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Toward "Stable-on-the-Table" Enzymes: Improving Key Properties of Catalase by Covalent Conjugation with Poly(acrylic acid)

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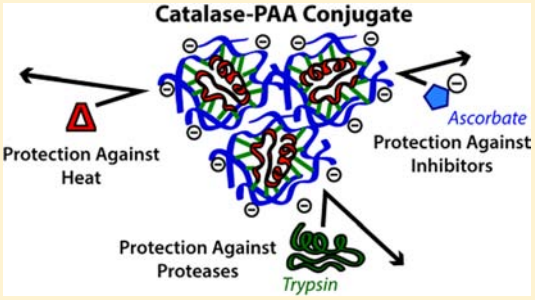
Supporting Information

ABSTRACT: Several key properties of catalase such as thermal stability, resistance to protease degradation, and resistance to ascorbate inhibition were improved, while retaining its structure and activity, by conjugation to poly(acrylic acid) (PAA, M_w 8000) via carbodiimide chemistry where the amine groups on the protein are appended to the carboxyl groups of the polymer. Catalase conjugation was examined at three different pH values (pH 5.0, 6.0, and 7.0) and at three distinct mole ratios (1:100, 1:500, and 1:1000) of catalase to PAA at each reaction pH. The corresponding products are labeled as Cat-PAA(x)-y, where x is the protein to polymer mole ratio and y is the pH used for the synthesis. The coupling reaction consumed about 60−70% of the primary amines on the catalase; all samples were completely water-soluble and formed nanogels, as evidenced by gel electrophoresis and electron microscopy. The UV circular dichroism (CD) spectra indicated substantial retention of protein secondary structure for all samples, which increased to 100% with increasing pH of the synthesis and polymer mole fraction. Soret CD bands of all samples indicated loss of ∼50% of band intensities, independent of the reaction pH. Catalytic activities of the conjugates increased with increasing synthesis pH, where 55−80% and 90−100% activity was retained for all samples synthesized at pH 5.0 and pH 7.0, respectively, and the K_m or V_{max} values of Cat-PAA(100)-7 did not differ significantly from those of the free enzyme. All conjugates synthesized at pH 7.0 were thermally stable even when heated to ∼85−90 °C, while native catalase denatured between 55 and 65 °C. All conjugates retained 40−90% of their original activities even after storing for 10 weeks at 8 °C, while unmodified catalase lost all of its activity within 2 weeks, under similar storage conditions. Interestingly, PAA surrounding catalase limited access to the enzyme from large molecules like proteases and significantly increased resistance to trypsin digestion compared to unmodified catalase. Similarly, negatively charged PAA surrounding the catalase in these conjugates protected the enzyme against inhibition by negatively charged inhibitors such as ascorbate. While Cat-PAA(100)-7 did not show any inhibition by ascorbate in the presence of 270 μM ascorbate, unmodified catalase lost ∼70% of its activity under similar conditions. This simple, facile, and rational methodology produced thermostable, storable catalase that is also protected from protease digestion and ascorbate inhibition and most likely prevented the dissociation of the multimer. Using synthetic polymers to protect and improve enzyme properties could be an attractive approach for making "Stable-on-the-Table" enzymes, as a viable alternative to protein engineering.



INTRODUCTION

In our quest to establish robust alternatives to protein engineering and enhance the physical, chemical, and biochemical properties of enzymes,[1−4] we evaluated the influence of covalent conjugation of catalase with poly(acrylic acid) on the properties of catalase, including catalytic activity, thermal stability, long-term storage, and resistance to ionic inhibitors as well as degradation by proteases. Our hypothesis is that (1) covalent entrapment of an enzyme within a polyelectrolyte matrix would improve its thermal stability due to stratification of the protein structure by the polymer as a direct result of a reduction in enzyme conformational entropy, (2) the polymer shroud around the enzyme would protect it from bacterial attack or protease-degradation by preventing direct access to the peptide backbone of the enzyme, and (3) the negative charge field of the poly(acrylic acid) surrounding the enzyme would also repel anionic inhibitors and endow the enzyme with considerable protection. These ideas were tested using catalase as a model enzyme, a commercially important enzyme[5] with a very high turnover number. Catalase is an antioxidant[6] and converts hydrogen peroxide to water and molecular oxygen with a high rate and specificity.[7] Catalase is used extensively in the textile industry to remove toxic bleaching effluents and improve water recyclability.[8] These applications, however, are severely limited by catalase's poor thermal and storage stabilities, partly due to the dissociation of its subunits,[9] and

