, which has been shown to improve the activity of cytochrome c (cyt c), trypsin, and chymotrypsin in a broad range of bulk solution pH. Non-covalent binding of substrate or inhibitor molecules to conjugated polymers can influence catalytic rates by enriching local concentrations of these species, as demonstrated with horseradish peroxidase-DNA, chymotrypsin-poly(quaternary ammonium), and catalase-poly(acrylic acid) conjugates. The polyanionic surfaces of DNA nanocages significantly improved turnover number of several encapsulated enzymes. Finally, local charge supplied by grafted polyelectrolytes can affect the catalytic cycle of trypsin by perturbing the free energy of reactants and intermediates.

The above insights demonstrate that controlling the enzyme microenvironment of polymer conjugates deserves further research. We have previously demonstrated that conjugation to PAA improves the thermal and chemical stability of enzymes. The effect of PAA molecular weight and polyelectrolyte features on the catalytic activity of enzymes has not been examined. For this reason cytochrome c-poly(acrylic acid) (cyt c-PAA) conjugates were selected as our model system: (1) The cyt c catalytic cycle features several cationic and anionic intermediates, the influence of polyelectrolytes on the relative stability of these intermediates, and their mechanistic influence enzyme turnover has not been explored and (2) the effect of PAA molecular weight and cyt c:COOH ratio on the kinetics of the resulting conjugates may be examined, which is an often overlooked design consideration. With these factors in mind, we report here cyt c-PAA conjugates with up to 34-fold enhancement in turnover number ($k_{cat}$), afforded by PAA influence.
on the pH microenvironment and PAA charge-induced destabilization of wasteful reaction intermediates.
Figure 2.1. Synthesis of cyt c-PAA by carbodiimide chemistry (top). Conjugates showed enhanced peroxidase activity, tracked by the conversion of guaiacol (200 µM) to 3,3'-dimethoxy-4,4'-biphenoquinone ($\lambda_{\text{max}} = 470$ nm).
2.3. Results and Discussion

2.3.1. Synthesis and characterization of cyt c-PAA

Cyt c is a 12.5 kDa soluble, globular hemoprotein found in mitochondria, where it shuttles electrons during respiration and acts as a peroxidase during apoptosis. Cyt c-PAA conjugates were formed by crosslinking PAA carboxyl groups with the 20 primary amines of cyt c (19 lysine, 1 N-terminus) using carbodiimide chemistry (Figure 2.1). PAA (10 μM) was activated by stirring with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 5-10 mM) in sodium phosphate buffer (20 mM, pH 7.0) for 10 minutes at 25 °C. Cyt c (10 μM) was then added, and the solution was stirred for four hours. Conjugates were successfully produced using PAA with average molecular weights (Mw) 1.8k, 8k, 50k, 100k, and 250k g mol⁻¹. Reaction by-products were routinely removed by dialysis. Cytochrome c-poly(acrylic acid) conjugates were named as cyt c-PAA(XXX), where the value denoted by XXX gives the molecular weight of PAA. For example, the conjugate of cyt c with 250k g mol⁻¹ PAA is indicated by cyt c-PAA(250k). Successful conjugation was validated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE, 12.5% polyacrylamide), after analysis by agarose gel electrophoresis proved inconclusive (Figure 2.2). All cyt c-PAA derivatives showed several bands or wide smears, indicating the broad molecular weight distribution expected from protein-polymer nanogels. The disappearance of bands corresponding to free cyt c confirmed the covalent conjugation to cyt c. The degree of crosslinking between cyt c and PAA was estimated using the trinitrobenzene sulfonic acid (TNBSA) assay to quantitate the primary amines in cyt c and cyt c-PAA (Figure 2.3). After conjugation to PAA, the number of primary amines of cyt c was reduced from 22.9(±0.8) to 10-13 (Table 2.1) for all cyt c-PAA conjugates. No significant difference was found among the cyt c-PAA with different Mw PAA (Welch’s t-test, p<0.05). Thus, ~10 crosslinks were made per cyt c during the EDC condensation.
Figure 2.2. (A) Agarose gel of cyt c/PAA physical mixtures revealed strong complexation between cationic cyt c and anionic PAA for PAA(8k) and larger $M_w$. (B) SDS-PAGE gel of cyt c-PAA conjugates synthesized with 5 mM EDC. Significant unconjugated cyt c was found in cyt c-PAA(250k) with 5 mM EDC. (C) SDS-PAGE gel of cyt c-PAA(250k) with increasing [EDC]. 10 mM EDC was sufficient to obtain complete crosslinking.
Figure 2.3. (A) Absorbance spectra of glycine standards incubated (37 °C, 2h) with trinitrobenzene sulfonic acid (TNBSA). (B) Calibration curve for concentration of primary amines produced from spectra in panel A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1° amines/cyt c</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt c</td>
<td>22.9(±0.8)</td>
</tr>
<tr>
<td>cyt c-PAA(1.8k)</td>
<td>9.7(±0.6)</td>
</tr>
<tr>
<td>cyt c-PAA(8k)</td>
<td>10.2(±0.4)</td>
</tr>
<tr>
<td>cyt c-PAA(50k)</td>
<td>12(±1)</td>
</tr>
<tr>
<td>cyt c-PAA(100k)</td>
<td>11(±2)</td>
</tr>
<tr>
<td>cyt c-PAA(250k)</td>
<td>13.1(±0.6)</td>
</tr>
</tbody>
</table>

Table 2.1. Number of primary amines per cyt c as determined from the TNBSA assay.
2.3.2. Michealis-Menten kinetics

Next, we assessed the effect of PAA conjugation on cyt c peroxidase kinetics. In the catalytic cycle, hydrogen peroxide is reduced to water, and single electron oxidation of two equivalents of guaiacol regenerates the ferric heme state. These two guaiacol radicals combine to form 3,3'-dimethoxy-4,4'-biphenooquinone, which is orange in color (Figure 2.1).\(^{52}\) Oxidation of guaiacol is the rate determining step of peroxidase activity, so the Michaelis-Menten kinetics were examined by varying the [guaiacol] (1-200 μM) for a fixed [H\(_2\)O\(_2\)] (50 mM).\(^{53}\) The constants \(K_M\) and \(v_{\text{max}}\) of cyt c and cyt c-PAA of all molecular weights were obtained by fitting the Michaelis-Menten model to the guaiacol-dependent initial rate data. The non-linear least squares fits are represented by the dashed lines in Figure 2.4. The \(K_M\) value of cyt c for guaiacol oxidation was 7(±1) μM, orders of magnitude smaller than that of other heme enzymes but in good agreement with literature reports.\(^{53}\) The \(K_M\) values of cyt c-PAA(1.8k), cyt c-PAA(8k), and cyt c-PAA(50k) did not differ significantly (Welch’s t-test, p<0.05) from cyt c, but \(K_M\) increased with larger molecular weight PAA, reaching up to 38(±4) μM.

The turnover number (\(k_{\text{cat}}\)) is the rate constant for conversion of enzyme-substrate complex to product, and was determined using the fitted \(v_{\text{max}}\). For native cyt c, \(k_{\text{cat}}\) was 0.077(±0.002) s\(^{-1}\), again in good agreement with literature reports.\(^{53}\) As molecular weight of PAA increased, \(k_{\text{cat}}\) of cyt c-PAA increased as well, ranging from 0.21(±0.01) s\(^{-1}\) in the case of cyt c-PAA(1.8k) to 2.66(±0.08) s\(^{-1}\) for cyt c-PAA(250k). This represented a dramatic increase in the enzymatic activity of cyt c after conjugation to PAA, with the forward rate constant enhanced between 3- and 34-fold.

Physical mixtures of cyt c and PAA 50k, 100k, and 250k (no EDC) were examined next to determine if covalent conjugation was required for activity enhancements (Figure 2.4). The physical mixture of cyt c and 50k M\(_w\) PAA had a similar \(k_{\text{cat}}\) to the covalent adduct, which was ~4-fold greater than that of cyt c alone. Agarose gel electrophoresis of cyt c/PAA demonstrated strong
non-covalent complexation of cyt c and PAA (Figure 2.2), so the enhanced activity of these species was expected. Unlike the covalent conjugates, no further enhancement was noted with 100k or 250k M₆ PAA physical mixtures. The full complement of Michaelis-Menten parameters for cyt c-PAA and cyt c/PAA physical mixtures are in Table 2.2.
**Figure 2.4.** (A) Michealis-Menten kinetics of cyt c-PAA conjugates. (B) Michealis-Menten kinetics of physical mixtures of cyt c and PAA 50k, 100k, or 250k. The behavior of cyt c/PAA(50k) was similar to its corresponding covalent conjugate. No additional kinetic enhancement was noted in the physical mixtures with 100k or 250k relative to cyt c/PAA(50k), unlike the covalent conjugates with the same $M_w$ PAA.

<table>
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<th>$K_M$ (M)</th>
<th>$k_{cat}$ (M s$^{-1}$)</th>
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<tr>
<td>cyt c</td>
<td>7(±1)x10$^{-6}$</td>
<td>0.077(±0.002)</td>
</tr>
<tr>
<td>cyt c-PAA(1.8k)</td>
<td>7(±2)x10$^{-6}$</td>
<td>0.21(±0.01)</td>
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<tr>
<td>cyt c-PAA(8k)</td>
<td>5.1(±0.9)x10$^{-6}$</td>
<td>0.245(±0.005)</td>
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<td>cyt c-PAA(50k)</td>
<td>4.8(±0.5)x10$^{-6}$</td>
<td>0.289(±0.004)</td>
</tr>
<tr>
<td>cyt c-PAA(100k)</td>
<td>12(±1)x10$^{-6}$</td>
<td>0.71(±0.016)</td>
</tr>
<tr>
<td>cyt c-PAA(250k)</td>
<td>38(±4)x10$^{-6}$</td>
<td>2.66(±0.08)</td>
</tr>
<tr>
<td>cyt c/PAA(50k)</td>
<td>6.8(±0.9)x10$^{-6}$</td>
<td>0.285(±0.004)</td>
</tr>
<tr>
<td>cyt c/PAA(100k)</td>
<td>10(±1)x10$^{-6}$</td>
<td>0.281(±0.006)</td>
</tr>
<tr>
<td>cyt c/PAA(250k)</td>
<td>9(±3)x10$^{-6}$</td>
<td>0.29(±0.01)</td>
</tr>
</tbody>
</table>

**Table 2.2.** Michealis-Menten parameters of cyt c-PAA covalent conjugates and cyt c/PAA physical mixtures (shaded cells).
2.3.3. Cyt c-PAA structural studies

The enhancement of cyt c peroxidase activity upon binding anionic cardiolipin has been well documented in the literature. However, partial unfolding of cyt c and dissociation of the Fe-Met80 bond are key to the enhanced peroxidase activity in the presence of cardiolipin. A ~6-fold increase in $k_{cat}$ for the oxidation of guaiacol has also been reported for horse cyt c dimerized by treatment with ethanol, while binding to CTAB decorated gold nanoparticles increased $k_{cat}$ of pyrogallol oxidation ~14-fold. Binding to functionalized carbon nanomaterials increased $v_{max}/K_M$ dramatically. Again, these rate enhancements by others are accompanied by the breakage of the Met80-Fe bond and/or partial denaturation of cyt c. Thus, we examined cyt c-PAA to determine if the heme ligation had been affected by the conjugation reaction.

Circular dichroism (CD) and visible absorbance spectroscopy were employed to determine if the $k_{cat}$ enhancement was caused by changes to cyt c secondary structure or heme ligation. Cyt c is primarily alpha helical, which was reflected in the UV CD by negative peaks at 222 and 208 nm (Figure 2.5A). The 208 nm band was weaker than the 222 nm band in all cases, indicative of monomeric cyt c. Only small changes in molar ellipticity were observed, demonstrating that there was little perturbation in the protein secondary structure. Similar changes were observed in the CD spectra of cyt c/PAA mixtures lacking EDC, as well as in experiments where cyt c and PAA were placed in separate cells and measured in tandem (Figure 2.6). These data suggest the observed changes in the CD spectra of cyt c-PAA could simply be artifacts caused by PAA absorbance in the UV.

The spectra of the heme prosthetic group are highly sensitive its ligation and binding pocket structure, which are reflected in the Soret band (~400 nm) and Q band (~550 nm). The spectra of all cyt c-PAA samples in these regions could be nearly overlaid on that of native cytochrome c, clearly demonstrating that the conjugation reaction only minimally perturbed the sensitive structure of the protein (Figure 2.5B-C). The Soret CD spectra of cyt c and all cyt c-PAA
derivatives showed a negative peak at 415 nm and a positive peak at 398 nm, indicative of native ferric cyt $c$ with His/Met in the axial positions.$^{60}$ The Soret band of native ferric cyt $c$ has a maximum at 409 nm in the visible absorbance spectrum,$^{58,59}$ which was observed in all cyt $c$-PAA except cyt $c$-PAA(250k). The Soret band of cyt $c$-PAA(250k) shifted to 407 nm, which could be caused by minor changes in the secondary structure.$^{58}$ The Q band of native cyt $c$ had a maximum at 528 nm, which was maintained in all cyt $c$-PAA derivatives. Finally, a weak charge transfer band at 695 nm demonstrated the native ligation of His/Met for cyt $c$ and all cyt $c$-PAA.$^{61}$ Taken together, the data indicated that the secondary structure and heme ligation of cyt $c$-PAA was not significantly changed and thus not responsible for the enhancement in $k_{\text{cat}}$. 
Figure 2.5. (A) UV circular dichroism (CD) spectra of cyt c and cyt c-PAA conjugates. Conjugate spectra are labelled with their corresponding PAA molecular weight. (B) Soret CD spectra of cyt c and cyt c-PAA conjugates. (C) Soret (left), Q band (center), and Met charge transfer band (right) absorbance spectra of cyt c (solid curve) and cyt c-PAA conjugates (dashed lines).
Figure 2.6. (A) Circular dichroism spectra of cyt $c$ and PAA in tandem cells. Cyt $c$ and PAA were present in separate cuvettes, which were placed together in the spectrometer. This revealed that scattering or absorbance from PAA in the UV region caused an increase in the observed molar ellipticity of cyt $c$, even when PAA was present in a separate cell and thus had no direct interaction with cyt $c$. (B) UV CD spectra of physical mixtures of cyt $c$ and PAA (no EDC).
2.3.4. Mechanistic studies

The influence of anionic polyelectrolytes on enzyme activity by controlling the microenvironment may provide insight into the enhanced kinetics of cyt c-PAA. For example, encapsulation of enzymes such as glucose oxidase in DNA nanocages enhanced the catalytic activity because the phosphates ordered the protein hydration layer. Glucose oxidase and horseradish peroxidase bound to DNA exhibit increased cascade reaction efficiency because the phosphate backbone maintains a favorable local pH. Additionally, conjugation of cyt c to poly(methacrylic acid) was reported to change the optimum pH of cyt c by acidifying the pH microenvironment. We also studied local enrichment of guaiacol by partitioning to the PAA phase, but guaiacol appeared to have only weak affinity for PAA (Figure 2.7).

To test the effect of PAA on the pH activity profile, we measured the rate of guaiacol oxidation (200 μM guaiacol, 50 mM H₂O₂, 20 mM sodium phosphate) at pH 6-8. Cyt c and cyt c-PAA(50k) were examined in this manner. The peroxidase activity of cyt decreased as the pH was increased, while the pH profile of cyt c-PAA was relatively flat (Figure 2.8A). The Soret CD and absorbance spectra did not change over this pH range, indicating no changes to heme ligation (Figure 2.8B-C). These observations were in line previously reports of cyt c-PMMA conjugates with broadened pH response. Thus, modulation of the local pH by the weakly acidic COOH groups of PAA may explain enhanced activity of cyt c-PAA.
Figure 2.7. (A) Absorbance spectra of guaiacol retained in the aqueous phase after extraction with chloroform (initial [guaiacol] in aqueous phase was 2 mM). The solid line represents the aqueous phase containing 250k PAA in sodium phosphate buffer, pH 7.0, while the dashed line represents the aqueous phase which contained only the buffer. (B) Percent retention of guaiacol in aqueous phase as a function of PAA molecular weight. Percent retention was calculated from the absorbance spectra after extraction with chloroform.
Figure 2.8. (A) Variation of cyt c and cyt c-PAA(50k) rate of guaiacol oxidation with pH. Activity of cyt c decreased as pH increased, while that of cyt c-PAA increased over the same range. (B) Soret CD of cyt c-PAA(50k) at pH 6.0 (orange curve) and pH 8.0 (green curve). Changes in the relative intensities of the peaks indicates small changes in heme solvent exposure, while the presence of both positive and negative peaks indicated heme ligation did not change when the pH was changed. (C) Heme absorbance of cyt c-PAA(50k) at pH 6.0 (orange curve) and pH 8.0 (green curve). Soret and Q band maxima were found in the same position at each pH, indicating heme ligation was unchanged and heme iron was in the native, ferric state.
Examining the catalytic cycle of cyt c revealed an additional mechanism for improved catalysis by cyt c-PAA (Scheme 2.1). In heme peroxidases, oxidation of guaiacol by occurs in three steps. First, \( \text{H}_2\text{O}_2 \) is reduced and the heme iron is oxidized from Fe(III) to Fe(IV)-oxo (Compound I). Next, the oxidation of guaiacol produces a guaiacol radical and converts the heme to Compound II, which is still in the Fe(IV) state. Finally, oxidation of a second equivalent of guaiacol converts the heme back to the native ferric state. Critically, Compound II can react with \( \text{H}_2\text{O}_2 \) to produce Compound III, a negatively charged Fe(III)-peroxo state. Formation of Compound III can be reversible, but reaction of Compound III with peroxide results in enzyme inactivation by auto-oxidation and heme bleaching. Even when the enzyme is not inactivated, Compound III is a “sink” for guaiacol oxidation, as it consumes an equivalent of hydrogen peroxide and completes a catalytic cycle without a concomitant oxidation of guaiacol.

We hypothesized that the high negative charge density of PAA could suppress formation of Compound III by unfavorable electrostatic interactions. It is possible that PAA charge may also stabilize the cationic Compounds I and II. However, we examined formation of Compound III because its accumulation can be conveniently observed spectroscopically without time-resolved techniques under the appropriate conditions. To rationalize the interaction of charged groups of PAA with heme reactive intermediates, we estimated the Debye length in 20 mM sodium phosphate buffer at room temperature. Under these conditions, the Debye length was \(~1.6\) nm, comparable to the diameter of cyt c. Thus, it was reasonable to expect electrostatic interaction between PAA charged groups and reactive heme intermediates.