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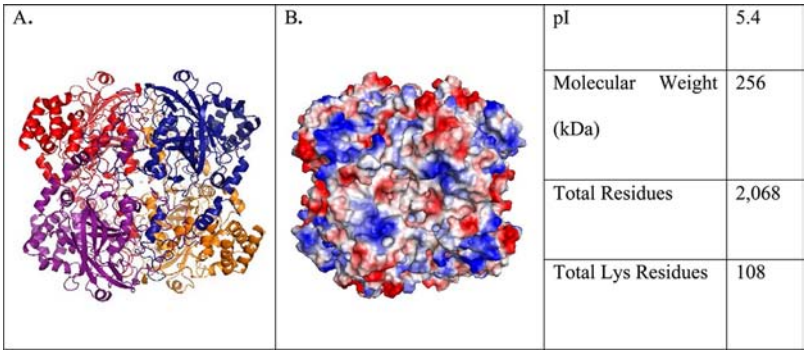
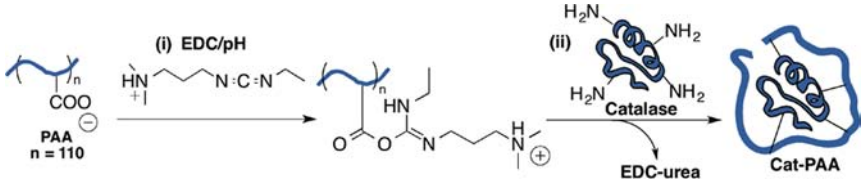
Chart 1. Key Properties of Catalase



Scheme 1. Synthesis of Cat-PAA Bioconjugate by Activation with a Water-Soluble Carbodiimide



sensitivity to proteases, bacteria, and inhibitors such as ascorbate or fluoride.[10] For example, it has a short half-life of only 6.7 h at 50 °C[11] or 2 weeks at 4 °C.[12,13]

Catalase has been immobilized and stabilized on alumina[8] and polyelectrolytes[14] by noncovalent interactions, while synthetic polymers,[15] silicates,[16] and polysaccharides[17] have been used for covalent conjugation chemistries. Covalent conjugation of catalase with polymers via site-specific attachment, in which certain protein residues are modified prior to attachment to the polymer, has been reported.[18−22] Binding of the enzyme on a flat surface may not prevent multimeric enzymes from dissociating into their subunits, and the effect of a support matrix or the appended polymer on the thermal stability, storage stability, or long-term activities of catalase, where the three-dimensional scaffold around the enzyme might stabilize it by inhibiting dissociation of the tetramer (Chart 1) has never been investigated. Going forward, random chemical attachment of polymers may afford catalase conjugates in scalable quantities. Additionally, the influence of random chemical conjugation of catalase with polyelectrolytes like PAA on stabilization of the 3D structure of catalase due to entropic effects, inhibiting the dissociation of its multimeric structure, protecting it from bacterial and proteolytic attack or inhibition by anionic inhibitors have never been investigated. Physical mixtures of the enzyme and PAA may improve the enzyme properties via confinement effects, but this advantage is lost quickly when the samples are diluted. Thus, stabilization by covalent conjugation with a polymer in a 3D environment can be useful to improve stability and lower operational costs by recycling the enzyme preparation.

In this work, we have chosen to synthesize catalase-poly(acrylic acid) bioconjugates to harness properties of both poly(acrylic acid) (PAA) and catalase. PAA (M_w 8000) is a negatively charged[23] polyelectrolyte that is hydrophilic, water-soluble,[24] and commercially available in large quantities.[25] PAA has one carboxyl group per monomer, which is suitable for conjugation with lysine side-chains of proteins using water-soluble carbodiimides.[26] Catalase-PAA (Cat-PAA) conjugates were synthesized by conjugation chemistry, and the influence of reaction pH and mole ratios of catalase to PAA on the properties of the conjugate was evaluated. Short/long-term stability, thermal stability, and protection against proteases were also investigated, and several Cat-PAA conjugates retained native-like structures and activities while also demonstrating significant improvement in these stabilities. Catalase activity is inhibited by fluoride,[27] azide,[28] ascorbate,[29] and copper(II).[30] We show that inhibition of Cat-PAA activity by anionic ascorbate is dramatically reduced, while that of cationic copper(II) was not. A number of evidences are presented to show that key properties of catalase are improved significantly by covalent conjugation with PAA.