We examined the formation of Compound III by monitoring the Q bands of cyt c and cyt c-PAA(250k) in the presence of 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). (Figure 2.9). Upon introduction of 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) to a solution of 10 \( \mu \text{M} \) cyt c (20 mM sodium phosphate, pH 7.4), the shoulder at 550 nm disappeared while the absorbance at 533 nm and 563 nm increased, which indicate the appearance of Compound III. These spectral changes were complete within 30 seconds, and persisted for
more than 30 minutes. Subsequent addition of guaiacol did not produce 3,3'-dimethoxy-4,4'-biphenoquinone, which suggests that the catalyst was deactivated. Under similar conditions, cyt c-PAA(250k) did not show any spectral changes to indicate the appearance of Compound III, and continued to perform the catalytic cycle.

Next, heme bleaching studies were conducted to examine the inactivation of cyt c and cyt c-PAA under the conditions of the kinetic assays (20 mM sodium phosphate, pH 7.0, 20 °C). Cyt c and cyt c-PAA(250k) were exposed to 50 mM H$_2$O$_2$, and the disappearance of the Soret absorbance at 410 nm was monitored. Interestingly, heme bleaching of cyt c-PAA(250k) was initially more rapid that that of cyt c (Figure 2.9). However, as the reaction proceeded, the rate of cyt c-PAA(250k) bleaching slowed relative to cyt c. After the reaction was complete, cyt c-PAA(250k) retained 13.1(±0.9)% of its initial Soret absorbance, while cyt c retained only 6(±1)% (Figure 2.10). In the absence of guaiacol, conjugation to PAA offered mild protection from inactivation by heme bleaching. Inactivation by peroxide is further inhibited by the presence of substrates guaiacol or phenol, so this gain in stability can be relevant during guaiacol oxidation.

Taken together, the modulation of local pH and suppression of wasteful intermediates could have a synergistic effect, which result in the 34-fold enhancement in cyt c-PAA turnover number reported here (Scheme 2.2). This may also help to explain the broadened pH profile, as the ionization of PAA changes >50% over the range studied. Additionally, both effects explain the dependence of $k_{cat}$ on PAA $M_w$ – increasing the $M_w$ at a fixed cyt c:PAA mole ratio increases the concentration of acidic groups and magnitude of the negative charge.
Scheme 2.1. Schematic representation of cyt c catalytic cycle.
Figure 2.9. (A) Changes in Q band absorbance of cyt c and cyt c-PAA(250k) (offset) in the presence of 100 μM H₂O₂. The shoulder at ~550 nm disappeared, while the absorbance at 533 and 553 nm increased, indicative of the formation of Compound III in cyt c. No such changes were noted for cyt c-PAA(250k). (B-C) Spectra of the Q band of cyt c (B) and cyt c-PAA(250k) (C) in the presence of 100 μM H₂O₂.
Figure 2.10. (A) Kinetic trace of cyt c heme bleaching (20 mM phosphate, pH 7.0, 20 °C). In the presence of 50 mM H₂O₂ but the absence of guaiacol, auto-oxidation and heme bleaching occurred, indicated by a decrease in the Soret absorbance at 410 nm. Decay of the heme in cyt c-PAA(250k) was initially more rapid than cyt c, consistent with the increased peroxidase activity caused by acidification of the local environment. However, bleaching of cyt c-PAA(250k) slowed significantly relative to cyt c as the reaction progressed. (B) After completion of the heme bleaching reaction, cyt c-PAA(250k) retained a greater degree of its Soret absorbance, indicating a lesser extent of inactivation in cyt c-PAA(250k).
Scheme 2.2. Schematic representation of dual influences of PAA on cyt c peroxidase activity.
2.3.5. Extension to other heme enzymes

We next examined if these concepts could be extended to other heme peroxidases, such as horseradish peroxidase (HRP) or hemoglobin (Hb). PAA conjugates of HRP and Hb were prepared and characterized in a similar manner to cyt c-PAA, after which Michaelis-Menten kinetics of guaiacol oxidation were measured (Figure 2.11, Table 2.3). HRP-PAA had similar \( K_M \) and \( k_{\text{cat}} \) to native HRP (Welch’s t-test, \( p<0.05 \)). The rate constant of inactivation of HRP by \( \text{H}_2\text{O}_2 \) via Compound III is at least 10-fold smaller than that of cyt c,\(^{63,67} \) so the lack of improvement was not unexpected. Hb, a typically inefficient peroxidase,\(^ {68} \) was improved by conjugation to PAA. \( k_{\text{cat}} \) of Hb-PAA(50k) and Hb-PAA(250k) were 1.1(±0.1) s\(^{-1} \) and 0.62(±0.04) s\(^{-1} \), both larger than that of Hb, 0.43(±0.06) s\(^{-1} \) (Table 2.3). Together, the kinetics of HRP-PAA, cyt c-PAA, and Hb-PAA indicated that the PAA could improve the efficiency of certain enzymatic processes when the enzymes were unstable in the presence of excess \( \text{H}_2\text{O}_2 \).
Figure 2.11. (A) Agarose gel (0.5% agarose w/w, pH 7.0) of HRP and HRP-PAA derivatives. (B) Agarose gel (0.5% agarose w/w, pH 7.0) of Hb and Hb-PAA derivatives. (C) Michaelis-Menten kinetics of HRP-PAA (20 nM HRP, 200 μM H₂O₂) were nearly unchanged when conjugated to PAA. (D) Marked changes were noted in Hb-PAA Michaelis-Menten kinetics. Kᵦ decreased sharply, while vₘₐₓ increased with Mₚ PAA.
<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_M$ (mM)</th>
<th>$v_{\text{max}}$ (M s$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$ M$^{-1}$)</th>
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</thead>
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<td>Hb</td>
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<tr>
<td>Hb-PAA(100k)</td>
<td>0.8(±0.1)</td>
<td>6.2(±0.4)x10$^{-7}$</td>
<td>0.62(±0.04)</td>
<td>7.9(±0.1)x10$^2$</td>
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</table>

**Table 2.3.** Michealis-Menten parameters of HRP-PAA and Hb-PAA (shaded cells).
2.4. Conclusions

The results presented here hint at ways in which the physical characteristics of polyelectrolytes such as PAA could be used to tune the catalytic activity and efficiency of enzyme-polymer conjugates. In this case, PAA destabilized a wasteful intermediate while acidifying the local pH, thus improving the activity of heme peroxidases, cyt c and Hb, and leading to the 34-fold increase in $k_{\text{cat}}$ of cyt c-PAA(250k). Significantly, by suppressing Compound III formation, PAA influenced the enzyme mechanism. These effects could be systematically controlled by varying the PAA M$_w$, allowing for precise tuning of peroxidase activity. Additionally, these findings suggest that, with a good understanding of the catalytic mechanism and careful polymer selection, this concept could be extended to other enzyme-polymer systems.
2.5. Materials and Methods

2.5.1. Materials

Cytochrome c and horseradish peroxidase (230 U mg\(^{-1}\)) were purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). Poly(acrylic acid) 1.8k, 8k, 100k, and 250k g mol\(^{-1}\) (weight average molecular weight), sodium phosphate, Brilliant blue R250, bromophenol blue, guaiacol, agarose Type I (low EEO), glycine, chloroform, acetic acid, isopropanol, acrylamide, sodium dodecyl sulfate, and trinitrobenzene sulfonic acid (5% w/v in water) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(acrylic acid) 50k (weight average molecular weight) was purchased from Polysciences, Inc (Warrington, PA). Tris base, N,N’-methylenebis(acrylamide), ammonium persulfate, tetramethylenediamine, and 2-mercaptoethanol were purchased from Thermo Fisher Scientific (Waltham, MA). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from TCI America (Portland, OR). Poly(ethylene glycol), 20000 g mol\(^{-1}\), was purchased from Alfa Aesar (Haverhill, MA). Hemoglobin was purchased from MP Biomedicals (Santa Ana, CA). Dialysis membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA).

2.5.2. Synthesis of enzyme-PAA conjugates

Protein-enzyme conjugates were prepared per previously reported methods,\(^1\) with a few minor modifications. First, activated poly(acrylic acid) solution was prepared by stirring PAA with EDC in sodium phosphate buffer (20 mM pH 7.0) for 10 minutes. Next, cyt c, HRP, or Hb were added while stirring, and the resulting mixture was reacted at room temperature for four hours. The final concentration of enzyme and PAA were 10 μM. The concentration of EDC was maintained at 5 mM for all \(M_w\) of PAA to avoid excessive enzyme-enzyme crosslinking, with the exception of 250k PAA. Here, 10 mM EDC was required to obtain complete crosslinking and ensure there was no free cyt c, as determined by gel electrophoresis. The EDC reaction byproducts were removed by dialysis against 20 mM sodium phosphate, pH 7.0, using a 200-500
Da cutoff membrane for cyt c-PAA(1.8k) and cyt c-PAA(8k), and a 15 kDa cutoff membrane for all other Mw PAA.

2.5.3. Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose (0.5% w/v) in Tris-acetate buffer (40 mM, pH 7.0). Solutions were heated in the microwave for 30 s to dissolve agarose. Samples were prepared for electrophoresis by combining 20 μL of 10 μM cyt c or cyt c-PAA solution with 10 μL of loading buffer (50% v/v glycerol, 0.01% w/v bromophenol blue). Gels were run in a horizontal gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc., Grand Island, NY) for 30 minutes, with 100 V constant voltage and using 40 mM Tris acetate, pH 7.0 as the running buffer. Gels were stained for at least four hours with an aqueous solution of 20% v/v acetic acid and 0.03 g L⁻¹ Brilliant blue R250, then destained for four hours with 10% v/v acetic acid prior to imaging.

2.5.4. SDS-PAGE

12.5% w/w acrylamide separating gels and 5% w/w acrylamide stacking gels were used for SDS-PAGE. The separating gel was prepared by combining 4.2 mL 30% acrylamide solution (18.75 g acrylamide, 0.5 g N,N'-methylenebis(acrylamide), 64 mL DI water), 2.5 mL 4x lower gel buffer (9.35g Tris base, 0.2 g SDS, 50 mL DI water, pH 8.8), 3.3 mL DI water, 50 μL 10% ammonium persulfate, and 5 μL tetramethylenediamine. The stacking gel was prepared by combing 0.85 mL 30% acrylamide solution, 1.25 mL 4x upper gel buffer (3.025 g Tris base, 0.2 g SDS, 50 mL DI water, pH 6.8), 2.9 mL DI water, 25 μL 10% ammonium persulfate, and 5 μL tetramethylenediamine. The separating gel was poured into a 1 mm mold and allowed to polymerize for 30 minutes, after which the stacking gel was poured and similarly allowed to polymerize.

Samples were prepared by combining 20 μL of ~50 μM cyt c with 15 μL SDS-PAGE loading buffer (2% w/v SDS, 10% w/w 2-mercaptoethanol), after which the solutions were heated at 90 °C for 2 minutes. 15-20 μL of sample solution was loaded per well. Gels were run in a vertical
Bio-Rad Mini-PROTEAN electrophoresis apparatus at 150 V for 15 minutes, then 200 V until the dye front reached the bottom of the gel (~20 minutes). The running buffer was 3.03 g L\(^{-1}\) Tris base, 14.41 g L\(^{-1}\) glycine, and 1 g L\(^{-1}\) SDS. The gel was stained with an aqueous solution of 10% v/v acetic acid, 10% v/v isopropanol, and 0.02 g L\(^{-1}\) Brilliant blue R250 for four hours, then with an aqueous solution of 20% v/v acetic acid and 0.03 g L\(^{-1}\) Brilliant blue R250 for four hours. Gels were destained with 10% acetic acid prior to imaging.

2.5.5. Trinitrobenzene sulfonic acid assay

Quantitation of primary amines using trinitrobenzene sulfonic acid (TNBSA) was performed by a previously reported method.\(^2\) 250 µL of 0.01% (w/v) TNBSA was added to 500 µL of protein or glycine solution and incubated for 2 hours at 37 °C. After incubation, 250 µL 10% (w/v) sodium dodecyl sulfate and 125 µL of 1 M HCl were added to each sample. The absorbance at 335 nm was then obtained. To produce the standard curve, glycine solutions with concentrations of 1, 3, 5, 7, 10, 15, and 20 µM were analyzed in this way. Protein samples were prepared at 0.8 µM. The concentration of primary amines in each protein sample was determined using the glycine calibration plot (one 1° amine per glycine), and the number of primary amines per protein was determined by dividing the concentration of primary amines by the protein concentration.

2.5.6. Dynamic light scattering

Conjugate hydrodynamic radius was measured by dynamic light scattering (DLS). Samples were filtered with a 0.22 µm syringe filter to remove dust particles and large aggregates prior to analysis. Precision Detectors PDDLS/CoolBatch 40T and PD4047 dynamic light scattering detectors with 658 nm laser and 90° laser and monitoring optics were employed for DLS measurements. Measurements were completed in triplicate.
2.5.7. Circular dichroism spectroscopy

Circular dichroism spectra were measured with a JASCO J-710 spectropolarimeter. UV CD spectra were scanned from 195-260 nm using a 0.05 cm path length quartz cuvette and the following parameters: 1 nm data pitch, continuous scanning mode, 1 second response speed, 2 nm bandwidth, 6 scans per spectrum. Soret CD spectra were scanned from 300-550 nm using a 1 cm path length quartz cuvette and the following parameters: 1 nm data pitch, continuous scanning mode, 1 second response speed, 1 nm bandwidth, 10 scans per spectrum. Because the Soret CD features are weak, mild smoothing was applied (minimum data convolution width) to improve noise using JASCO Spectra Analysis software. Blank spectra (buffer solution only) were subtracted from all spectra, which were then normalized with respect to path length and protein concentration.

2.5.8. UV/Visible absorbance spectroscopy

Soret and Q band absorbance spectra were obtained using a HP 8453 UV/visible spectrophotometer and a 1 cm pathlength quartz cuvette. The change in Q band absorbance in the presence of H$_2$O$_2$ was monitored by injecting 100 μM H$_2$O$_2$ into a solution of 10 μM cyt c or cyt c-PAA, then recording the UV/Vis spectrum at intervals. Delta absorbance plots were produced by subtracting the spectrum of a given time point from the spectrum of the subsequent time point. Measurements were completed in triplicate.

2.5.9. Michealis-Menten kinetics and activity studies

Michealis-Menten kinetics. Solutions containing cyt c or cyt c-PAA, guaiacol, and sodium phosphate buffer (pH 7.4) were prepared and placed in a 1 cm glass cuvette. Absorbance at 470 nm ($A_{470}$) was monitored as a function of time using a HP 8453 UV/visible spectrophotometer. Once a stable baseline was established, H$_2$O$_2$ was injected into the cuvette while stirring at 1000 RPM, and the change in $A_{470}$ was recorded for 1 minute. The final concentrations of reactants were: [cyt c] = 1 μM, [guaiacol] = 5-200 μM, [H$_2$O$_2$] = 50 mM, and [sodium phosphate] = 20 mM.
The activity assay was performed in triplicate at 20 °C and atmospheric pressure for each concentration of guaiacol tested. Initial rates were obtained from the slope of the linear portion of the kinetic traces, where the change in absorbance with respect to time was the greatest. The variation of initial rate with guaiacol concentration was fit to the Michaelis-Menten model and the Michaelis constants were extracted, as described below.

The Michaelis-Menten model indicates that enzymatic catalysis occurs in two steps, a reversible binding of substrate to enzyme followed by conversion of substrate to product and subsequent release of product. The Michaelis-Menten equation

\[ v_0 = \frac{v_{\text{max}}[S]}{K_M + [S]} \]

is a hyperbolic function that describes how the initial rate of the reaction depends on the concentration of substrate [S], the maximum initial rate \( v_{\text{max}} \), and the affinity of the enzyme for the substrate \( K_M \) (the Michaelis constant). A decrease in \( K_M \) indicates stronger substrate binding and thus higher affinity. The turnover number, \( k_{\text{cat}} \), was equal to \( v_{\text{max}}/[	ext{cyt c}] \), and was the rate constant for the conversion of enzyme-substrate complex to product. The catalytic efficiency was equal to \( k_{\text{cat}}/K_M \).

The peroxidase activity assay employed here required two substrates, guaiacol and H$_2$O$_2$. In this case, H$_2$O$_2$ was kept in excess, and thus the observed \( K_M, v_{\text{max}}, \) and \( k_{\text{cat}} \) are apparent parameters with respect to guaiacol.

Michealis-Menten kinetics of HRP-PAA and Hb-PAA were obtained using the same method. Final activity assay concentrations were: [HRP] = 20 nM, [guaiacol] = 1-16 mM, [H$_2$O$_2$] = 0.2 mM, [sodium phosphate] = 20 mM; or [Hb] = 1 μM, [guaiacol] = 0.1-16 mM, [H$_2$O$_2$] = 50 mM, [sodium phosphate] = 20 mM.

Activity studies at various pH. Full kinetic assays were not completed to study the effect of pH or local crowding agents. Instead, conditions were selected where [guaiacol]>>\( K_M \) so that the initial rate could be seen as an approximation of \( v_{\text{max}} \). As such, activity studies were performed
follow the same method as the kinetic studies, with the following concentrations: [cyt c] = 1 μM, [guaiacol] = 200 μM, [H₂O₂] = 50 mM, and [sodium phosphate] = 20 mM. For pH dependent studies, the pH of sodium phosphate buffer was varied from 6-8. In all cases, activity studies were completed in triplicate.

2.5.9. Guaiacol partitioning studies

Partitioning of guaiacol to the polymer phase was estimated by examining the partitioning of guaiacol between chloroform and buffered aqueous solutions of poly(acrylic acid). 2 mM guaiacol was dispersed in 20 mM sodium phosphate, pH 7.0 in a glass vial, an equal volume of chloroform was added. For testing of partitioning to PAA phase, 10 μM PAA was also added to the phosphate buffer and guaiacol solution. The vial was capped and shaken vigorously, after which the chloroform and aqueous layers were allowed to separate. This was repeated three times, then the upper aqueous layer was removed. The concentration of guaiacol in the aqueous layer was determined from the UV/Vis spectrum using the extinction coefficient at 274 nm, ε = 2550 M⁻¹ cm⁻¹. Samples were prepared and analyzed in this manner in triplicate.

2.5.10. Estimation of Debye length

When ions are dispersed in solution, their charges can be screened by other charged species in the solution. The strength of electrostatic interactions with a charge decay approximately exponentially with distance, and the Debye length (κ⁻¹) is the characteristic length of this decay. The Debye length can be calculated theoretically using the equation:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_r \varepsilon_0 k_b T}{\sum n_i z_i^2 e^2}}$$

where $\varepsilon_r$ is the dielectric constant of the solvent, $\varepsilon_0$ is the permittivity of free space, $k_b$ is the Boltzmann constant, T is the temperature, e is the electronic charge, and $n_i$ and $z_i$ are the number density concentration and charge of the $i$th species in solution. This equation was employed to estimate the Debye length under the conditions of the kinetic assay (20 mM sodium phosphate,
pH 7.0, 20 °C). Contributions from carboxylate groups of PAA were neglected, as the concentration of these groups was one or more orders of magnitude smaller than that of the buffer ions in all cases (1 µM cyt c-PAA).

2.5.11. Heme bleaching studies

Bleaching of the cyt c heme was examined by addition of hydrogen peroxide to cyt c in the absence of guaiacol. 1 µM cyt c or cyt c-PAA was prepared in 20 mM sodium phosphate, pH 7.0. Solutions were stirred at 20 °C, and 50 mM H₂O₂ was added. The absorbance at 410 nm was monitored as a function of time after addition of peroxide.
2.6. Acknowledgements

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Chapter 3. Dramatic improvement in thermal and chemical stability of cytochrome c by conjugation to brush-like polymers

3.1. Abstract

Cytochrome c was conjugated to brush-like poly(norbornene)-graft-poly(acrylic acid) (PNPAA, PN DP = 5, PAA DP = 38, $M_n = 16.1$ kDa) by carbodiimide chemistry. Conjugation to brush-like PNPAA greatly restricts the enzyme conformational entropy, and was expected to significantly improve stability to thermal and chemical denaturation. Circular dichroism studies demonstrated the resulting cyt c-PNPAA conjugates could retain their secondary structure when maintained at 90 °C, well above the $T_M$ of cyt c. Additionally, cyt c-PNPAA did not denature even when exposed to 8.45 M urea at 45 °C, as monitored by tryptophan emission studies. Finally, cyt c peroxidase-like activity was enhanced by conjugation to PNPAA. Taken together, these data indicated that polymer conjugation resulted in dramatic stabilization of the protein without hampering the enzyme-like function.
3.2. Introduction

Enzymes are attractive catalysts for industrial and biomedical applications for their high catalytic efficiency and specificity. However, their utility is limited by their tendency to be denatured or otherwise inactivated by challenging environmental conditions, such as high temperature, acidic or basic pH, dehydration, or the presence of chemical denaturants such as organic solvents or surfactants.\textsuperscript{69,70} As such, considerable attention has been devoted to stabilizing enzymes to improve their longevity and recyclability. The most popular approaches include chemical modification of side chains, functionalization with synthetic polymers, directed evolution/protein engineering, encapsulation in or immobilization on nanoparticles, immobilization on surfaces, or immobilization in matrices or frameworks such as hydrogels or metal-organic frameworks.\textsuperscript{7,69–75} Each approach has its own advantages and limitations. For example, immobilization or encapsulation approaches may limit diffusion of substrate to the active site, thus reducing catalytic efficiency. Excessive chemical modification or functionalization can disrupt the structure of and access to the active site, again reducing enzymatic activity. Additionally, the immobilization techniques or modification chemistries must be carefully selected, as the conditions used for these reactions can disrupt the sensitive structure of the enzyme. Finally, care must be taken to avoid restricting the enzyme mobility too much, as the reduced structural dynamics can improve stability but may reduce turnover as well.\textsuperscript{76}

In our previous work, we have endeavored to stabilize enzymes by multi-point conjugation of poly(acrylic acid) (PAA) to various enzymes using carbodiimide chemistry.\textsuperscript{2,3,77} Our hypothesis is that the multiple crosslinks between enzyme and polymer restricts the number of microstates available to the denatured state, thus reducing the conformational entropy change associated with denaturation. This, in turn, increases the free energy of denaturation, making it more unfavorable and shifting the equilibrium towards the native state. Additionally, the negatively charged polymer shell helps to prevent aggregation of enzyme-polymer conjugates because of
electrostatic repulsion among PAA-conjugated molecules. We have also demonstrated that this approach can be extended to improve stability and recyclability of enzymes interlocked in cellulose, with or without PAA.\textsuperscript{78}

In the current work, we have improved the stability of enzyme-polymer conjugates by modifying the polymer morphology. We replaced linear PAA with the brush-like poly(norbornene)-\textit{graft}-poly(acrylic acid) (PNPAA), which has a short norbornene backbone (DP = 5) with 5 PAA grafts (DP = 38). Here, conjugation to multiple PAA chains that are linked to the same norbornene backbone is expected to further reduce the enzyme’s conformational entropy and rigidify its structure, thereby improving the stability against denaturing conditions. We demonstrated this principle by conjugation of cytochrome \textit{c} (cyt \textit{c}) to PNPAA using established carbodiimide chemistry methods. Cytochrome \textit{c} is a basic hemoprotein that can act as an electron shuttle and has peroxidase-like activity.\textsuperscript{49} The resulting cyt \textit{c}-PNPAA conjugates retained their native-like secondary structure, and their peroxidase activity was enhanced, in line with previously reported conjugates of cyt \textit{c} and anionic polyelectrolytes.\textsuperscript{10,77} Importantly, cyt \textit{c}-PNPAA could be heated to 90 °C without significant disturbance to its secondary structure, as demonstrated by circular dichroism spectroscopy, and could resist denaturation even in 8.5 M urea. Details are enumerated below.
3.3. Results and discussion

3.3.1. Synthesis and characterization of cyt c-PNPAA

PNPAA was prepared following reported methods, with a few modifications.\textsuperscript{79,80} The structure and composition of PNPAA was verified by gel permeation chromatography and nuclear magnetic resonance spectroscopy. The $M_n$ was 16.1 kDa (PDI = 1.27), consisting of a norbornene backbone (DP = 5) with 5 PAA grafts (3.2 kDa each).\textsuperscript{†} Cyt c-PNPAA conjugates were prepared using carbodiimide chemistry, similar to our previously reported enzyme-PAA conjugates (Scheme 3.1).\textsuperscript{2,77} Conjugates were routinely purified by dialysis or through the use of ultracentrifugation filters (15 kDa cutoff). Synthesis was validated by agarose gel electrophoresis (pH 7.0) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.1). In the agarose gel, cationic cyt c migrated toward the negative electrode, while cyt c-PNPAA migrated toward the positive electrode. This reversal in electrophoretic mobility indicated the presence of anionic PNPAA, rendering the conjugates negatively charged. Analysis by SDS-PAGE indicated an increase in molecular weight relative to native cyt c (12 kDa, lane 2). The majority of cyt c-PNPAA was excluded from the gel (lane 3), indicating formation of large crosslinked networks, as observed in previous enzyme-linear PAA conjugates.\textsuperscript{2,77}

\textsuperscript{†} PNPAA was prepared and characterized by Ian Martin (Kasi group, University of Connecticut).
Scheme 3.1. (A) Chemical structure of poly(norbornene)-graft-poly(acrylic acid) (PNPAA). (B) Schematic representation of cyt c-PNPAA synthesis.
Figure 3.1. (A) Agarose gel electrophoresis of cyt c-PNPAA indicated complete charge reversal following conjugation to PNPAA. (B) SDS-PAGE of cyt c and cyt c-PNPAA.
3.3.2. Thermal stability studies

The thermal stability of cyt c-PNPAA was assessed by circular dichroism (CD) spectroscopy. The UV CD spectrum is diagnostic of the protein secondary structure, and the transition from native folding state to the denatured state as a consequence of thermal denaturation can be clearly observed.\textsuperscript{81} Under standard conditions (25 °C, 1 atm, pH 7.0), cyt c has primarily α-helical structure,\textsuperscript{50} which was reflected by the negative peaks at 222 and 208 nm in the CD spectrum. Importantly, the CD spectrum of cyt c-PNPAA at 25 °C was similar to that of cyt c, indicating that conjugation to PNPAA did not significantly disturb the protein secondary structure (Figure 3.2). In all cases, the 208 nm band was weaker than the 222 nm band, which was indicative of monomeric cyt c.\textsuperscript{57}

Thermal denaturation of cyt c was observed by monitoring the CD spectrum with increasing temperature (Figure 3.3A). As the temperature was increased from 55 to 75 °C, the ratio of the 222 and 208 nm bands decreased until the intensity of the two features was roughly equal. This may demonstrate the formation of domain swapped oligomers, driven by thermal unfolding of the protein.\textsuperscript{57} When the temperature was further increased, definition of both α-helical features was lost while the ellipticity below 210 nm increased, which was attributed to denaturation of cyt c. Additionally, the ellipticity at 222 nm decreased sharply above 75 °C. On the other hand, such dramatic changes were not observed in the CD spectrum of cyt c-PNPAA, even at 90 °C (Figure 3.3B). From 40 to 90 °C, the ellipticity at 222 nm of cyt c-PNPAA only decreased by ~15%, compared to the ~60% loss observed for cyt c (Figure 3.3C). Additionally, the ratio of the 222 and 208 nm features did not vary as much as that of cyt c (Figure 3.3D). The denaturation temperature could not be calculated from the CD data given that the spectral changes indicate both denaturation and oligomerization, but taken together, these data clearly indicated that cyt c-PNPAA was stabilized against thermal denaturation.

Comparison of cyt c-PNPAA to cyt c conjugated to linear PAA (cyt c-PAA) revealed the improved stabilizing effects of the comb-like morphology of PNPAA (Figure 3.3E). With increasing
temperature, cyt c-PAA showed a steady decrease in ellipticity at 222 nm, losing ~33% of its initial value. Interestingly, the concomitant increase in ellipticity at 208 nm observed in cyt c did not occur in cyt c-PAA, indicating that PAA helped to prevent oligomerization of cyt c similar to our previous reports of catalase-PAA and hemoglobin-PAA.\(^2,3\) Unlike cyt c, cyt c-PAA still retained some residual α-helical structure at 95 °C, as the feature at 222 nm was not completely lost. However, cyt c-PAA showed more dramatic changes in the CD structure at elevated temperature compared to cyt c-PNPAA, qualitatively demonstrating the additional stabilization afforded by the brush-like polymer architecture.
Figure 3.2. Comparison of cyt c (black) and cyt c-PNPAA (red) UV circular dichroism spectra at 25 °C. Some changes in the relative intensities of the 222 and 208 nm peaks were observed, but cyt c-PNPAA still retained its mainly α-helical structure following conjugation.
Figure 3.3. (A) Changes in UV CD of cyt c with temperature (25-95 °C). The decrease in ellipticity at 222 nm ($\theta_{222}$) and increase in ellipticity at 208 nm ($\theta_{208}$) indicated formation of cyt c oligomers up to 75 °C. Denaturation and loss of secondary structure followed, as demonstrated by the loss of both features. (B) CD spectra of cyt c-PNPAA demonstrated minor changes over 40-90 °C. (C) Retention of $\theta_{222}$ during thermal denaturation studies of cyt c (black squares) and cyt c-PNPAA (red circles). Ellipticity was normalized relative to $\theta_{222}$ at 25 °C. (D) Variation of $\theta_{222}/\theta_{208}$ with temperature for cyt c (black squares) and cyt c-PNPAA (red circles). (E) CD spectra of cyt c conjugated with linear PAA at various temperatures (25-95 °C).
3.3.3. Chemical denaturation studies

We next conducted chemical denaturation studies to further probe the stability of cyt c-PNPAA. Urea is known to denature proteins by disrupting the hydrogen bonding interactions that stabilize the secondary and tertiary structure, and cyt c is denatured in the presence of \( > \sim 7 \text{ M} \) urea.\(^{82}\) The denaturation can be conveniently tracked by changes in fluorescence from cyt c aromatic residues (\( \lambda_{ex} = 280 \text{ nm}, \lambda_{em} = 340-350 \text{ nm} \)), particularly tryptophan (Trp59). In the folded state, emission of the lone Trp in cyt c is quenched because of its close proximity to the heme prosthetic group.\(^{50,83}\) As cyt c unfolds, the distance between the heme and Trp increases, leading to decreased quenching and an increase in Trp emission (Figure 3.4).\(^{83}\)

Denaturation of cyt c in the presence of urea was studied at 25, 30, 35, 40, and 45 °C at pH 7.4 (Figure 3.5A). The Trp fluorescence at 345 nm was normalized with respect to the maximum emission observed (8.45 M urea), and the denaturation curves were fit to a logistic function to determine the urea concentration at the midpoint of the denaturation. At 25 °C, Trp fluorescence began to increase with \( \sim 6 \) M urea, and the midpoint urea concentration was 7.34(±0.05) M. As the temperature was increased, the amount of urea required to denature cyt c decreased, with the midpoint reaching 6.82(±0.04) M at 45 °C.

The urea denaturation curves were also employed to assess the thermodynamics of cyt c denaturation. Because the Trp fluorescence is proportional to the relative populations of folded and unfolded cyt c, it can be used to calculate the conditional equilibrium constant, \( K_D \), for each concentration of urea using the following expression: 

\[
K_D = \frac{P_{min} - P_{obs}}{P_{obs} - P_{max}}
\]

where \( P_{max}, P_{min}, \) and \( P_{obs} \) are the minimum, maximum, and observed Trp fluorescence.\(^{82,84}\) The free energy change in the absence of urea, \( \Delta G^* \) was determined from a plot of \( K_D \) versus [urea] using the empirical expression 

\[
\Delta G_D = -RT \ln K_D = \Delta G^* - m[\text{urea}]
\]

where \( m \) is an empirical constant describing the variation of \( K_D \) with [urea] (Figure 3.5B).\(^{82,84}\) Thus, the \( \Delta G^* \) of denaturation was determined from the intercept of the plot of ln\( K_D \) versus [urea]. At 25 °C, \( \Delta G^* \) of denaturation for cyt c was 9.4(±0.4)

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\( \Delta G^* \) generally decreased with temperature, reaching \( 7.8(\pm0.4) \text{ kcal mol}^{-1} \) at \( 45 \, ^\circ \text{C} \), indicating cyt c was easier to denature at increased temperature. The equilibrium constant for denaturation in the absence of urea, \( K_{D^*} \), was also found from the intercept of the plot of \( \ln K_D \) versus [urea], and a van't Hoff plot was employed to determine the denaturation enthalpy, \( \Delta H_D \), and entropy, \( \Delta S_D \), at the median temperature of the experiment, \( 35 \, ^\circ \text{C} \) (Figure 3.5C). \( \Delta H_D \) was \( 28(\pm5) \text{ kcal mol}^{-1} \) and \( \Delta S_D \) was \( 65.8(\pm0.1) \text{ cal mol}^{-1} \text{ K}^{-1} \), again in good agreement with literature reports.\(^85,86\)

Urea induced denaturation of cyt c-PNPAA was similarly examined by Trp fluorescence. Interestingly, only a mild increase in Trp fluorescence was observed, even at \( 45 \, ^\circ \text{C} \) and \( 8.45 \text{ M} \) urea. Cyt c-PNPAA Trp fluorescence was normalized relative to the maximum emission noted for cyt c, and with \( 8.45 \text{ M} \) urea this only reached \( \sim20\% \) of the emission of fully denatured cyt c (Figure 3.5D). Thus, we concluded that cyt c-PNPAA could not be denatured by urea, and conjugation to brush-like PNPAA significantly stabilized the structure of cyt c.
Figure 3.4. Emission spectrum of cytochrome c (4 µM, 280 nm excitation) in the presence of increasing urea (20 mM sodium phosphate, pH 7.4).
Figure 3.5. (A) Urea denaturation curves of cyt c at 25-45 °C. (B) Determination of the reference free energy change, ΔG*, and denaturation equilibrium constant, K_D, of cyt c at 25-45 °C. (C) van't Hoff plot of cyt c denaturation. (D) Denaturation of cyt c-PNPAA was minimal at 45 °C, even in the presence of 8.45 M urea.
3.3.4. Enzymatic activity studies

Enzyme-PNPAA and enzyme-PAA conjugates are stabilized by multi-point attachment of the polymer to the enzyme, restricting the conformational entropy in an effort to make denaturation more unfavorable. This stabilization thus comes with reduced structural dynamics, which could negatively affect the enzymatic activity.\(^{76}\) With this in mind, we assessed the peroxidase activity of cyt c by tracking the oxidation of guaiacol in the presence of hydrogen peroxide (Scheme 3.2). Progress of the reaction can be tracked by the absorbance of the product 3,3'-dimethoxy-4,4'-biphenoquinone at 470 nm. Activity studies were carried out at room temperature and pH 7.0, with 50 mM H\(_2\)O\(_2\) and 100 µM guaiacol, which was well above the reported K\(_M\) of cyt c (~10 µM guaiacol) or any previously reported cyt c-PAA derivatives.\(^{53,77}\) Consistent with our previous findings, where conjugation of PAA to cyt c improves peroxidase activity by modulating the local pH and suppressing wasteful intermediates, cyt c-PNPAA exhibited enhanced activity relative to cyt c (Figure 3.6). Comparing the initial rates, the activity of cyt c-PNPAA was 530(±70)% greater than that of cyt c under these conditions. Thus, conjugation to PNPAA provided enhanced stability while simultaneously enhancing cyt c activity in a similar manner to our previously reported linear PAA conjugates.\(^{77}\)
Scheme 3.2. Cytochrome c catalyzes the oxidation of guaiacol in the presence of hydrogen peroxide. The reaction progress is tracked by appears of 3,3'-dimethoxy-4,4'-biphenoquinone, which has an absorbance maximum at 470 nm.
Figure 3.6. Guaiacol oxidation kinetic traces of cyt c (blue) and cyt c-PNPAA (red). Activity study conditions were 1 µM cyt c or cyt c-PNPAA, 100 µM guaiacol, 50 mM H$_2$O$_2$, 20 mM sodium phosphate, pH 7.0.
3.4. Conclusions

Cyt c-PNPAA conjugates were successfully synthesized using carbodiimide chemistry. We hypothesized that multi-site attachment to the brush-like polymer would restrict the conformational entropy of cyt c-PNPAA, and thus further enhance the stability of cyt c-PNPAA relative to the conjugates prepared with linear PAA. In thermal denaturation studies, cyt c-PNPAA retained its secondary structure at 90 °C, where cyt c was completely denatured and cyt c-PAA lost a significant amount of its α-helicity. When exposed to 8.45 M urea, cyt c was denatured while cyt c-PNPAA retained its structure, as demonstrated by tryptophan fluorescence. Taken together, these data demonstrated that multi-site conjugation to brush-like PNPAA significantly improved stability of cyt c towards both thermal and chemical denaturation. Critically, the improved stability did not come at the expense of the enzymatic activity, as conjugation to PNPAA lead to a ~5-fold enhancement in cyt c peroxidase activity, consistent with previous reports of cyt c-PAA conjugates. Thus, conjugation of enzymes to brush-like polymers is an attractive strategy to stabilize enzymes for use in aggressive conditions.
3.5. Materials and Methods

3.5.1. Materials

Cytochrome c was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). Sodium phosphate, Brilliant blue R250, bromophenol blue, guaiacol, agarose Type I (low EEO), glycine, acetic acid, isopropanol, acrylamide, sodium dodecyl sulfate, and trinitrobenzene sulfonic acid (5% w/v in water) were purchased from Sigma-Aldrich (St. Louis, MO). Tris base, N,N'-methylenebis(acrylamide), ammonium persulfate, tetramethylenediamine, and 2-mercaptoethanol were purchased from Thermo Fisher Scientific (Waltham, MA). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from TCI America (Portland, OR). Dialysis membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA).