RESULTS

The influence of reaction conditions on covalent conjugation of catalase to PAA and the resultant properties of the conjugate are presented here. Gel electrophoresis, electron microscopy, optical spectroscopy, stability, peptidase digestion, and inhibition studies showed that high stabilities are achieved while retaining native-like activity and structure.

Nomenclature. For clarity, each sample was labeled in terms of the mole ratio of protein to polymer and the pH of the reaction. For example, Cat-PAA(100)-6 refers to the conjugate obtained by reacting the enzyme with the polymer at a mole ratio of 1:100, at pH 6.0. In control studies, the corresponding physical mixtures were denoted with a slash, such as Cat/PAA(100)-6.

Synthesis of Cat-PAA Conjugates. PAA was activated with EDC (Scheme 1, step (i)) and then reacted with catalase (Scheme 1, step (ii)) at specific mole ratios (Supporting Information Table S1) for 4 h to form Cat-PAA conjugates. By tuning the pH, and by adjusting the concentration of EDC, the net charge on PAA-EDC intermediate was controlled for favorable interaction with anionic catalase. The reaction mixture was dialyzed to remove byproducts and unreacted EDC. Conjugation was carried out with increasing mole ratios of catalase:PAA (1:100, 1:500, or 1:1000) and at pH 5.0, 6.0, or 7.0 (see Supporting Information Table S2 for the ratios of primary amines to the carboxyls). All conjugates and their

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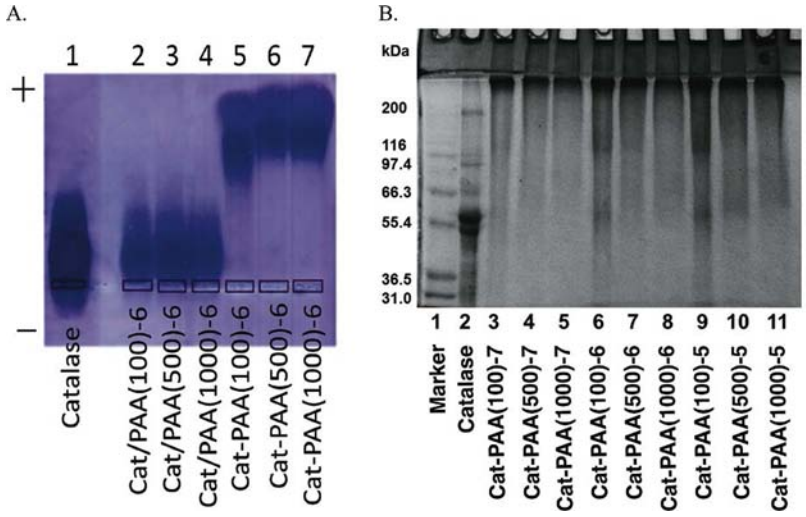

Figure 1. (A) Agarose gel of the Cat-PAA-6 (lanes 5−7), corresponding controls of catalase (lane 1, 10 μM), catalase/PAA mixtures (lanes 2−4), and (B) SDS/PAGE of the Cat-PAA conjugates synthesized at pH 7.0 (lanes 3−5), pH 6.0 (lanes 6−8), pH 5.0 (lanes 9−11), catalase (lane 2), and standard molecular weight marker (lane 1).