3.5.2. Synthesis of enzyme-PNPAA conjugates

Protein-enzyme conjugates were prepared per previously reported methods, with a few minor modifications. First, activated poly(acrylic acid) solution was prepared by stirring PNPAA (10 μM) with EDC (5 mM) in sodium phosphate buffer (20 mM pH 7.0) for 10 minutes. Next, cyt c (10 μM) was added while stirring, and the resulting mixture was reacted at room temperature for four hours. The EDC reaction byproducts were removed by dialysis against 20 mM sodium phosphate, pH 7.0, using a 15 kDa cutoff membrane.

3.5.3. Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose (0.5% w/v) in Tris-acetate buffer (40 mM, pH 7.0). Solutions were heated in the microwave for 30 s to dissolve agarose. Samples were prepared for electrophoresis by combining 20 μL of 10 μM cyt c or cyt c-PAA solution with 10 μL of loading buffer (50% v/v glycerol, 0.01% w/v bromophenol blue). Gels were run in a horizontal gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc., Grand Island, NY) for 30 minutes, with 100 V constant voltage and using 40 mM Tris acetate, pH 7.0 as the running buffer.
Gels were stained for at least four hours with an aqueous solution of 20% v/v acetic acid and 0.03 g L\(^{-1}\) Brilliant blue R250, then destained for four hours with 10% v/v acetic acid prior to imaging.

3.5.4. SDS-PAGE

12.5% w/w acrylamide separating gels and 5% w/w acrylamide stacking gels were used for SDS-PAGE. The separating gel was prepared by combining 4.2 mL 30% acrylamide solution (18.75 g acrylamide, 0.5 g N,N'-methylenebis(acrylamide), 64 mL DI water), 2.5 mL 4x lower gel buffer (9.35g Tris base, 0.2 g SDS, 50 mL DI water, pH 8.8), 3.3 mL DI water, 50 μL 10% ammonium persulfate, and 5 μL tetramethylenediamine. The stacking gel was prepared by combining 0.85 mL 30% acrylamide solution, 1.25 mL 4x upper gel buffer (3.025 g Tris base, 0.2 g SDS, 50 mL DI water, pH 6.8), 2.9 mL DI water, 25 μL 10% ammonium persulfate, and 5 μL tetramethylenediamine. The separating gel was poured into a 1 mm mold and allowed to polymerize for 30 minutes, after which the stacking gel was poured and similarly allowed to polymerize.

Samples were prepared by combining 20 μL of ~50 μM cyt c with 15 μL SDS-PAGE loading buffer (2% w/v SDS, 10% w/w 2-mercaptoethanol), after which the solutions were heated at 90 °C for 2 minutes. 15-20 μL of sample solution was loaded per well. Gels were run in a vertical Bio-Rad Mini-PROTEAN electrophoresis apparatus at 150 V for 15 minutes, then 200 V until the dye front reached the bottom of the gel (~20 minutes). The running buffer was 3.03 g L\(^{-1}\) Tris base, 14.41 g L\(^{-1}\) glycine, and 1 g L\(^{-1}\) SDS. The gel was stained with an aqueous solution of 10% v/v acetic acid, 10% v/v isopropanol, and 0.02 g L\(^{-1}\) Brilliant blue R250 for four hours, then with an aqueous solution of 20% v/v acetic acid and 0.03 g L\(^{-1}\) Brilliant blue R250 for four hours. Gels were destained with 10% acetic acid prior to imaging.

3.5.5. Circular dichroism spectroscopy

Circular dichroism spectra were measured with a JASCO J-710 spectropolarimeter. UV CD spectra were scanned from 195-260 nm using a 0.05 cm path length quartz cuvette and the
following parameters: 1 nm data pitch, continuous scanning mode, 1 second response speed, 2
nm bandwidth, 6 scans per spectrum. For thermal denaturation studies, 1 μM cyt c was prepared
in 20 mM sodium phosphate, pH 7.0 and 1 cm pathlength cuvette was used. The temperature
was controlled using a sample cell connected to a thermostat-controlled water heater. Spectra
were first measured at 25 °C, then heated to the desired intervals. Solutions were retained in the
cell for the duration of the experiment, and maintained at each temperature analyzed for 5 minutes
before measuring the spectrum.

3.5.6. Urea denaturation

Cytochrome c (4 μM) was combined with 0-8.45 M urea in 20 mM sodium phosphate, pH
7.4. Tryptophan (Trp) fluorescence was collected in a FLEXstation 3 plate reader with the
following settings: 280 nm excitation, 325-500 nm monitoring, 325 nm cutoff filter, low PMT
sensitivity. Urea denaturation curves were prepared using normalized Trp fluorescence, \( \frac{I_{\text{obs}} - I_{\text{min}}}{I_{\text{max}} - P_{\text{min}}} \),
where \( I_{\text{max}}, I_{\text{min}}, \) and \( I_{\text{obs}} \) are the maximum, minimum, and observed Trp emission intensities. The
midpoint urea concentration, where denaturation was 50% complete, was determined by fitting
the denaturation curves to a logistic function: \( I_{\text{norm}} = \frac{1}{1 + e^{-k([\text{urea}] - [\text{urea}]_0)}} \) where \( I_{\text{norm}} \) is the
normalized Trp emission, \([\text{urea}]_0\) is the midpoint urea concentration, and \( k \) is a parameter related
to the steepness of the curve.

3.5.7. Enzymatic activity studies

Solutions containing cyt c or cyt c-PNPAA, guaiacol, and sodium phosphate buffer (pH
7.4) were prepared and placed in a 1 cm glass cuvette. Absorbance at 470 nm (A470) was
monitored as a function of time using a HP 8453 UV/visible spectrophotometer. Once a stable
baseline was established, \( \text{H}_2\text{O}_2 \) was injected into the cuvette while stirring at 1000 RPM, and the
change in A470 was recorded for 1 minute. The final concentrations of reactants were: \([\text{cyt c}] = 1 \mu\text{M}, \ [\text{guaiacol}] = 100 \mu\text{M}, \ [\text{H}_2\text{O}_2] = 50 \text{mM}, \) and \([\text{sodium phosphate}] = 20 \text{mM}. \) The activity assay
was performed in triplicate at 20 °C and atmospheric pressure for each concentration of guaiacol
tested. Initial rates were obtained from the slope of the linear portion of the kinetic traces, where the change in absorbance with respect to time was the greatest. These conditions were selected where [guaiacol] >> $K_M$ so that the initial rate could be seen as an approximation of $v_{\text{max}}$. 
3.6. Acknowledgements

This work was supported by NSF EAGER Award DMR-1441879 and by UCONN VPR Research Excellence Award. The authors thank Ian Martin for synthesis and characterization of PNPAA, as well as Julie Taing, Shelby McCormick, and Joseph Gorecki for technical support.
Chapter 4. Protein BioPhosphors: Multi-functional, Protein-based Hydrogel for White Emission, Sensing, and pH Detection

4.1. Abstract

A highly efficient, multi-functional, bioderived white light emitting hydrogel (biophosphor) consisting of crosslinked bovine serum albumin (BSA) and three fluorescent dyes, Coumarin 460 (C460), fluorescein (F), and 5(6)-carboxy-x-rhodamine (ROX) is reported here. White light emission was obtained upon excitation of the biophosphor at 365 nm with appropriate mole ratios of the above dyes. The CIE 1931 chromaticity coordinates of white emission with 365 nm excitation were (0.36, 0.37) and the correlated color temperature was 5300 K. Multi-functional nature of the biophosphor was also demonstrated. A UV LED (361 nm) coated with the above biophosphor, for example, indicated white emission (CIE 0.28, 0.31), with a half-life of 106(±5) h. The white emission was also highly sensitive to pH over a broad range (pH 1-11). Incorporation of glucose oxidase and peroxidase in the biophosphor allowed for the detection of glucose over a physiologically relevant range of 1.8 mg dL\(^{-1}\) to 288 mg dL\(^{-1}\). This is a unique advanced biophosphor with LED and sensing applications, and it is the first example of a multi-functional, proteinaceous white emitter.
4.2. Introduction

The design and synthesis of an inexpensive and efficient white light emitting hydrogel of bovine serum albumin (BSA) embedded with blue (Coumarin 460, C460), green (fluorescein, F), and red (5(6)-carboxy-x-rhodamine, ROX) emitting dyes is reported here. White light generation is of current interest in response to the demands of biodegradable materials for the lighting and display industries. Currently, white light emitters include nanomaterials, quantum dots, polymers, metal-organic frameworks, inorganic-organic hybrids, metal complexes, lanthanide doped phosphors, and organic fluorophores. Each material has its associated challenges, such as efficiency, cost, ease of synthesis, biodegradability, and toxicity. In particular, balancing the molar absorptivity and quantum efficiency of emissive components is a significant challenge for systems which combine direct emission and sensitized emission via Förster resonance energy transfer (FRET) to produce white light.

Hydrogels are hydrophilic polymer networks, and are emerging as versatile new matrices for high efficiency generation of white light using inter-molecular energy transfer processes. The gel matrix improves energy transfer efficiency by rigidifying the locations of the donor-acceptor pairs and preventing their aggregation which can lead to quenching. Despite the advantageous properties of white emitting hydrogels, implementation in a functional device and photostability was not systematically studied, and biodegradability has not been demonstrated. Additionally, white emitting protein-based hydrogels are not known other than a report of a white emitting gelatin hydrogel with chromaticity coordinates [0.26, 0.33], far from being coordinates of pure white emission [0.33, 0.33]. To the best of our knowledge, there are no reports of a multifunctional, non-toxic, biodegradable, white emitting protein hydrogel.

BSA is inexpensive and readily available as a waste product of the meat industry. BSA has a large number of primary amines (59 lysine) and carboxylic acids (99 aspartic acid/glutamic acid), which can be crosslinked under controlled conditions by carbodiimide chemistry to form a
network of amide bonds without disrupting the intricate secondary structure of the protein. Protein secondary structure plays an important role for dye binding at the intended site and for enzyme activity retention, when enzymes are incorporated in the matrix for sensing or catalytic applications. We envisioned that this molecular network of BSA would result in a water-rich hydrogel with discrete binding sites for dye binding which would be suitable for the construction of a white emitting gel.

Previously reported BSA gelation strategies included crosslinking of BSA with polymers, thiol-ene click chemistry, and glutaraldehyde crosslinking. The current strategy for BSA gelation is advantageous with respect to these earlier methods because no prior modification of BSA is required. The crosslinking chemistry used here has been proven to be benign for the retention of native-like structures of biological macromolecules. The retention of the native-like structure of proteins/enzymes is essential to realize the anticipated binding of the dyes and to retain the catalytic activities of enzymes when incorporated in the matrix. Finally, the amide bonds that make-up the hydrogel are stable to hydrolysis yet biodegradable by proteases, which are ubiquitous in the environment. Biodegradability is a significant challenge for LED coatings, particularly among rare earth-doped inorganic phosphors, and this is an important aspect of our biological materials design.
Scheme 4.1. BSA hydrogel formed by crosslinking protein loaded with Coumarin 460 (blue), fluorescein (green), and (5,6)carboxy-x-rhodamine (red) dyes. The amide bond crosslinks are indicated by purple lines. The CIE 1931 chromaticity coordinates were (0.36, 0.37) when excited at 365 nm. The material was used for LED coating (361 nm LED) to produce a white LED. Emission of the biophosphor gel was sensitive to pH over a broad range (1-11). Incorporation of enzymes glucose oxidase and peroxidase permitted specific detection of glucose when white emission was quenched by the enzymatic reaction product.
We hypothesized that BSA would be an ideal host for the dense packing of red, green and blue dyes due to its ability to bind numerous dyes at discrete, single occupancy binding sites. Such binding sites would control inter-dye distances, while the moderate to high affinity binding of the dyes to the protein would also translate to high local concentrations without dye aggregation or self-quenching. Control over inter-dye distances will be useful to attenuate FRET and generate simultaneous emission from all three dyes. When appropriately proportioned, these three primary emission colors will produce white light emission, as sensed by the human eye.

A simple method to prepare a white emitting BSA hydrogel (biophosphor) is described here. White light was generated when excited at 365 nm by using an optimum mixture of C460 to produce blue emission, F to produce green emission, and ROX to produce red emission. These dyes bind to pristine BSA with moderate to high affinities at discrete, single occupancy binding sites and have high fluorescence quantum yields (Table 4.1). The resulting biohydrogel was suitable for LED coatings. Because white emission originates from three different dyes, any changes in intensities of one of the three emission components would generate off-white emission. This sensitivity to the local environment in the biohydrogel was exploited to construct a small molecule sensor for glucose and a pH sensor (pH 1 to 11), which demonstrated the versatility of the novel biohydrogel (Scheme 4.1). Thus, white emissive, bioderived, stimuli-responsive, sensing hydrogel from inexpensive, environmentally friendly, sustainable and earth-abundant elements was produced, as enumerated below.
<table>
<thead>
<tr>
<th></th>
<th>Coumarin 460</th>
<th>Fluorescein</th>
<th>(5,6)carboxy-x-rhodamine</th>
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</thead>
<tbody>
<tr>
<td>$\varepsilon$ ($\text{cm}^{-1} \text{M}^{-1}$)</td>
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<td>$76900$ at $490$ nm (pH$&gt;$6)$^{23}$</td>
<td>$36000$ (580 nm, aqueous buffer)$^{104}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$58300$ (sodium phosphate, pH 7.0)$^\dagger$</td>
</tr>
<tr>
<td>$\lambda_{\text{em}}$ (nm)</td>
<td>$456$ (H$_2$O), $451$ (EtOH)$^{105}$</td>
<td>$514$ (sodium phosphate)$^{23}$</td>
<td>$604$ (5-ROX), $605$ (6-ROX)$^{104}$</td>
</tr>
<tr>
<td>$\phi_f$</td>
<td>$0.055$ (H$_2$O), $0.73$ (EtOH)$^{105}$</td>
<td>$0.93$ $^{23}$</td>
<td>$0.94$ (5-ROX), $0.96$ (6-ROX)$^{104}$</td>
</tr>
<tr>
<td>$K_b$ ($\text{M}^{-1}$ at pH 7)</td>
<td>$8.1 \times 10^5$ (human serum albumin)$^{21}$ $7(\pm 1) \times 10^5$ M$^{-1}$$^\dagger$</td>
<td>$2.8 \times 10^4$$^{22}$ $4.0(\pm0.5) \times 10^4$ $^\dagger$</td>
<td>$7.1(\pm0.6) \times 10^4$ M$^{-1}$$^\dagger$</td>
</tr>
<tr>
<td>Binding site</td>
<td>Site I, Site II$^{21}$</td>
<td>Site I$^{102}$</td>
<td>N/D</td>
</tr>
</tbody>
</table>

$^\dagger$ Presented in the current work.

**Table 4.1.** Dye photophysical properties and BSA binding constants.
4.3. Results and Discussion

4.3.1. Synthesis of BSA hydrogel

BSA was crosslinked in deionized water at ~25 °C using a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). EDC is used to form amide bonds between the amine groups of lysine side chains and carboxyl groups of aspartic/glutamic acid residues. This intricate 3-dimensional network of BSA resulted in a transparent hydrogel with >80% water (w/w) (Scheme 4.2).

Gelation conditions were optimized to be 175 mg mL\(^{-1}\) BSA (2.64 mM) and 100 mM EDC in order to achieve short gelation time and low swelling by systematically varying EDC and BSA concentrations over range of 100 to 200 mg mL\(^{-1}\) (1.51 to 3.02 mM) BSA and 60 to 200 mM EDC (Table 4.2, Figure 4.1A). Gelation was confirmed by the gel inversion test, where the solutions flowed under these conditions while hydrogels did not.\(^{106}\) At ≤150 mg mL\(^{-1}\) (2.26 mM) BSA or ≤80 mM EDC, several hours were required for gelation. The gelation time decreased with increasing concentrations of these reagents, reaching 40 minutes or less at ≥185 mg mL\(^{-1}\) (2.79 mM) BSA with 100 mM EDC or ≥150 mM EDC with 175 mg mL\(^{-1}\) (2.64 mM) BSA. There was no phase separation or precipitation. Additionally, hydrogels were transparent at all concentrations of EDC or BSA used here, which is essential for making efficient light converting materials. All future studies were conducted using 175 mg mL\(^{-1}\) (2.64 mM) BSA and 100 mM EDC.
Scheme 4.2. White emitting BSA hydrogel was formed by crosslinking the protein with a water-soluble carbodiimide (EDC). Blue (Coumarin 460), green (fluorescein), and red (5(6)-carboxy-x-rhodamine) dyes were embedded during gelation.
<table>
<thead>
<tr>
<th>[BSA] (mg mL⁻¹)</th>
<th>[EDC] (mM)</th>
<th>Gelation time (minutes)</th>
</tr>
</thead>
<tbody>
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<td>175</td>
<td>60</td>
<td>336(±5)</td>
</tr>
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<td>175</td>
<td>80</td>
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</tr>
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<td>175</td>
<td>100</td>
<td>70(±1)</td>
</tr>
<tr>
<td>175</td>
<td>120</td>
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<td>200</td>
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<tr>
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<td>185</td>
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</tr>
<tr>
<td>200</td>
<td>100</td>
<td>33(±1)</td>
</tr>
</tbody>
</table>

**Table 4.2.** Gelation time as a function of EDC or BSA concentration.
Figure 4.1. (A) Gelation time decreased with increasing [BSA] (100 mM EDC) and with increasing [EDC] (175 mg mL\(^{-1}\) BSA). Samples were prepared in triplicate, and some error bars are too small to see. (B-C) Swelling of BSA gel as a function of BSA concentration (B) and EDC concentration (C). The equilibrium swelling ratio (inset) varied inversely with concentration of EDC and was independent of BSA concentration. (D) The shear storage (G') and loss (G'') moduli of the biophosphor were measured as a function of angular frequency. G' was greater than G'' and both were independent of angular frequency, indicating an elastic response typical of hydrogels.
4.3.2. Characterization of the hydrogel

Swelling studies were used to qualitatively estimate the crosslink density of the gel. The extent of swelling of a chemically crosslinked hydrogel in contact with water is inversely related to its crosslink density, as described by the Flory-Rhener swelling equation. Therefore, water uptake after immersing vacuum-dried gels in water was observed, and the change in mass as a function of soaking time was noted until no further water uptakes was observed. The degree of swelling was calculated as \( \frac{m_{\text{swollen}} - m_{\text{dry}}}{m_{\text{dry}}} \).

The degree of swelling decreased from 7.0(±0.8) to 1.6(±0.13) as [EDC] was increased at 175 mg mL\(^{-1}\) of BSA, with little change at >100 mM EDC (Figure 4.1B-C). Reduced degree of swelling indicated qualitatively that increasing [EDC] lead to a greater degree of crosslinking in the gel. In contrast, the degree of swelling after 70 hours demonstrated no change when [BSA] was varied (100 mM EDC). Thus, hydrogels with low degree of swelling were obtained at [EDC] ≥ 100 mM, a key design parameter to ensure hydrogel phosphors would not be sensitive to relative humidity changes. Taken together with the gelation time data, 175 mg mL\(^{-1}\) BSA and 100 mM EDC gave near minimum equilibrium swelling with about one hour gelation time, and these conditions were used for all further studies.

To further probe the mechanical properties of the biophosphor, the shear storage (G’) and loss (G”) moduli were measured as a function of angular frequency (Figure 4.1D). Samples for rheological measurements were prepared with 175 mg mL\(^{-1}\) BSA and 100 mM EDC. Both G’ and G” were independent of angular frequency up to 10 rad s\(^{-1}\), a typical response of hydrogels which indicates an elastic material. Additionally, G’ was greater than G”, again indicating a dominantly elastic response to the applied shear force. Taken together with the inversion tests, these evidences help confirm the formation of an elastic hydrogel.

The morphology of the hydrogel was examined by scanning electron microscopy (SEM). Gels formed with 175 mg mL\(^{-1}\) BSA and 100 mM EDC were flash frozen in liquid nitrogen,
lyophilized, and ground to a powder with mortar and pestle prior to imaging. SEM micrographs of the flash frozen swollen gel indicated a honeycomb-like structure with pores approximately 5 to 20 µm in diameter (Figure 4.2A-B), clearly demonstrating the porous nature of the biophosphor gel. These were compared to SEM micrographs of BSA hydrogels that were slowly dried under vacuum at room temperature, which indicated a monolithic, nearly featureless solid (Figure 4.2C-D). Slow drying of hydrogels at ambient temperature without flash freezing causes the pores to collapse,96 which was observed here. Thus, the vacuum dried gel for LED coating had a collapsed structure, while the porous swollen gel was suitable for sensing applications which require diffusion of an analyte into the matrix.
Figure 4.2. (A,B) Scanning electron microscopy micrographs of swollen BSA gel (175 mg mL\(^{-1}\) BSA, 100 mM EDC) after flash freezing and lyophilization clearly indicate the porous nature of the hydrogel. (C,D) Scanning electron microscopy micrographs of BSA gel (175 mg mL\(^{-1}\) BSA, 100 mM EDC) after drying under vacuum at room temperature. Slow drying under caused the pores to collapse.
4.3.3. White emitting biophosphor compositions

The white emitting biophosphor film was produced by embedding suitable concentrations of three fluorescent dyes, C460 ($\lambda_{\text{ex}}=370$ nm, $\lambda_{\text{em}}=422/430$ nm), F ($\lambda_{\text{ex}}=506$ nm, $\lambda_{\text{em}}=540$ nm), and ROX ($\lambda_{\text{ex}}=578$ nm, $\lambda_{\text{em}}=602$ nm) (Figure 4.3A). Overlap between donor emission and acceptor absorbance is an important prerequisite for efficient FRET, and the absorbance and emission spectra of C460, F, and ROX in the BSA gel illustrated that both C460-F and F-ROX could be donor-acceptor pairs (Figure 4.3B). Additionally, the dyes had reasonable affinity for BSA. The association constant of ROX to BSA was measured by fluorescence polarization anisotropy (FPA) to be $7.1(\pm0.6) \times 10^4$ M$^{-1}$, (Figure 4.3C-E) which was not previously reported in the literature. The association constants of F and C460 to BSA were also measured by FPA to validate the methodology, and found to be $4.0(\pm0.5) \times 10^4$ M$^{-1}$ (F) and $7(\pm1) \times 10^5$ M$^{-1}$ (C460), which are in good agreement with literature reports.\textsuperscript{21,22}

White emission was obtained by adjusting the mole ratios of the three dyes and two optimum conditions were obtained (Figure 4.4A, Table 4.3). Gels suitable for LED applications required vacuum drying at room temperature and a composition of 75 µM C460, 450 µM F, and 22 µM ROX to produce white light when excited at 365 nm, a common wavelength of a UV LED (Figure 4.4B, solid line). The emission contained contributions from each of the chromophores, with peaks at 422/430, 540, and 602 nm corresponding to C460, F, and ROX.
Figure 4.3. (A) Chemical structures of Coumarin 460 (C460), fluorescein (F), and 5(6) carboxy-x-rhodamine (ROX). (B) Overlap of absorbance (solid lines) and emission (dashed lines) of C460 (blue), F (green), and ROX (red) in vacuum dried biophosphor. The absorbance and emission intensity were normalized to 1 at their maximum value. (C-E) Fluorescence polarization anisotropy titration of fluorescein (A), 5,6-carboxy-x-rhodamine (B), and Coumarin 460 (C) by increasing [BSA] (up to 128 μM). [Dye] was fixed at 10 μM. The binding constant of F to BSA was 4.0(±0.5)x10^4 M^-1, while that of ROX to BSA was 7.1(±0.6)x10^4 M^-1 and that of C460 to BSA was 1.4(±0.2)x10^5 M^-1.
Figure 4.4. (A) Example of emission spectra (365 nm excitation) obtained during dye concentration optimization. C460, F, and ROX concentrations were set initially based on the ratios of their molar absorptivities and quantum yields, and BSA and EDC concentrations were 175 mg mL$^{-1}$ and 100 mM. (B) Dashed emission spectrum of C460, F, and ROX in vacuum dried biophosphor was obtained by assuming no FRET and summing individual dye spectra. Sensitization of green and red emitters indicated FRET when compared to biophosphor emission (solid line).
Table 4.3. An example set of chromaticity data from white emission optimization experiments for hydrated, *de novo* gels. Chromaticity coordinates were calculated from the emission spectra (365 nm excitation, examples in Figure S3). Dye concentrations were set initially based on the ratios of their molar absorptivities and quantum yields, and the relative amounts of the blue (C460), green (F), and red (ROX) were adjusted based on the chromaticity coordinates until white emission was reached (coordinates close to [0.33, 0.33]).

<table>
<thead>
<tr>
<th>[BSA] (mg mL⁻¹)</th>
<th>[EDC] (mM)</th>
<th>[C460] (µM)</th>
<th>[F] (µM)</th>
<th>[ROX] (µM)</th>
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<td>75</td>
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</table>
White emitting gels suitable for wet applications such as small molecule detection or pH sensing were made with 30 µM C460, 450 µM F, and 75 µM ROX and were used de novo without drying. These also gave white emission when excited at 365 nm. (Figure 4.5). The difference in dye concentrations required for white emission from hydrated and dehydrated gels was attributed to differences in FRET efficiency in the two types of gels. The emission of each dye in the vacuum dried gel was more intense when compared to the de novo gel because removal of water from the matrix reduced the total volume and increased the effective concentration of dyes in the vacuum dried gels.101

Next, we examined the extent of covalent attachment of the dyes to BSA, as the dyes were added to the reaction mixture before EDC crosslinking. The degree of covalent labeling was determined by dialyzing the powdered gel prior to trypsin digestion. The mole percent (100x[moles of dye attached/total moles of dye]) of dye attached to the gel was determined by absorbance (Figure 4.6), and 4.4(±0.3)% of F and 60(±4.8)% of ROX were covalently linked to the gel. C460 does not have a carboxylate or a reactive amino group to link with the protein side chains, so C460 was non-covalently associated with the gel by hydrophobic effects and hydrogen bonding interactions.21

Finally, the reversible association of dyes with the gel was demonstrated by dye leeching experiments. Gels were incubated at room temperature in 12-fold excess volume of phosphate buffer, pH 7.0. Under these conditions, there was some release of the dyes from the gel and the emission spectrum of the supernatant revealed two peaks for Coumarin 460, one at ~420 nm and another at ~455 nm, in addition to those of F (540 nm) and ROX (602 nm) (Figure 4.7A). The degree of leeching could not be quantitated due to the overlap of multiple spectra from various species (Figure 4.7B), but the leeching experiment indicated that a fraction of the dye molecules are bound to the gel via non-covalent interactions.
**Figure 4.5.** (A) Emission spectrum (365 nm excitation) of hydrated, *de novo* biophosphor containing 175 mg mL\(^{-1}\) BSA, 100 mM EDC, 30 µM C460, 450 µM F, and 75 µM ROX. (B) Excitation spectrum (630 nm monitoring) of hydrated, *de novo* biophosphor.
Figure 4.6. (A) Absorbance spectra of 5,6-caboxy-x-rhodamine (ROX, 1-16 μM) in 20 mM sodium phosphate, pH 7.0. (B) The molar extinction coefficient of ROX at 575 nm was determined from the slope the plot of absorbance vs. concentration. Solutions were prepared in triplicate, and some error bars are smaller than the points. (C) Schematic representation of tryptic digestion of the biophosphor used to assess the extent of covalent attachment of fluorescein (F) and 5,6-carboxy-x-rhodamine (ROX). The vacuum dried sample was ground to powder, dialyzed to remove non-covalently bound dyes, then digested by trypsin for 4 hours at 37 °C. The absorbance of the resulting solution was used to calculate F and ROX concentrations.
Figure 4.7. (A) Emission spectrum of supernatant over BSA hydrogel (175 mg mL\(^{-1}\) BSA, 100 mM EDC, 75 μM C460, 450 μM F, 22 μM ROX) incubated at room temperature in 12.5 fold excess volume of 20 mM sodium phosphate, pH 7.0. Coumarin peaks at ~420 nm and ~455 nm demonstrated the presence of free and bound species in solution, indicating release of loosely associated BSA from the gel in addition to the dyes. (B) Emission spectrum of 10 μM C460 in 20 mM sodium phosphate, pH 7.0 in the presence of 0 to 64 μM BSA. The arrow indicates the change in [BSA]. As [BSA] increased, the emission was blue shifted and the fluorescence intensity increased, demonstrating that the quantum yield of bound C460 is greater than that of C460 free in solution.
4.3.4. Chromaticity coordinates

CIE 1931 color space provides a quantitative measure of how visible light is perceived by the human eye and are therefore employed to assess the quality or “whiteness” of emissive materials. Emission color is represented by chromaticity coordinates in CIE 1931 color space, which are derived by weighted integration of the blue, green, and red components of the emission spectrum.\textsuperscript{110} The ideal (X, Y) coordinates of pure white light are (0.33, 0.33). The biophosphor emission (365 nm excitation, \textbf{Figure 4.8A}, solid line) was used to calculate the chromaticity coordinates, and these were (0.36, 0.37), indicating white light.\textsuperscript{110} These results are comparable to other white light emitting hydrogels, such as poly(aryl ether) dendron-based gel [(0.33, 0.32)],\textsuperscript{90} nanoclay hydrogels [(0.33, 0.32)],\textsuperscript{91} melamine derived hydrogel [(0.31, 0.36)],\textsuperscript{88} a pyridinium hydrogel [(0.36, 0.35), (0.33, 0.39)],\textsuperscript{111,112} and a gelatin hydrogel [(0.26, 0.33)].\textsuperscript{87}

The correlated color temperature (CCT) of a light source, defined as the temperature of a blackbody radiator emitting the same hue of light as the source, was calculated using the chromaticity coordinates.\textsuperscript{113,114} The CCT of the biophosphor film was 5300 K, corresponding to cool white light (4200-6500 K).\textsuperscript{115}
4.3.5. Fluorescence studies and FRET

Fluorescence emission spectrum of the biophosphor was compared to the sum of the spectra of gels containing C460, or F, or ROX alone to examine the role of FRET in white emission. The contribution of emission from C460 (422/430 nm) was diminished while the emission from F (540 nm) and ROX (602 nm) was enhanced by 78% and 370% in the biophosphor (Figure 4.8A, solid line, 365 nm excitation) when compared to the summed spectrum (Figure 4.8A, dashed line). The increased intensities of F and ROX peaks indicated energy absorbed by C460 was emitted by F or ROX, providing evidence for energy transfer from excited states of C460.

The biophosphor excitation spectrum was also compared to the sum of the spectra of single dye gels containing C460, or F, or ROX. The biophosphor spectrum had peaks corresponding to the absorption spectra of C460 at 370 nm and F at 512 nm in addition to a peak for direct excitation of ROX at 580 nm (Figure 4.8B, solid line, 630 nm monitoring). The position of dye peaks in the excitation spectrum were in good agreement with the biophosphor absorption spectrum (Figure 4.8C). Taken together with the emission data, this provided strong evidence for energy transfer from the excited state of C460 to the ground state of F (C460→F), from the excited state of F to the ground state of ROX (F→ROX), and from excited state of C460 to ROX via the intermediary F (C460→F→ROX). The difference between the summed spectrum of the component dyes (Figure 4.8B, dashed line) and that of the biophosphor can be readily accounted for by the contributions of FRET in the biophosphor film. The importance of BSA was demonstrated by control experiments in which the emission spectrum of a mixture of 75 µM C460, 450 µM F, and 22 µM ROX in deionized water was obtained. The emission of C460 and ROX was significantly quenched while that of F was enhanced when compared to that of the biophosphor gel with the same dye composition. The color coordinates were (0.29, 0.60), indicating green emission and demonstrating that BSA was required for white emission (Figure 4.8D). In another control experiment, a solution of 175 mg mL⁻¹ BSA, 75 µM C460, 450 µM F, and 22 µM ROX but
lacking EDC was dried to form a non-covalent film. The emission of each chromophore diminished at least 10% relative to that of the biophosphor, and emission of C460 decreased disproportionately more, which resulted in color coordinates of (0.32, 0.41). Unlike the transparent hydrogels, the drop-cast films were opaque. Scattering likely reduced the penetration of photons into the matrix, resulting in fewer dye excited states and reduced emission of the film relative to the gel. Thus, a crosslinked hydrogel was advantageous (Figure 4.8D).
Figure 4.8. (A) Dashed emission spectrum of C460, F, and ROX in vacuum dried biophosphor was obtained by assuming no FRET and summing individual dye spectra. Sensitization of green and red emitters indicated FRET when compared to biophosphor emission (solid line). (B) Excitation spectra were obtained by monitoring emission of ROX at 630 nm. Summed excitation spectrum (dashed line) was determined as in panel A. Increased intensity of C460 (370 nm) and F (512 nm) in vacuum dried biophosphor (solid line) indicated FRET. (C) Absorption spectrum of vacuum dried featured peaks corresponding to C460 (365 nm), F (507 nm), and ROX (578 nm). Blue (C460), green (F), and red (ROX) curves are absorption spectra of individual dyes in vacuum dried BSA gel. (D) Various fluorescence emission spectra to probe the role of the BSA hydrogel matrix in promoting FRET in the biophosphor (black solid curve).
4.3.6. Quenching studies

Quenching studies were conducted to further examine energy transfer in the biophosphor. Vacuum dried gels containing the donor (D)-acceptor (A) pairs C460(D)-F(A), C460(D)-ROX(A) and F(D)-ROX(A) demonstrated sensitization of acceptor emission and quenching of donor emission as the acceptor concentration increased (Figure 4.9A-B). The quenching radius was determined from the y-intercept of plots of $\ln(I_0/I)$ versus quencher concentration, as described by the Perrin equation:

$$\ln \left( \frac{I_0}{I} \right) = VN_A [Q]$$

where $I_0$ and $I$ are the intensities of the donor in the absence and presence of the quencher, $N_A$ is Avogadro’s number, $[Q]$ is the quencher concentration, and $V$ is the quenching volume (Figure 4.9C-D). The quenching radius of the C460-F pair was $40(\pm 13)$ Å, as determined from these data, while that of the F-ROX pair was $50(\pm 20)$ Å. C460 emission was not quenched by ROX (Figure 4.10A), and all emission from ROX/BSA hydrogel embedded with C460 and ROX was due to direct excitation of ROX at 365 nm (Figure 4.10B-C). Taken together, these data demonstrated that the energy transfer pathway in the biophosphor was C460→F→ROX (Figure 4.10D).

The efficiency of energy transfer was estimated from the quenching data, using the equation $E = 1 - \frac{I_{DA}}{I_D}$, where $E$ is the fractional energy transfer efficiency, $I_D$ is the intensity of donor emission in the absence of the acceptor, and $I_{DA}$ is the intensity of donor emission in the presence of the acceptor. The FRET efficiencies of the C460-F and F-ROX pairs were 0.63 and 0.41. The overall FRET efficiency of C460→F→ROX was 0.25, the product of the efficiencies of each step. This efficiency is greater than 11.9% reported for a hydrogel containing organic dyes and Eu(III). Because no quenching of C460 emission by ROX was observed, the pathway C460→ROX was not used when calculating the efficiency.
Figure 4.9. (A) Quenching of Coumarin 460 (C460) emission by fluorescein (F) in BSA hydrogel. (B) Quenching of F emission by 5(6)-carboxy-X-rhodamine (ROX) in BSA hydrogel. (C) The ratio of C460 emission in the absence of F to C460 emission in the presence of F at each F concentration was plotted and the Förster radius was determined from the slope of the plot. (D) The ratio of F emission in the absence of ROX to F emission in the presence of ROX at each ROX concentration was plotted and the Förster radius was determined from the slope of the plot.
Figure 4.10. (A) Emission of C460 was unaffected by increasing concentrations of ROX. The observed ROX emission was nearly identical to that of ROX in the absence of C460. (B) The ratio of C460 emission in the absence of ROX to C460 emission in the presence of ROX at each ROX concentration was plotted. No quenching of C460 was observed as ROX concentration was increased. (C) Comparison of ROX emission in the presence (solid curves) and absence (dashed curves) of C460. Curves of the same color indicate experiments with the same ROX concentration. Only a small increase in ROX emission in the presence of C460, indicating that ROX emission was primarily caused by direct excitation rather than sensitization by C460. (D) Schematic representation of FRET pathway in the biophosphor (C460→F→ROX). Excited states of fluorescein and 5(6)-carboxy-X-rhodamine could be populated by FRET and direct absorption.
4.3.7. White emitting bio-LED and its photostability

A white emitting hybrid LED was produced by coating a UV-LED (361 nm) with a mixture of BSA/dye/EDC before it formed a gel. The average thickness of the coating was ~0.5 mm, estimated geometrically from the volume of the gel and the LED surface area coated. The biophosphor converted 361 nm UV to white light, as evidenced by the emission spectrum of the coated LED, which had chromaticity coordinates (0.28, 0.31) (Figure 4.11A). The shift in chromaticity towards the blue region was caused by contributions from the LED emission below 400 nm that was not completely absorbed by the coating. However, the biophosphor coating was successfully used to produce a white emitting LED (Figure 4.11B), and it can be further tuned.