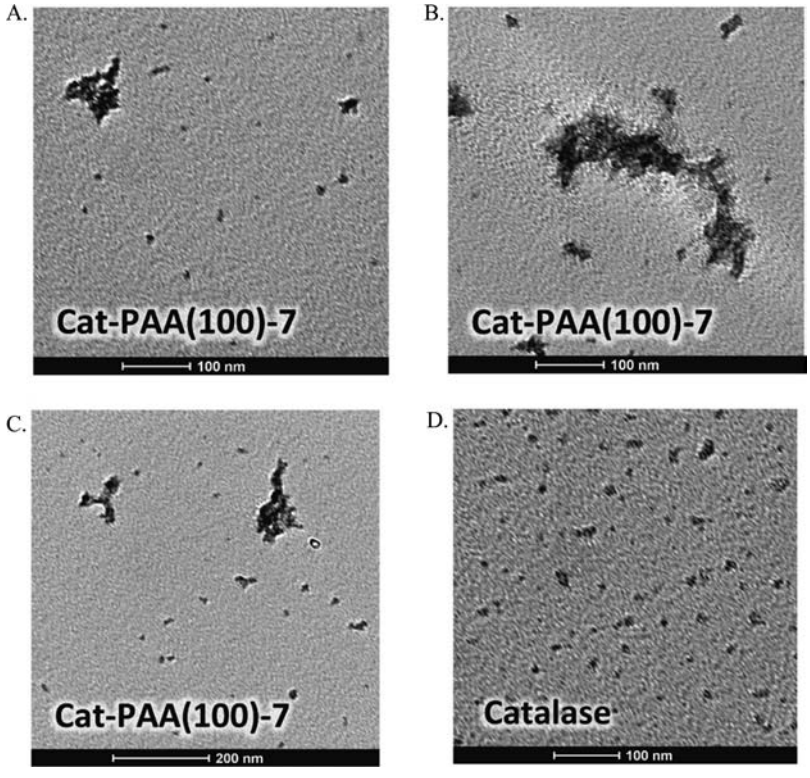

Figure 2. TEM of (A,B) Cat-PAA(100)-7 (0.01 μM, scale bar 100 nm magnification), and (C) Cat-PAA(100)-7 (0.01 μM, scale bar 200 nm) and (D) catalase (0.01 μM, scale bar 100 nm). Samples were dialyzed, dried, and then stained with Uranyl acetate for 30 min.

physical mixtures were completely water-soluble. Progress of the conjugation reaction was monitored by gel electrophoresis, as described below.

Agarose Gel Electrophoresis and SDS/PAGE. Covalent attachment of negatively charged PAA to catalase was expected to increase the net charge and increase the electrophoretic mobility, which was observed in agarose gel electrophoresis (Figure 1A). Mobilities of Cat-PAA(100)-6, Cat-PAA(500)-6, and Cat-PAA(1000)-6 (lanes 5−7, 10 μM protein) were greater than that of catalase (lane 1) or the corresponding physical mixtures (lanes 2−4). The electrophoretic mobilities of conjugates synthesized at pH 5.0 and 7.0 gave essentially the same results and they indicated near complete conversion to the conjugates (Supporting Information Figure S1).

SDS PAGE was also used to test the formation of the conjugates (Figure 1B), where the conjugates are not expected to move freely through the gel due to the covalently attached long chain polymer and also due to the possibility of covalent linking of the individual subunits of the multimeric enzyme. The SDS PAGE of catalase (lane 2), Cat-PAA synthesized at pH 7.0, 6.0, and 5.0 and with mole ratios of catalase:PAA of 1:100, 1:500, and 1:1000 (lanes 3−11, respectively; Figure 1B)

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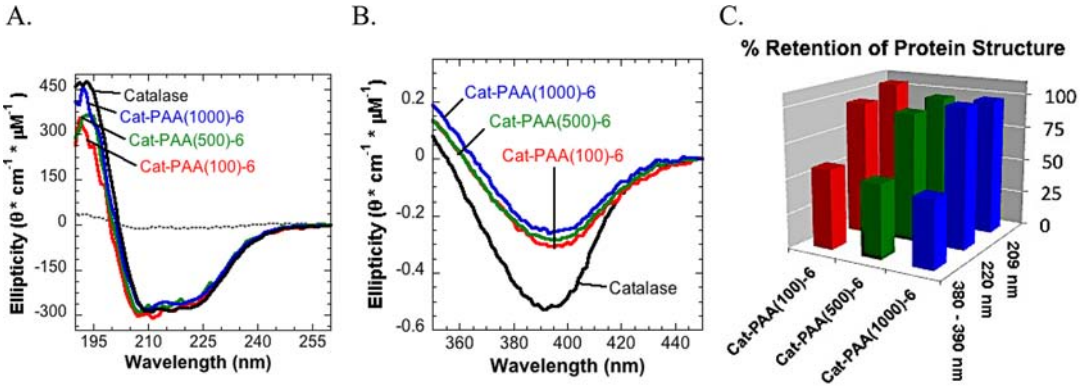