Biophosphor photostability is an important metric for successful implementation in and LED and this was examined. Samples (175 mg mL\(^{-1}\) BSA, 100 mM EDC, 75 µM C460, 450 µM F, 22 µM ROX, vacuum dried) were illuminated by 361 nm LEDs and emission spectra were recorded at specific time intervals (Figure 4.11C). F and ROX diminished more rapidly than C460 (422 nm), leading to a shift in color towards blue over the lifetime of the film (Figure 4.11D). The chromaticity coordinates of the biophosphor shifted from (0.36, 0.37) (cool white) to (0.24, 0.27), indicative of light blue emission, over time. The overall intensity I was obtained by integration of the emission spectrum from 380 to 680 nm. A plot of I/I\(_0\) (intensity at time t/initial intensity) vs time (Figure 4.11E) was employed to determine the half-life, the time taken for the initial intensity to drop to half its value. The half-life of emission of the biophosphor was 106(±5) hours, which is short for lighting applications but reasonable for a sensor that need not be operated continuously.
Figure 4.11. (A) Emission spectrum of 361 nm LED coated with vacuum dried biophosphor (175 mg mL⁻¹ BSA, 100 mM EDC, 75 µM C460, 450 µM F, 22 µM ROX). The chromaticity coordinates were (0.28, 0.31). The shift in chromaticity towards the blue region was caused by contributions from the LED emission below 400 nm, which was not completely screened by the coating. (B) Photograph of bare 361 nm LED (right) and 361 nm LED coated with the biophosphor (left). (C) Emission spectra of biophosphor with increasing irradiation time (361 nm LED, ambient conditions). (D) Chromaticity coordinates of biophosphor as a function of irradiation time. The arrow indicates the direction of change with time. (E) Integrated emission intensities of the biophosphor film as a function of irradiation time, normalized with respect that at 0 h irradiation time. The half-life estimated from this plot was 106(±5) h.
For comparison, a 10% loss of efficiency after 100 h of LED irradiation (385 nm) and a shift toward cooler white was reported for a white emitting protein rubber consisting of green, red, and blue fluorescent proteins embedded in poly(ethylene oxide).\textsuperscript{117,118} However, the photostability studies were based on the direct emission from the LED and emission from the protein rubber while our studies include only the sensitized emission and excludes direct LED emission. Other than this example, photostabilities of white emissive materials is often not reported or reported to be poor.\textsuperscript{117} LED coating and photostability was not established for other white emitting hydrogels. The biophosphor photostability can be improved by sealing in inert atmosphere, as molecular oxygen can react with excited dyes and damage them.\textsuperscript{119}
4.3.8. pH sensing

The white emitting, stimuli-responsive hydrogels are also unique for sensing applications. Stimuli-responsive hydrogels, which experience a change in swelling behavior in response to an environmental stimulus such as pH, temperature, or the presence of a ligand, have been studied extensively.\textsuperscript{89,120–123} White emitting hydrogels, however, have not been explored for sensing. White emission can be highly sensitive to stimuli because of the delicate balance of red, green, and blue emission required to produce white light. If an analyte or stimulus disrupts the outcome in any way, a change in color from white to non-white occurs. This can be readily detected as change in the ratios of the intensities of the principal color components. Ratiometric analysis of the emission improves reproducibility and protects against baseline shift.

Sensing of pH was explored to demonstrate the benefits of white light emission for sensing and the applicability of the biophosphor gel as a sensing platform. BSA has 59 acidic residues (Asp and Glu) and 82 basic residues (Arg and Lys),\textsuperscript{92} so we expected the gel to demonstrate pH responsive swelling, which would modulate emission characteristics by changing dye-dye distances in the gel. Fluorescein has four different protolytic forms with varying molar absorptivity, and its quantum yield increases with pH.\textsuperscript{23} Thus, the emission of the biophosphor gel was expected to respond to pH for multiple reasons.

Samples for pH sensing were prepared with 175 mg mL\textsuperscript{−1} BSA, 100 mM EDC, 30 \(\mu\)M C460, 450 \(\mu\)M F, and 75 \(\mu\)M ROX. As previously discussed, the dye composition of hydrated gels for sensing was different from that of dried gels for LED coating because of the variation in FRET efficiencies in the two states. After gelation was complete, the gels were incubated for one hour in 0.1 M HCl (pH 1), 0.1 M NaOH (pH 13), or a buffer containing 25 mM citric acid, 25 mM sodium phosphate, 25 mM Tris base, and 25 mM sodium bicarbonate (pH 2-11). The front face emission spectra (365 nm excitation) of the gel in the pH-adjusted solution was obtained (Figure 4.12A). Emission shifted from red in acidic pH to white in neutral conditions and finally to green in basic pH, and corresponding chromaticity plots are shown in Figure 4.12B.
A calibration curve was created by plotting the ratio of emission intensity of F to ROX (Figure 4.12C). The F:ROX ratio varied linearly with pH from 1-11, while the F:C460 ratio was only linear from pH 2-5 and thus was not employed for sensing. To the best of our knowledge, this is among the first examples of pH sensing using a white emissive material. The only other example of pH sensing white emissive materials reported in literature are protein nanoparticles and an aqueous cocktail of dyes from vegetable extracts. Additionally, the linear range is significantly broader than other white emitting systems. BSA nanoparticles had a range of pH 4-11, while the aqueous mixture of vegetable extracts used chromaticity coordinates to sense pH over a range of 1-12.5 and a linear range was not reported. The F:ROX ratio also provided good sensitivity, with a slope of 0.16 per pH unit. Taken together, these data demonstrate the applicability of the biophosphor as a pH sensor.

Three main factors are likely responsible for the observed pH sensitivity of the biophosphor. First, pH responsive swelling was qualitatively observed (Figure 4.13A), with gels in highly acidic solutions swelling to significantly larger volumes than those at neutral pH. Such swelling increases the distance between donors and acceptors, disrupting the energy transfer and offsetting the ratios of intensities of blue, green and red colors. Second, the absorbance spectra of the solution showed increasing absorbance at 500 nm as the pH increased from 7-11, indicating that absorbance of fluorescein was changing with pH or that the dye was released from the gel (Figure 4.14B). These changes can disrupt the FRET from F to ROX, demonstrated above. Third, the quantum yield of fluorescein is known to increase with pH, with significant increases above ~pH 6. Thus, multiple factors contribute to the observed high sensitivity to pH of the solution in contact with the white emissive gel.
Figure 4.12. (A) Emission spectra of biophosphor gel as a function of pH from 1 to 11. The emission from the fluorescein chromophore (525 nm) increased with increasing pH. Each curve is labeled with the pH of the experiment. (B) CIE chromaticity coordinates were calculated from the front face emission spectra of the combined gel and supernatant after incubation in 0.1 M HCl (pH 1) or 25 mM citrate, 25 mM sodium phosphate, 25 mM Tris HCl, and 25 mM sodium carbonate (pH 2-11) for one hour. Coordinates shifted from pink to white to green regions as pH increased, with the majority of the change in the X coordinate. The arrow indicates increasing pH. (C) The ratio of emission of fluorescein to 5(6)-carboxy-X-rhodamine (F/ROX, red points) varied linearly with pH from 1 to 11.
Figure 4.13. (A) Qualitative observation of pH responsive swelling of the biophosphor. Gels with the same initial volume were incubated in 0.1 M HCl (pH 1) or 25 mM citrate, 25 mM sodium phosphate, 25 mM Tris HCl, and 25 mM sodium carbonate (pH 2-11) for one hour. The gels incubated at acidic pH qualitatively attained a greater final volume than those incubated at neutral or basic pH. (B) Absorbance spectra of the supernatants above gels were collected. Release of C460 (325 nm), F (490 nm), and ROX (575 nm) was observed, as demonstrated by dye absorbance peaks in the supernatant. The release of F (490 nm) was sensitive to pH, as evidenced by the increase in intensity of the F peak at alkaline pH (green and blue curves).
4.3.9. Glucose sensing

We tested the possibility of including enzymes into the hydrogel matrix to detect small molecule analytes, using glucose oxidase and its substrate glucose as a model system. Biophosphor gels were prepared as previously described (175 mg mL\(^{-1}\) BSA, 100 mM EDC, 30 µM C460, 450 µM F, and 75 µM ROX) containing glucose oxidase (GOx, 5 mg mL\(^{-1}\)) and horseradish peroxidase (HRP, 0.5 mg mL\(^{-1}\)). Glucose oxidase catalyzes the oxidation of glucose with ambient oxygen and produces hydrogen peroxide. HRP uses the hydrogen peroxide produced by GOx to oxidize guaiacol and produce a colored compound.\(^{124}\) The gel formation was unaffected by the addition of these two enzymes to the reaction mixture, and this was supported by the inversion test described above.

The BSA/GOx/HRP hydrogels were incubated at room temperature with 0.1 mM (1.8 mg dL\(^{-1}\)) to 32 mM (576 mg dL\(^{-1}\)) glucose in the presence of excess guaiacol (30 mM) in 20 mM sodium phosphate, pH 7.0. In the glucose sensing assay, the glucose solution was added to the gel. After 30 minutes, the solution was separated from the gel and front face emission recorded (Figure 4.14A). Here, the emission of all dyes was quenched as the concentration of glucose was increased, but the visual changes to emission color were minor. The emission ratio of F:ROX and C460:ROX varied linearly with concentration of glucose from 0.1 mM (1.8 mg dL\(^{-1}\)) to 8 mM (144 mg dL\(^{-1}\)) (Figure 4.14B) with a slope of 0.054 mM\(^{-1}\) (F:ROX) or 0.067 mM\(^{-1}\) (C460:ROX). The linear range could be extended to 288 mg dL\(^{-1}\) if log [glucose] was plotted on the X-axis. The physiological range of glucose is 80-120 mg dL\(^{-1}\), so the biophosphor could detect clinically relevant concentrations of glucose by the incorporation of GOx and HRP in the biophosphor, thus demonstrating the detection of small molecules using the enzymatic biophosphor gel.\(^{125}\)
Figure 4.14. (A) Emission spectra of biophosphor gel containing 5 mg mL\(^{-1}\) glucose oxidase and 0.5 mg mL\(^{-1}\) horseradish peroxidase after incubation at room temperature with 0-32 mM glucose and 30 mM guaiacol in 20 mM sodium phosphate, pH 7.0. The emission of all dyes was quenched as concentration of glucose increased. Each curve is labeled with the concentration of glucose tested. (B) The ratio of emission of fluorescein to 5(6)-carboxy-x-rhodamine (F/ROX, red points) and Coumarin 460 to ROX (C460/ROX, blue points) varied linearly with glucose concentration from 0.1 to 8 mM glucose. (C) Visual detection of glucose was possible by forming the enzyme incorporated hydrogel within filter paper. The appearance of the brown color of the oxidized guaiacol product was proportional to the concentration of glucose.
Visual detection of glucose using the enzyme incorporated hydrogel was also possible. Here, a hydrogel containing 175 mg mL\(^{-1}\) BSA, 5 mg mL\(^{-1}\) GOx, and 0.5 mg mL\(^{-1}\) HRP was formed within the voids of Whatman 1 filter paper by soaking the paper with the protein/EDC solution prior to gelation. In order to improve the appearance of brown color from the oxidized guaiacol dimer, the fluorescent dyes were not incorporated in the gel. After gelation was complete, a hole-punch was used to obtain small, regular sections of the paper for analysis. These were incubated with 0.1 mM (1.8 mg dL\(^{-1}\)) to 16 mM (288 mg dL\(^{-1}\)) glucose in the presence of excess guaiacol (30 mM) and 20 mM sodium phosphate, pH 7.0. Color appeared after five minutes, and the darkness of the color was qualitatively proportional to the concentration of glucose (**Figure 4.14C**). The response could be quantitated using a scanner and image analysis software such as ImageJ per a previously reported method.\(^{13}\) In this manner, a simple method for visual detection of glucose without the need for fluorescence spectrscoopy was also demonstrated using the BSA hydrogel.
4.4. Conclusions

The multifunctional, biomaterial based hydrogel described here is the first example of a white light emitting hydrogel with demonstrated applications in both lighting and sensing, and the biophosphor is produced from sustainable materials with minimal toxic byproducts. The relative crosslink density and swelling behavior of the gel were under complete chemical control. BSA continued to serve as an excellent host for small molecules, and the gel matrix promoted FRET amongst the incorporated dyes, Coumarin 460, fluorescein, and 5(6)-carboxy-X-rhodamine. When excited at 365 nm, white emission was obtained from hydrated, de novo gels (chromaticity coordinates = [0.31, 0.35]) and vacuum dried gels (chromaticity coordinates = [0.36, 0.37]) by changing the dye composition. Significantly, proteolytic degradation of the matrix indicated that the material was also biodegradable. The final white-emissive material was also inexpensive, costing only 2.08 cents to coat one LED or 20.8 cents per gram based on retail prices of the components.

The resulting white emitting gel was suitable for a variety of applications. A white LED was produced by coating the biophosphor on a 361 nm LED, after which the biophosphor photostability in ambient air was studied ($t_{1/2}=106\pm5$ h). Additionally, multi-chromophoric white light emitting systems are ideally suited to sensing applications because of ratiometric analysis of the emission data leads to high reproducibility and protection against baseline shifts. The biophosphor gel was responsive to pH because of the sensitivity of both the BSA gel matrix and the dyes to pH. The linear range of detection, pH 1-11, was significantly broad when compared to other white emissive pH sensing systems. Finally, glucose sensing over a clinically relevant range (1.8-144 mg dL$^{-1}$) was realized by inclusion of glucose oxidase and horseradish peroxidase into the biophosphor gel, demonstrating that specific enzymes can be embedded in the gel. This also demonstrated the successful detection of small molecules by incorporation of enzymes into the matrix. Taken together, the biophosphor could be used in an integrated sensing device, where white emission
is driven by a 361 nm LED and changes in fluorescence are detected upon addition of an analyte. In this manner, the white light emitting, biodegradable, sustainably produced biophosphor gel serves as a robust platform for further exploration of biomaterial based sensing and lighting.
4.5. Materials and methods

4.5.1. Materials

Bovine serum albumin was purchased from Equitech Bio (Kerville TX). 7-Diethylamino-4-methylcoumarin (Coumarin 460), fluorescein, and sodium phosphate were purchased from Sigma-Aldrich (Milwaukee, WI). 5(6)-carboxy-x-rhodamine was purchased from AnaSpec (Fremont, CA). Greiner CELLSTAR black polystyrene 96 well plates were purchased from Fisher Scientific (Atlanta, GA). Spectra/Por 6 dialysis membranes (pre-wetted RC tubing, 25 kD molecular weight cutoff) were purchased from Spectrum Labs (Rancho Dominguez, CA). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from TCI American (Portland, OR). UV Light emitting diodes (5 mm, 361 nm, 750 µM luminous intensity, part# L5-0-U5TH15-1) were purchased from LED Supply (Randolph, VT).

4.5.2. Synthesis and optimization of white emitting hydrogel

A solution of 225 mg mL⁻¹ BSA was prepared in deionized water by slow addition of solid BSA with stirring. The final concentration of the stock solution was verified spectrophotometrically, using the molar extinction coefficient at 280 nm, 43824 M⁻¹ cm⁻¹. The BSA stock was then diluted to the desired [BSA] using deionized water. Solid EDC was added to produce a final [EDC] of 100 mM. Gelation was confirmed by the inversion test, and was observed for [BSA] ranging from 75-225 mg mL⁻¹.

To optimize gelation condition, hydrogels were prepared at either fixed [BSA] (175 mg mL⁻¹) and varying [EDC] (40-200 mM) or fixed [EDC] (100 mM) with varying [BSA] (75-200 mg mL⁻¹). The gelation time, the time from addition of EDC to confirmation of gelation by inversion, was obtained.

White light emission was obtained by adding Coumarin 460 (C460, blue), fluorescein (F, green), and 5(6)-carboxy-x-rhodamine (ROX, red) to the BSA solution prior to addition of EDC. The BSA/C460/F/ROX solution was prepared from a concentrated stocks of C460 (5 mM in
ethanol), F (10 mM in DI water), and ROX (1 mM in methanol) and added to BSA solution in water to give 75 µM C460, 450 µM F, and 75 µM ROX or less. No precipitation was noted when the samples were prepared, most likely because the dye binds to BSA with good affinity. To produce the vacuum dried gels for LED coating, the gels were dried under vacuum at room temperature for ~4 hours until no change in mass was observed. Hydrated gels for sensing were used de novo. White emission was obtained by systematically varying dye concentrations, and validated using CIE 1931 chromaticity coordinates. Chromaticity coordinates were calculated using MatLab 2013A and the open source “CIE Coordinate Calculator” by Prashant Patil.

The optimized solution phase dye concentrations were [C460] = 30 µM, [F] = 450 µM, and [ROX] = 75 µM for hydrated gels or [C460] = 75 µM, [F] = 450 µM, and [ROX] = 22 µM for vacuum dried films. The volume of the vacuum dried film was calculated from the density of the BSA/dye solution (1.059 g/mL at 21 °C), the mass of the dry components, and the water content of the dry film (16(±2)% by mass). After drying, the volume of the gel was reduced 5.5 fold, and the dye concentrations in the dried film were: [C460] = 410 µM, [F] = 2.5 mM, and [ROX] = 120 µM.

4.5.3. Swelling studies

Sets of hydrogels were prepared at either fixed [BSA] (175 mg mL⁻¹) and varying [EDC] (40-200 mM) or fixed [EDC] (100 mM) with varying [BSA] (75-200 mg mL⁻¹). After gelation was complete, the gels were dried under vacuum at room temperature. The mass was measured at intervals, and drying continued until no further change in mass was observed.

The dried gels were immersed in a 10-fold excess volume of deionized water. Periodically, the gels were removed from the water, blotted to remove surface water, and the mass was obtained. This process was repeated until no change in mass was observed. The swelling ratio was then calculated as \( \frac{\text{mass swollen} - \text{mass dried}}{\text{mass dried}} \).
4.5.4. Rheological measurements

Shear storage and loss moduli were measured using a TA Instruments AR-G2 stress-controller rheometer. Swollen hydrogels were prepared from 175 mg mL\(^{-1}\) BSA and 100 mM EDC with a thickness of 1.7 mm. Angular frequency sweeping tests were performed using parallel plates (d = 20 mm) and were kept at 25 °C with an aluminum Peltier plate. The angular frequency was scanned from 0.1 to 10 rad s\(^{-1}\) with an axial force of 0.3 N.