Figure 3. (A) UV CD of Cat-PAA(100)-6 (red), Cat-PAA(500)-6 (green), Cat-PAA(1000)-6 (blue), and unmodified catalase (black) in phosphate buffer (pH 6.0, 20 mM). Baseline is shown as the dashed gray line. (B) Soret CD of the samples as in panel A in phosphate buffer (pH 6.0, 20 mM). (C) Secondary structure retention as estimated from the CD data shown in panels A and B. All spectra were normalized with respect to protein concentrations and path lengths (1 μM and 0.05 cm, or 10 μM and 1 cm).

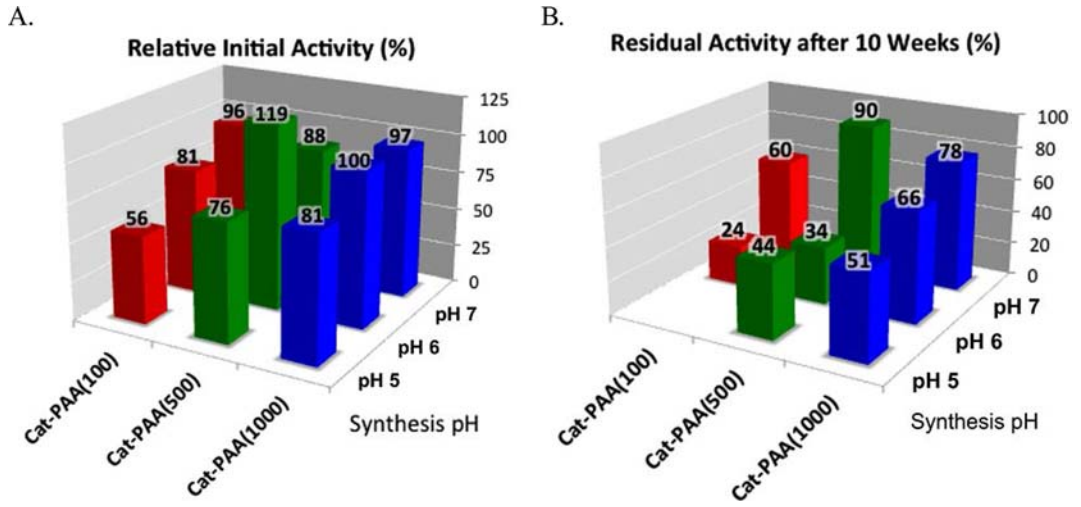

Figure 4. (A) Relative activities of Cat-PAA conjugates (0.01 μM enzyme, 5 mM H_2O_2 (5 mM, 20 mM phosphate buffer pH 7.0)); and activities estimated from the initial rate of the disappearance of H_2O_2, at room temperature. (B) Long-term activities of Cat-PAA samples after storing for 10 weeks at 8 °C in phosphate buffer (pH 5.0, 6.0, or 7.0, 20 mM). All have <15% error, as estimated from triplicate measurements.

indicated dark bands at the very top of the gel. Catalase had two major bands below 67 kDa (lane 2, expected M_w of ~64 kDa).[31] Cat-PAA(100)-5 and Cat-PAA(100)-6 (lanes 6 and 9) indicated small amounts of unreacted catalase, but all other samples indicated complete conversion to the conjugate which migrated more slowly than the unmodified catalase.

Transmission Electron Microscopy (TEM). The morphology and phase characteristics of Cat-PAA conjugates were analyzed by examining TEM images. Conjugation of catalase to PAA gave rise to nanogels, which appeared as dark protein clusters of variable shape and with sizes of 100−250 nm in diameter (Figure 2A−C, Cat-PAA(100)-7). Unmodified catalase had discrete protein clusters of uniform size that were approximately 20−30 nm diameter (Figure 2D). An image of the bare chip did not indicate any nanogel-like structures or artifacts (Supporting Information Figure S2).