4.5.5. Scanning electron microscopy

Samples of swollen gels for scanning electron microscopy (SEM) were flash frozen and lyophilized, while those of collapsed gels for LED coating were dried under vacuum at room temperature. The dried gel was then section with a razor blade, coated with Au-Pd in Edwards E306A Coating System and taken directly for imaging. SEM images of vacuum dried gels were obtained using a JEOL FESM 6335 using 5 kV accelerating voltage. SEM images of flash frozen swollen gels were obtained using a FEI Teneo LVSEM using 10 kV accelerating voltage. All images were of the sectioned face. Scale bars on images were redrawn to identical size using Adobe Illustrator to improve visibility.

4.5.6. Spectroscopy

UV/Visible absorbance spectra were captured using an HP 8450 diode array spectrophotometer (Varian Inc., Santa Clara, CA). Samples were prepared as described above. Single dye samples were prepared with identical concentrations of BSA, EDC, and dye, except the other two dyes were omitted. Gels were formed and dried on a glass microscope slide, with final thickness of ~0.5 mm.

Fluorescence emission and excitation spectra were obtained using a Molecular Devices FlexStation 3 Microplate Reader. Gels were formed in the wells of a Greiner CELLSTAR black polystyrene 96 well plate (75 μL solution). Vacuum dried gels were dried under vacuum at room temperature until no change in mass was observed, while hydrated gels were measured
immediately. Emission spectra were collected with the following parameters: 365 nm excitation, low PMT setting, 6 readings per well, front face detector. Excitation spectra were collected with the following parameters: 630 nm monitoring, low PMT setting, 6 readings per well, front face detector. All spectra were normalized with respect to a solution of 10 μM ROX in 10 mM sodium phosphate, pH 7.0, which was collected at the same time.

4.5.7. Fluorescence quenching studies

Gels were prepared (175 mg mL\(^{-1}\) BSA, 100 mM EDC) containing donor-acceptor pairs (C460-F, F-ROX, and C460-ROX) and dried under vacuum. The volume of the film was calculated using the total mass of each component and the density of the solution prior to gelation, and this volume was used to calculate the acceptor concentration in the film. The concentration of the donor was kept constant (0.42 mM C460 or 2.5 mM F) while that of the acceptor was varied over a range of 0-2.5 mM. Perrin plots were constructed by plotting \(\ln(I_0/I)\) vs [acceptor], where \(I_0\) and \(I\) are the emission intensities of the donor in the absence and presence of the acceptor. The quenching volume was determined from the slope of the plot and used to calculate the quenching radius.

4.5.8. Dye binding studies

The binding constant of F and ROX to BSA were determined using fluorescence polarization anisotropy (FPA). Solutions of 10 μM F or ROX in 20 mM sodium phosphate, pH 7.0, with increasing concentrations of BSA (1-128 μM) for one hour. Samples were prepared in triplicate. FPA was measured using a Molecular Devices FlexStation 3 Microplate Reader with the following parameters: low PMT, high sensitivity, and \(\lambda_{\text{excitation}} = 485\) nm, \(\lambda_{\text{emission}} = 515\) nm, 495 nm cutoff filter for F or \(\lambda_{\text{excitation}} = 570\) nm, \(\lambda_{\text{emission}} = 595\) nm, 590 nm cutoff filter for ROX.

A simple equilibrium model was used to obtain the dissociation constant (\(K_d\)) and association constant (\(K_b = K_d^{-1}\)) from the data, using Kaleidagraph 4.1.3 for non-linear regression.

\[
\frac{[D]_b}{[D]_T} = f_b = \frac{[\text{BSA}]}{K_d + [\text{BSA}]} \tag{1}
\]
In equation 1, \([D]\) is the dye concentration, the subscripts \(b\) and \(T\) indicate bound or total concentration, and \(f_b\) is the mole fraction bound. In FPA experiments, the observed anisotropy is equal to the anisotropy of each species multiplied by its fractional abundance:

\[
r_{\text{observed}} = \sum_{i=1}^{n} f_i r_i = f_f r_f + f_b r_b \quad (2)
\]

where \(f\) is mole fraction, \(r\) is fluorescence anisotropy, and the subscripts \(f\) and \(b\) indicate free and bound states.[1] Equation 2 can be solved in terms of fraction bound by substituting \(f_f = 1 - f_b\). Combining this with equation 1, the dissociation constant can be obtained by directly fitting a plot of anisotropy versus dye concentration (equation 3) if we assume that BSA is in excess and \([\text{BSA}]\) = \([\text{BSA}]_T\).

\[
r_{\text{observed}} = r_f + (r_b - r_f) \frac{[\text{BSA}]_T}{K_D + [\text{BSA}]_T} \quad (3)
\]

4.5.9. Determination of covalently bound dyes

Vacuum dried gels (175 mg mL\(^{-1}\) BSA, 100 mM EDC) were crushed to powder using a mortar and pestle. A known mass of gel powder was suspended in 5 mL deionized water and dialyzed six times in the dark against 20 mM sodium phosphate, pH 7.0 (400-fold excess volume), using Spectra/Por 6 dialysis membrane (25 kDa cutoff). The solid was recovered, washed with deionized water, and dried under vacuum. After drying, the gel powder was incubated in 50 \(\mu\)M trypsin and 20 mM sodium phosphate, pH 7.0, for four hours at 37 °C. Complete dissolution of the powder was observed. The absorbance spectrum of the digested gel was collected and concentrations of \(F\) and ROX were determined using their extinction coefficients.

4.5.10. Determination of molar absorptivity of (5,6)carboxy-x-rhodamine

ROX solutions were prepared in 20 mM sodium phosphate, pH 7.0, using volumetric glassware and serial dilution from a concentrated stock. Solutions were prepared in triplicate. The absorbance of ROX at 575 nm was obtained using an HP 8450 diode array spectrophotometer (Varian Inc., Santa Clara, CA), and the baseline was subtracted from the observed spectrum. The
extinction coefficient was obtained from the slope of the plot of absorbance at 575 nm versus [ROX].

4.5.11. LED coating

Gel solution was prepared with 175 mg mL\(^{-1}\) BSA, 100 mM EDC, 75 µM C460, 450 µM F, and 22 µM ROX. The solution became a viscous liquid ~45 minutes after EDC addition, before gelation was complete. This solution was coated on a UV LED (5 mm, 361 nm, 750 µM luminous intensity) by successive drop-casting of 30 µL aliquots until the desired thickness was obtained. The coated LED was then dried under vacuum until no change in mass was observed. The thickness of the LED coating was calculated using the volume of the film and by approximating the LED as a cylinder.

4.5.12. Photostability measurements

Vacuum dried gels were prepared in triplicate as described above within a 96 well plate. The plate was placed at a fixed height and orientation above 365 nm LEDs so that the dried gels were 2.5 mm above and light from any LED fully illuminated one gel. The black walls of the plate prevented LEDs from illuminating neighboring wells. Gels were illuminated continuously in a dark room for 120 h and removed periodically to obtain the emission spectrum.

4.5.13. pH sensing

Hydrated gels were prepared in a 96 well plate. Samples were incubated at room temperature for 1 h with 4x excess volume of 0.1 M HCl (pH 1), 0.1 M NaOH (pH 13), or a broad range buffer containing 25 mM sodium citrate, 25 mM sodium phosphate, 25 mM Tris, and 25 mM sodium bicarbonate (pH 2-11). After incubation, the front-face emission spectrum of each well containing both the supernatant and gel was obtained.

4.5.14. Glucose sensing

Hydrated gels were prepared in a 96 well plate. Solid glucose oxidase (5 mg mL\(^{-1}\)) and peroxidase from horseradish (1 mg mL\(^{-1}\)) were added to the reaction mixture for making the above
BSA gels, prior to addition of EDC. Samples were incubated at room temperature for 30 minutes with 4x excess volume of glucose solutions in 20 mM sodium phosphate, pH 7.0. Guaiacol was added to a final concentration of 30 mM and the gels were incubated for another 30 minutes. The supernatant was removed, and the gels were washed three times with deionized water. The front-face emission spectra of the gels were then obtained.
4.6. Acknowledgements

This work was supported by NSF EAGER Award DMR-1441879 and by UCONN VPR Research Excellence Award. We thank Professor M. Shaw for help with the rheological studies. We also thank D. Lakheram, M. Anuganti, and C. M. Riccardi for technical help.
Chapter 5. Cationic nanoparticles regulate the aggregation of poly(glutamic acid)

5.1. Abstract

We report here regulation of poly(glutamic acid) (PLG) aggregation into amyloid fibers by cationic silica nanoparticles (NPs), SiO$_2$ modified with (3-aminopropyl)-triethoxysilane (SiO$_2$-NH$_2$). Binding between PLG and NPs is driven by their complementary electrostatic charges, and the presence of SiO$_2$-NH$_2$ during fibrillation at 75 °C and pH 4.0 accelerates fibril growth and eliminates the lag phase associated with nucleation. We systematically assessed the kinetics of PLG fibrillation at 45 °C using high throughput methods, and found that SiO$_2$-NH$_2$ can catalyze nucleation while simultaneously inhibiting fibril growth rate. Using model-based analysis, we proposed that PLG fibrillation in the presence of cationic silica NPs follows a modified heterogeneous nucleated polymerization model, with an additional pathway where reversible binding of NPs to growing fibers inhibits the fiber growth. Under the appropriate conditions, such as lower temperatures (45 °C), binding between PLG or PLG fibers and NPs is stronger, and inhibition of growth is observed. When the conditions are changed to reduce the binding affinity, such as by increasing the temperature to 75 °C, the acceleration of nucleation dominates and the NPs increase the rate of fibrillation. The experimental kinetics were successfully simulated with this model, demonstrating how the NPs can serve in a regulatory capacity.
5.2. Introduction

Protein supramolecular polymerization is efficiently regulated in natural systems. For example, actin polymerization can occur rapidly, and is controlled by both hydrolysis of ATP (chemical reactions) and interaction with actin binding proteins (macromolecular regulators).\textsuperscript{126,127} In the absence of such regulating events, polymerization of proteins by homogeneous nucleated polymerization, as first proposed by Oosawa, can be very slow.\textsuperscript{40,128} Both nucleation and growth phases require specific conditions and critical monomer concentrations to proceed. However, these can be influenced by regulatory factors in a predictable manner to enhance the rate of protein polymerization, as in the case of actin polymerization.

Drawing inspiration from nature, significant attention has been given to exploring the dynamic behavior of supramolecular polymers, and to understand the key design principles of supramolecular polymeric systems.\textsuperscript{129} The ultimate goal of such research is the rational design of natural, synthetic, or hybrid macromolecular systems that can produce functional supramolecular polymers in a predictable and regulated manner.\textsuperscript{130} We have previously reported that supramolecular polymerization of poly-L-glutamic acid (PLG) grafted comb polymers can be regulated using chemical reactions, by modification of PLG with benzyl amine.\textsuperscript{131,132} As an extension of this work, we sought to understand how nanoparticles can be used to regulate the aggregation of polyamino acids into amyloid fibrils.

Amyloid fibrils are formed by supramolecular assembly of misfolded, β-sheet rich proteins into highly stable fibrils as well as higher order structures.\textsuperscript{27–29,31} They have been implicated in several neurodegenerative diseases,\textsuperscript{30} and have also been employed as building blocks for functional materials with hierarchical nanostructure.\textsuperscript{130,133} With the growth of nanomedicine, the influence of nanoparticles on the aggregation behavior of proteins to form amyloid fibers has attracted significant attention. However, current studies on naturally amyloidogenic have revealed
the nanoparticles can have either inhibitory or promotive effects, which can be highly specific to the protein sequence and NP surface properties.\textsuperscript{32–36,38,134,135}

With this in mind, we endeavored to learn something fundamental about these processes by eliminating the sequence-specific complexities. We took advantage of the fact that any peptide can form amyloid fibrils under the correct conditions,\textsuperscript{28} and studied a simple model system consisting of homopolymers of amino acids with silicon dioxide (SiO\textsubscript{2}) nanoparticles. The SiO\textsubscript{2} NPs are naturally anionic, and can be readily functionalized to obtain cationic derivatives.\textsuperscript{136} We then examined the influence of these variably charged NPs on the aggregation kinetics of poly-L-(glutamic acid) (PLG), where the affinity of the acidic and basic polyamino acids is dictated principally by the electrostatic charge matching interactions.\textsuperscript{137} With this model system, we hoped to elucidate the role of NP-protein binding affinity in amyloidosis, and to use NPs as artificial regulators of protein aggregation.
5.3. Results and Discussion

5.3.1. Synthesis and characterization of cyt c-PAA

PLG with low polydispersity (<1.1 DPI) were prepared by ring opening polymerization of γ-(4-vinylbenzyl)-L-glutamate N-carboxyanhydride. The benzyl esters were removed in the subsequent deprotection step, which converted poly(γ-(4-vinylbenzyl)-L-glutamate) (PBLG) to PLG. PBLG molecular weight was characterized by gel permeation chromatography, and deprotection was confirmed by NMR (Figure 5.1). The resulting PLG molecular weights were 7.5, 12.8, and 20.0 kg mol⁻¹ (PLG₅₀, PLG₈₅, and PLG₁₃₃). Cationic silica NPs (SiO₂-NH₂) were prepared by modification of SiO₂ with (3-aminopropyl)triethoxysilane by established methods. Synthesis was validated by FTIR. Additionally, the zeta potential of SiO₂-NH₃ was +33 mV when dispersed in water, demonstrating successful cationization of the NPs (Figure 5.2). Finally, transmission electron micrographs of SiO₂-NH₂ revealed diameters of 15-20 nm, nearly unchanged from the unmodified SiO₂ (Figure 5.2).

‡ PBLG and PLG were prepared and characterized by our collaborator, Ziyuan Song, at University of Illinois, Urbana-Champaign.
§ SiO₂-NH₂ was prepared and its zeta potential measured by Ankarao Kalluri (Kumar group, University of Connecticut)
Figure 5.1. (A) Synthesis of poly-L-glutamic acid. (B) Gel permeation chromatography traces of poly-L-glutamic acid benzyl ester prior to deprotection. (C) Mass and polydispersity index of PBLG before deprotection. (D) Representative $^1$H NMR spectrum of PLG after deprotection.
Figure 5.2. (A) Schematic representation of cationization of SiO$_2$ nanoparticles. (B) FTIR spectra of SiO$_2$ (black) and SiO$_2$-NH$_2$ (red). The feature at ~2900 cm$^{-1}$ corresponded to the CH$_2$ stretch of the APTES propyl group, confirming the synthesis of SiO$_2$-NH$_2$. (C) TEM micrograph of SiO$_2$-NH$_2$. 

5.3.2. Kinetics of PLG aggregation are accelerated by cationic NPs

Next, kinetic studies were conducted to examine the effect of the cationic NPs on the aggregation behavior of PLG\textsubscript{50}. PLG is known to form fibrils in acidic environment (pH\leq4) and elevated temperatures.\textsuperscript{28,41} Here, PLG is α-helical, but its structure is destabilized by the reaction conditions and it can be converted to the characteristic β-sheet of amyloids.\textsuperscript{28} At higher pH, PLG adopts a coil structure because of the increased ionization and charge density, which can also inhibit fibrillation.\textsuperscript{41} The folding behavior of the synthesized PLG was examined by circular dichroism (Figure 5.3), and all PLGs were α-helical at pH\leq4. Thus, we selected pH 4.0 and 75 °C for PLG\textsubscript{50} fibrillation studies, in good agreement with literature reports.\textsuperscript{28,41}

The kinetics of PLG\textsubscript{50} amyloidosis were followed using Thioflavin T (ThT) staining. ThT binds selectively to the characteristic beta sheet structure of amyloid fibers, which is accompanied by a significant increase in fluorescence quantum yield.\textsuperscript{139} Briefly, solutions of 267 µM PLG\textsubscript{50} (2 mg mL\textsuperscript{-1}), 0.1 mg mL\textsuperscript{-1} SiO\textsubscript{2} or SiO\textsubscript{2}-NH\textsubscript{2}, and 60 µM ThT were prepared in 10 mM sodium acetate, pH 4.0. Samples were incubated at 75 °C and ThT emission (\lambda\textsubscript{ex} = 440 nm, \lambda\textsubscript{em} = 485 nm) was measured at intervals. In the absence of NPs, PLG\textsubscript{50} had a short lag phase, and the aggregation reaction was complete in about 4 hours (Figure 5.4). In the presence of cationic SiO\textsubscript{2}-NH\textsubscript{2}, on the other hand, the lag phase was virtually eliminated, and the rate of aggregation dramatically increased. Addition of SiO\textsubscript{2}-NH\textsubscript{2} reduced the time to complete the aggregation 4-fold, from \textasciitilde4 hours to \textasciitilde1 hour.
Figure 5.3. (A) UV circular dichroism spectra of PLG$_{50}$ at 75 °C and pH 3.6-5.6. (B) Helix to coil transition of PLG$_{50}$ at 75 °C. Fraction alpha helix was calculated using the ellipticity at 222 nm. The midpoint of the transition was pH 4.70(±0.04).
Figure 5.4. Kinetics of PLG₅₀ aggregation at pH 4.0 and 75 °C. The presence of SiO₂-NH₂ (blue curve) reduced the length of the lag phase and increased the rate of fibrillation when compared to PLG₅₀ alone (black curve).
5.3.3. Characterization of PLG₅₀ fibrils

The secondary structure of PLG₅₀ in the amyloid fibers was confirmed using FTIR, as the increase in ThT emission alone is not enough to prove the presence of β-sheet structure.¹⁴⁰ In particular, we examined the Amide I band (1600-1700 cm⁻¹), which is sensitive to the protein folding and amide backbone configuration.¹⁴¹ The FTIR spectrum of PLG₅₀ had a major feature at 1655 cm⁻¹, characteristic of α-helix (Figure 5.5A). After incubation, this feature disappeared, and a new peak appeared at 1608 cm⁻¹, which demonstrated conversion to β-sheet during fibrillation. Significantly, the FTIR spectra of PLG₅₀ fibrils prepared with and without SiO₂-NH₂ could be nearly overlaid in the Amide I and II regions, indicating the secondary structure of the aggregates was no affected by the NPs.

The morphology of the PLG fibrils was probed by transmission electron microscopy (TEM). PLG₅₀ aggregates had twisted, lamellar structures, as previously reported (Figure 5.5B).⁴¹,¹⁴² These twisted fibers were several tens of nanometers wide, and could be greater than 500 nm in length. In the presence of SiO₂-NH₂, excessive aggregation was noted, with NPs and PLG forming into large irregular clusters, though this aggregation was likely driven by drying effects and was thus an artifact of sample preparation and not the in situ fiber morphology (Figure 5.5C).¹⁴³ Additional dispersed fibers with morphologies identical to those observed in PLG₅₀ alone were also observed.
Figure 5.5. (A) FTIR spectra of PLG$_{50}$ before (grey) and after (blue) amyloidosis. The FTIR spectrum of PLG fibrils prepared in the presence of SiO$_2$-NH$_2$ could be nearly overlaid with that of PLG$_{50}$ fibers, indicating the presence of the NPs did not alter the fiber morphology. (B) TEM micrograph of PLG$_{50}$ fibrils revealed a twisted, lamellar morphology. (C) TEM micrograph of PLG fibrils formed in the presence of SiO$_2$-NH$_2$. 
5.3.4. Model based analysis of PLG$_{50}$ aggregation

Aggregation of polypeptides into amyloid fibrils is typically described using nucleated polymerization models. The simplest model is the homogeneous nucleation model, first proposed by Oosawa.\cite{40,128} Here, fibril growth follows only from the formation of the primary nucleus. The heterogenous nucleated polymerization model can be employed when secondary pathways, such as surface induced nucleation, can also generate nuclei to initiate fibril growth.\cite{144,145} A convenient analytical solution to the differential rate equations is:

\[
\frac{M(t)}{M_\infty} = 1 - e^{-\left(\frac{\lambda}{\kappa}\right)^2 \left(\cosh(\kappa t) - 1\right)}
\]

\[
\lambda = \sqrt{2k_n k_+ m_{tot} n^*}
\]

\[
\kappa = \sqrt{2k_+ k_2 m_{tot} n_2 + 1}
\]

where $M(t)/M_\infty$ is the time progress of conversion of monomer from soluble to aggregated state, $k_+$ is the forward elongation rate constant, $k_n$ is the rate constant of formation of the primary nucleus, $k_2$ is the secondary nucleus formation rate constant, $m_{tot}$ is the total monomer concentration, $n^*$ is the critical number of monomers in the primary nucleus, and $n_2$ is the number of monomers in the secondary nucleus.\cite{40,128,144,145} The parameters $\lambda$ and $\kappa$ represent the apparent rate constants of homogeneous and heterogeneous nucleated polymerization, respectively.

In order to rigorously apply this model-based analysis to PLG$_{50}$ aggregation, we employed a 384-well plate reader to track the kinetics in high throughput. The reaction temperature was reduced to 45 °C because of the limitations of this method, but this did allow for better resolution of the early stages of the reaction, which are crucial for the model fitting. First, we examined the PLG concentration dependence at 45 °C and pH 4.0. In the absence of NPs, PLG$_{50}$ kinetic traces exhibited the characteristic sigmoidal curve of heterogeneous nucleated polymerization,\cite{144,145} with a long lag phase followed by a rapid growth phase. As the [PLG] was increased, the lag time decreased and the elongation rate during the growth phase increased (Figure 5.6A). As was
observed at 75 °C, addition of SiO$_2$-NH$_2$ nearly eliminated the lag phase and reduced $t_{1/2}$, the time at which aggregation is 50% complete \((\text{Figure 5.6B, Table 5.1})\). In the early stages (<20% monomer conversion), the growth was nearly linear and the rate was nearly independent of [PLG]. However, in the later stages of aggregation, the growth rate actually slowed relative to PLG alone, and the time to complete the aggregation was unchanged by the addition of the NPs. This suggests that addition of the NPs increases the rate of heterogeneous nucleation early while simultaneously decreasing the growth rate in the later stages.

The kinetic data was fit to the analytical solution to the heterogenous nucleated polymerization model (solid lines in \text{Figure 5.6A-B}) and the apparent rate constants of homogenous nucleation, $\lambda$, and heterogeneous nucleation, $\kappa$, were extracted (\text{Table 5.1}). Examining the data and the model fitting together, a few things are apparent. First, $t_{1/2}$ is reduced by addition of SiO$_2$-NH$_2$ for each [PLG] tested, and $t_{1/2}$ has a weaker dependence on [PLG] in the presence of the NPs (\text{Figure 5.6C}). Second, the apparent rate constants of homogeneous nucleation, $\lambda$, are similar between samples with and without NPs, and the dependence of $\lambda$ on [PLG] is essentially unchanged. Third, the apparent rate constant of heterogeneous nucleation, $\kappa$, decreased in the presence of NPs, and had a stronger dependence on [PLG] than PLG alone (\text{Figure 5.6D}). Finally, while the heterogeneous nucleated polymerization model could be fit to the kinetic traces for aggregation in the presence of NPs, the fits did not describe the rapid rise in ThT emission in the first ~100 minutes. Additionally, 133 µM PLG with NPs could not be fit by this model. Taken together, these observations suggest that (1) generation of the heterogeneous nuclei at the NP surface occurs more rapidly than at the fibril surface; (2) the presence of the NPs reduces the average growth rate ($k_+$), especially later in the reaction; and (3) the acceleration in the early stages is likely caused by efficient nucleation rather than increased growth rate.
Figure 5.6. Kinetics of PLG aggregation in the absence (A) and presence (B) of cationic SiO$_2$-NH$_2$, fit to the heterogeneous nucleated polymerization model. (C) Dependence of aggregation $t_{1/2}$ (time to half completion) on [PLG] in the absence (black squares) and presence (blue half-squares) of SiO$_2$-NH$_2$. (D) Dependence of $\kappa$, the apparent rate constant of heterogeneous nucleation, on [PLG] in the absence (black squares) and presence (blue half-squares) of SiO$_2$-NH$_2$. 
<table>
<thead>
<tr>
<th>[PLG] (µM)</th>
<th>[NP] (mg mL⁻¹)</th>
<th>$t_{1/2}$ (h)</th>
<th>$\lambda$ (h⁻¹)</th>
<th>$\kappa$ (h⁻¹)</th>
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<td>0.331(±0.006)</td>
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**Table 5.1.** Variation of $t_{1/2}$, $\lambda$, and $\kappa$ with [PLG] in the absence and presence (shaded cells) of SiO₂-NH₂ nanoparticles.
Reversible binding on monomers to nanoparticles has been previously proposed to increase the rate of nucleation of naturally amyloidogenic proteins by locally increasing the concentration of monomers. For example, copolymeric nanoparticles of N-isopropylacrylamide and N-tert-butylacrylamide nanoparticles have been reported to increase the rate of aggregation of amyloid-β (Aβ) and β-microglobulin. Computational studies suggest that local crowding at the NP surface also changes the protein structural dynamics, which can aid the misfolding to β-sheet that is characteristic of amyloid fibers. This is in line with reports that the catalytic or inhibitory effect of NPs on protein aggregation may depend on the intrinsic protein stability. However, strong binding of monomers to NP may actually inhibit amyloid formation. Considering these factors may give some insight into how SiO$_2$-NH$_2$ can catalyze the nucleation of PLG amyloids while simultaneously slowing the growth rate. If the NPs can bind the monomeric PLG with a certain affinity, then it follows that the NPs should also have an affinity for the growing fibers, which will be proportional to the affinity for PLG. Thus, there may be competition at the growing ends of the fiber between binding of PLG, which leads to fiber growth, and binding of NPs, which does not lead to fiber growth. When PLG fibrils were formed in the absence of NPs and subsequently exposed to SiO$_2$-NH$_2$ after fibrillation, TEM micrographs revealed that NPs coated the surface of the fibers, which supported the ability of NPs to bind fibers (Figure 5.7A-B).

Based on the above observations, we propose a modification to the heterogeneous nucleated polymerization model to describe the effect of cationic NPs on PLG aggregation. Here, we consider the reversible binding of NPs to the fibers in addition to the homogeneous and heterogeneous nucleation pathways. Additionally, we assume that growth only occurs from fibers that are not bound to NPs, so that binding of NPs to fibers reduces the effective concentration of fibers and slows the growth rate. With this addition to the model, we simulated the kinetics of PLG aggregation in the presence and absence of NPs, using arbitrary rate constants. The simulated data was able to qualitatively reproduce the experimental data, where the presence of NPs (solid
lines) reduces lag time and \( t_{1/2} \) compared to PLG alone (hollow circles), but the time to complete the reaction is unchanged. The simulated data and experimental data can be found in Figures 5.7C and 5.7D, respectively. Additionally, we found that the binding constant, \( K_A \), of PLG to SiO\(_2\)-NH\(_2\) decreased with temperature (Figure 5.8, \( K_A = 21\times10^4 \) M\(^{-1}\) at 15 °C and 9.1\times10^4 M\(^{-1}\) at 25 °C). Because the affinity of fibers for NPs is proportional to that of PLG, this suggests that we did not observe the inhibitory effect of the NPs during the 75 °C kinetic experiments because the binding of NPs to growing fibers was weaker than at 45 °C. Thus, cationic SiO\(_2\)-NH\(_2\) nanoparticles can regulate the aggregation behavior of PLG based on the dual catalytic and inhibitory effects, where binding of PLG monomers increases nucleation efficiency in the earliest stages but binding of NPs to growing fibers can slow the growth rate (Scheme 5.1).
Figure 5.7. (A-B) TEM micrographs of PLG fibrils incubated in the absence of NPs. SiO$_2$-NH$_2$ was added after fibrillation and allowed to bind to the fibers. (C) Simulated kinetic data (arbitrary rate constants) from modified heterogeneous nucleation model, which incorporates inhibition of growth caused by NPs binding to growing fiber. Hollow circles are PLG alone, and solid lines are PLG with NPs. (D) Experimental data qualitatively agreed with the simulations. Hollow circles are PLG alone, and solid lines are PLG with NPs.
Figure 5.8. Standard curves were employed to quantitate free PLG remaining in the supernatant following binding of PLG$_{85}$ to SiO$_2$-NH$_2$. (A) UV absorbance standard curve ($\lambda = 222$ nm, 1 cm quartz cuvette). (B) Circular dichroism standard curve ($\lambda = 222$ nm, 0.2 cm quartz cuvette). (C) Scatchard plots were employed to determine the binding constant, $K_A$, of PLG$_{85}$ to SiO$_2$-NH$_2$ NPs (10 mM sodium acetate, pH 4.0, 15-25 °C). The slope of the plots is equal to $-K_A$. Binding was stronger at 15 °C (red, $K_A = 21 \times 10^4$ M$^{-1}$) than 25 °C (blue, $K_A = 9.1 \times 10^4$ M$^{-1}$).
Scheme 5.1. Schematic representation of PLG homogeneous nucleation, heterogeneous nucleation, and fiber growth (top) in the presence of cationic silica nanoparticles, as well as the inhibitory effects on nanoparticles on fibril growth (bottom).
5.4. Conclusions

In conclusion, we designed a system in which NPs could serve as regulatory species to control the aggregation behavior of poly(amino) acids. Binding interactions between PLG and cationic SiO$_2$-NH$_2$ NPs was driven by their complementary electrostatic charges, and allowed the NPs to function in both catalytic and inhibitory roles. Under the correct circumstances, i.e. 45 °C and pH 4.0, the presence of SiO$_2$-NH$_2$ could accelerate PLG nucleation in the early stages, but binding of the NPs to growing fibers could inhibit growth as the reaction proceeded. When the conditions were changed to ones where binding between PLG or PLG fibers and the NPs became weaker, such as by increasing the temperature to 75 °C, dramatic acceleration was observed. Thus, SiO$_2$-NH$_2$ NPs did not solely accelerate or slow the rate of PLG aggregation, but instead regulated the aggregation behavior by nature of its dual effect.
5.5. Materials and Methods

5.5.1. Materials

Sodium acetate, potassium chloride, (3-aminopropyl)-triethoxysilane, hydrochloric acid, sodium hydroxide, uranyl acetate, 0.22 μm syringe filters, and 384 well black polystyrene plates were purchased from Sigma-Aldrich (Milwaukee, WI). Thioflavin T and potassium bromide were obtained from Acros Organics (New Jersey, USA). Silicon dioxide nanopowder was purchased from US Research Nanomaterials, Inc. (Houston, TX).

5.5.2. Dynamic light scattering

The hydrodynamic radius of SiO$_2$-NH$_2$ and the complex of SiO$_2$-NH$_2$ with PLG was measured by dynamic light scattering (DLS). Samples were filtered with a 0.22 μm syringe filter to remove dust particles and large aggregates prior to analysis. Precision Detectors PDDLS/CoolBatch 40T and PD4047 dynamic light scattering detectors with 658 nm laser and 90° laser and monitoring optics were employed for DLS measurements. Measurements were completed in triplicate.

5.5.3. Zeta Potential

The zeta potential of SiO$_2$ and SiO$_2$-NH$_2$ was measured using the laser Doppler velocimetry method in a Brookhaven Instruments ZetaPlus zeta potential analyzer. NPs were dispersed in 1 mM KCl prior to analysis. Zeta potentials were measured in a 4 mL polystyrene cuvette, and three runs were performed for each sample. The zeta potential responses were calculated using Smoluchowski fits (software provided by the manufacturer).

5.5.4. FTIR and circular dichroism spectroscopy

Circular dichroism spectra were measured with a JASCO J-710 spectropolarimeter. UV CD spectra were scanned from 195-260 nm using a 0.05 cm path length quartz cuvette and the following parameters: 1 nm data pitch, continuous scanning mode, 1 second response speed, 2
nm bandwidth, 6 scans per spectrum. Blank spectra (buffer solution only) were subtracted from all spectra.

FTIR spectra were measured with a JASCO FTIR 480-Plus. Samples were dried under vacuum, then pressed into a KBr pellet for analysis. Mild smooth was applied (minimum convolution width) using JASCO spectra analysis software.

5.5.5. Transmission electron microscopy

TEM samples were deposited on carbon-coated copper grids, blotted by filter paper, and allowed to dry under ambient conditions. Uranyl acetate stained samples were rinsed first with 1% uranyl acetate aqueous solution before subsequent rinsing with DI water. Samples were analyzed with a Tecnai T12 G2 Spirit BioTWIN transmission electron microscope operating at an accelerating voltage of 80 kV.

5.5.6. Kinetic studies

PLG (4 mg mL⁻¹) and SiO₂-NH₂ (2 mg mL⁻¹) stock solutions were prepared in filtered (0.22 um syringe filter) 10 mM sodium acetate, pH 4.0. Where necessary, solutions were adjusted with filtered 0.1 M HCl or NaOH to achieve a final pH of 4.0. PLG solutions were sonicated for 1 minute break up preformed aggregates. SiO₂-NH₂ solutions were sonicated until the NPs were evenly dispersed in solution. 250 µM ThT was prepared in 10 mM sodium acetate, pH 4.0, and filtered with a 0.22 um syringe filter prior to use.

Reaction solutions were prepared by combining appropriate volumes of the stock solutions and diluting with 10 mM sodium acetate, pH 4.0. For 75 °C kinetics, a manual sampling approach was employed. Here, 15 µL aliquots were removed at intervals and combined with 75 µL of 60 µM ThT in 10 mM sodium acetate, pH 4.0. For high throughput kinetic assays, 60 µM ThT was included in the reaction solutions and ThT emission was monitored in situ. ThT emission spectra were obtained using a FLEXstation 3 plate reader (440 nm excitation, 455 nm cutoff filter, scan emission from 455 to 700 nm, low PMT sensitivity, 6 reads per well).
5.5.7. Model based analysis

PLG binding kinetics were fit to the heterogeneous nucleated polymerization model.\textsuperscript{144,145}
For individual fits, the analytical solution to the differential rate equations was employed:

\[
\frac{M(t)}{M_\infty} = 1 - e^{-\left(\frac{\lambda}{\kappa}\right)^2 (\cosh(\kappa t) - 1)}
\]

\[
\lambda = \sqrt{2k_nk_+m_\text{tot}n^*}
\]
\[
\kappa = \sqrt{2k_+k_2m_\text{tot}n_2+1}
\]

where \(\frac{M(t)}{M_\infty}\) is the time progress of conversion of monomer from soluble to aggregated state, \(k_+\) is the forward elongation rate constant, \(k_n\) is the rate constant of formation of the primary nucleus, \(k_2\) is the secondary nucleus formation rate constant, \(m_\text{tot}\) is the total monomer concentration, \(n^*\) is the critical number of monomers in the primary nucleus, and \(n_2\) is the number of monomers in the secondary nucleus.\textsuperscript{40,128,144,145} The fitting was completed using the non-linear regression functions of MATLAB and Kaleidagraph 4.01.

Simulations incorporating fiber growth inhibition by binding of NPs to growing fibers were completed using MATLAB. The relevant chemical equations are:

\[
n^*M \xrightarrow{k_n} M_n \quad \text{homogeneous nucleation}
\]
\[
n_2M \xrightarrow{k_2} M_{n_2} \quad \text{heterogeneous nucleation}
\]
\[
M_i + M \xrightarrow{k_+} M_{i+1} \quad \text{growth}
\]
\[
M_i + NP \xrightleftharpoons[K_A]{\text{K}} NP \bullet M_i \quad \text{fiber binding to NP}
\]

where \(M\) is a macromolecular monomer (PLG), \(M_n\) is the homogeneous nucleation, \(M_{n_2}\) is the heterogeneous nucleus, \(M_i\) and \(M_{i+1}\) are fibers containing \(i\) and \(i+1\) monomers, \(NP\) is the nanoparticle, \(NP \bullet M_i\) is the NP/fiber complex, \(k_n\) is the rate constant of homogeneous nucleation, \(k_2\) is the rate constant of heterogeneous nucleation, \(k_+\) is the fiber growth rate constant, and \(K_A\) is the association constant for the NP to the fibers. The differential equations were developed by
Hailin Fu, and the solutions to those equations were simulated in MATLAB with arbitrary rate constants.

5.5.8. Equilibrium binding studies

Equilibrium binding studies were employed to measure the association constant, \( K_A \), of PLG for SiO\(_2\)-NH\(_2\). Solutions of 10-300 \( \mu \)M PLG and 1 mg mL\(^{-1} \) SiO\(_2\)-NH\(_2\) were prepared in 10 mM sodium acetate, pH 4.0. Solutions were incubated for 1 hour in a temperature-controlled bath set to the desired temperature. SiO\(_2\)-NH\(_2\) and bound PLG were removed by centrifugation (10 minutes, 13.3 kDa). Free PLG in the supernatant was quantified by UV absorbance (222 nm) or UV CD (ellipticity at 222 nm). Standard curves were prepared by serial dilution of PLG standards, which were analyzed by UV absorbance and CD at 222 nm. Each standard or analyte solution was prepared at least in triplicate.

Bound PLG (PLG\(_{\text{bound}}\)) was determined by subtracting the free PLG concentration (PLG\(_{\text{free}}\)) from the starting concentration. The equilibrium constant was determined from a Scatchard plot. Here, [PLG\(_{\text{bound}}\)] was plotted versus [PLG\(_{\text{bound}}\)]/[PLG\(_{\text{free}}\)], and the slope of the plot was equal to – \( K_A \).
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References