Dynamic Light Scattering (DLS) Studies. The hydrodynamic radii of the conjugates were measured using DLS (Supporting Information Table S3) and all conjugates indicated a trimodal distribution of particle sizes, centered around 80(±18), 12(±3), and 2.4(±0.7) nm, independent of the synthesis pH or enzyme to PAA mole ratio. Unmodified catalase had a bimodal distribution of 55(±0.9) and 6(±3) nm, as reported.[10]

Protein Structure Retention. Circular dichroism (CD) spectra of the conjugates were measured in the UV (190−250 nm) and Soret (350−450 nm) regions to determine the secondary structure retention (Figure 3A) and perturbation of the heme environment (Figure 3B), respectively. Spectra of the conjugates (red, green, and blue lines) in 200−250 nm were all nearly identical to that of catalase (black line), with over 95% of structure retention.

Soret CD bands are sensitive to the protein structure, particularly around the heme-binding site. The Soret CD minima of Cat-PAA conjugates synthesized at pH 6 and pH 5 were red-shifted by 3 nm when compared to that of unmodified catalase (Figure 3B and Supporting Information Figure S3), and the depth of the minima decreased by ~40−60%, as compared in Figure 3C, while the remaining have been given in Supporting Information Table S4.

Number of Primary Amines Modified. The number of reactive amine groups consumed during the covalent conjugation was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) test.[32] A calibration curve was constructed using glycine standard (Supporting Information Figure S4A) and the

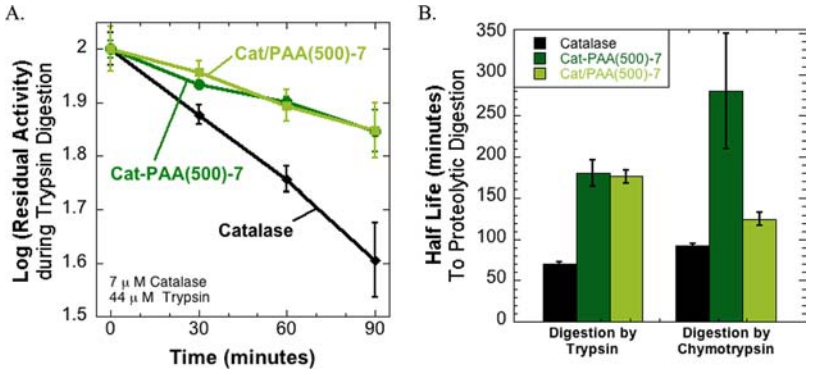

Figure 5. Resistance to trypsin (44 μM) digestion of catalase (7 μM) in phosphate buffer (pH 7.0, 20 mM) and Cat-PAA(500)-7 and the physical mixture. (A) Log of residual activities during digestion with trypsin plotted over time for Cat-PAA(500)-7 (dark green line), catalase/PAA(500)-7 (light green line), and catalase (black line). (B) Half-lives of Cat-PAA(500)-7 (dark green), catalase/PAA(100)-7 (light green), and catalase (black) while in the presence of trypsin or chymotrypsin.

calibration plot has been used to estimate the number of primary amines before and after covalent conjugation (Supporting Information Figure S4B). The relative numbers of primary amines estimated using 0.8 μM enzyme concentration were 10.6 for unmodified catalase, 3.2($\pm$0.8) for Cat-PAA(100)-7, 2.8($\pm$0.1) for Cat-PAA(500)-7, and 3.9($\pm$0.1) for Cat/PAA(1000)-7. Thus, there has been a considerable reduction (~60−70%) in the number of primary amines after PAA conjugation, supporting the notion that a polymeric network formed where the enzyme served as a cross-linker between multiple polymer chains.

Enzymatic Activities. To determine whether PAA adversely affected the functional properties of catalase, activities of Cat-PAA conjugates were measured and compared with unmodified catalase. The decomposition of H_2O_2 (substrate) by the protein was followed by monitoring the decrease in substrate absorbance at 240 nm as a function of time (Supporting Information Figure S5). Cat-PAA(100)-6 (red line) showed a slightly decreased initial activity compared to unmodified catalase (black line) and that of the catalase/PAA(100)-6 (pink line) physical mixture.

Specific activities were calculated by measuring the initial rates from the linear portions of the kinetic traces. The rates were then normalized against the specific activity of unmodified catalase to obtain relative activity. Relative activities of all Cat-PAA conjugates, measured in triplicates with <15% error, are compared with that of catalase in Figure 4A. Relative initial activities for the conjugates synthesized at pH 5.0 ranged from ~55% to 80% with increasing concentration of PAA. The activities of the samples synthesized at pH 6.0 and 7.0 ranged ~60−90%. Overall, conjugates synthesized at pH 7.0 had the highest activity with least variability.

The enzymatic activities depend on the rates of several steps in the catalytic cycle and insight into these steps was gained by measuring the Michaelis constant (K_M) and maximum initial velocity (V_{max}). Using the initial rates determined with increasing substrate concentrations, Lineweaver−Burk plots (plot of inverse of initial rate vs inverse of substrate concentration) were constructed using the equation

$$1/V = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

where $[S]$ = substrate concentration, V = initial reaction rate, K_M = Michaelis constant, and V_{max} = maximum initial velocity (Supporting Information Figure S6). The corresponding K_m and V_{max} values were found to be 57 mM and 0.011 s^{-1} for Cat-PAA(100)-7, 100 mM, and 0.016 s^{-1} for catalase/PAA(100)-7, and 45 mM and 0.011 s^{-1} for unmodified catalase. Thus, covalent modification had very little influence on substrate affinity or the V_{max}. These data clearly support the notion that the conjugated enzyme is nearly intact, and this will be possible only if the conjugated enzyme maintained its multimeric state.

Stability at 8 °C. Wrapping the enzyme in the PAA matrix was expected to enhance the long-term storage stability. Enzyme activities were measured after storage at 8 °C for 10 weeks in 20 mM phosphate buffer at the pH of synthesis (5.0, 6.0, or 7.0). Example kinetic traces of the Cat-PAA(100)-7 and catalase control after 10 weeks of storage are shown in Supporting Information Figure S7. The initial activities were compared with the activities of corresponding freshly synthesized Cat-PAA conjugates. All measurements were done in triplicate, which indicated errors <15%. Cat-PAA conjugates synthesized at pH 5.0 maintained ~50% of their original activity after 10 weeks, while those synthesized at pH 6.0 maintained 20−65% of their original activity. Those synthesized at pH 7.0, however, maintained 60−90% of their original activity. Additionally, the retention depended on polymer mole ratio. Cat-PAA(500)-7, for example, maintained nearly 90% of its original activity under these conditions. In sharp contrast, unmodified catalase had 0% activity after 2 weeks of storage at 8 °C, under the same conditions (Supporting Information Figure S6B). Thus, the bioconjugate had superior storability under ordinary refrigeration conditions.

The stabilities of Cat-PAA(100)-7 and catalase were also examined at two different concentrations (1 μM and 10 μM) and at 50 °C to further test if the enhanced stability is due to the prevention of the dissociation of the enzyme tetramer. We found that the deactivation rate has been independent of the conjugate initial concentration (Supporting Information Figure S8) which suggests that the enzyme may be present in its multimeric state, consistent with earlier observations. At both enzyme concentrations, Cat-PAA(100)-7 retained ~60% of its initial activity after 80 min at 50 °C, while catalase retained only ~25% of its original activity, under the same conditions.

Stability Against Proteolysis. Proteases, enzymes that degrade proteins by hydrolysis, are ubiquitous, and these often limit the solution phase stability of many proteins. We hypothesized that the synthetic polymer would resist protease digestion and prevent the access of proteases to the encased catalase. We therefore examined the rates of digestion of

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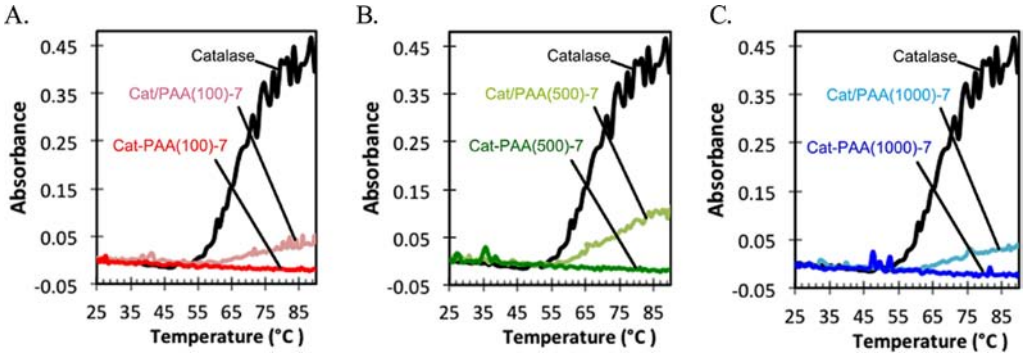

Figure 6. Resistance to thermal denaturation: Plots of enzyme absorbance at 280 nm as a function of temperature for Cat-PAA conjugates synthesized at pH 7.0 where the protein to polymer mole ratios were (A) 1:100, (B) 1:500, and (C) 1:1000. Comparison with that of catalase (black curve) and the corresponding physical mixtures (light pink, light green, and light blue) show that all three conjugates (red, green, and blue) were highly stable under these conditions.

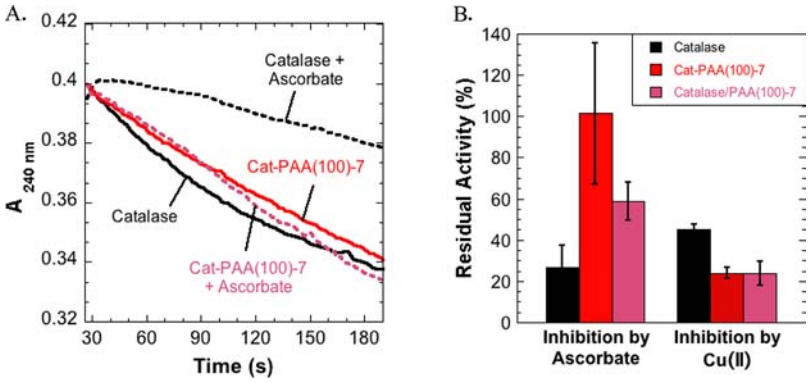

Figure 7. Protection against ascorbate inhibition: (A) Rate of decomposition of hydrogen peroxide (5 mM) by Cat-PAA(100)-7 in the presence (pink dashed line, 0.01 μM enzyme) or absence (red solid line) of ascorbate (270 μM) (Tris buffer, pH 7.0, 50 μM) incubated at ~37 °C for 40 min. Controls are represented by black thick and black dashed lines. (B) Relative residual activities of conjugate Cat-PAA(100)-7 (red), catalase (black), and the catalase/PAA(100)-7 physical mixture (pink) in the presence of anionic ascorbate and cationic copper(II).

conjugates by trypsin (Figure 5A) and chymotrypsin (Supporting Information Figure S9). The catalytic activities decreased logarithmically with time. Residual activity of any sample withdrawn at time t was calculated by comparing its activity with the activity of the same sample at 0 min. Half-lives were calculated from the slope of the plot of logarithm of residual activity vs incubation time (Figure 5B and Supporting Information Figure S9).

Resistance to proteolysis was determined from the slopes of the lines in Figure 5A for each sample and compared with that of catalase. After 90 min of digestion by trypsin, Cat-PAA(500)-7 retained more than 80% of its original activity, while catalase lost most of its activity. Similarly, after 90 min of digestion by chymotrypsin, Cat-PAA(500)-7 retained more than 80% of its original activity, while catalase retained about 45% of its original activity (Supporting Information Figure S9). The half-life of Cat-PAA(500)-7 conjugate against trypsin digestion increased to 176 min from the limited half-life of 70 min observed for the unmodified catalase. Similarly, the half-life of the conjugate for chymotrypsin digestion increased to 125 min from 90 min measured for unmodified catalase, which illustrates the protection due to wrapping of the enzyme in the polymer matrix.

Stability Toward Thermal Denaturation. Cross-linking the enzyme surface functional groups with PAA was expected to lower the conformational entropy of the denatured state and thereby increase thermal stability of the bioconjugate. The thermal denaturation curves were obtained by monitoring enzyme absorbance at 280 nm as a function of temperature for three of the conjugates, chosen as representative examples (Figure 6A–C).

The absorbance of catalase increased substantially around 68 °C (black line in all three panels), which corresponded to the denaturation temperature.[33] Cat-PAA(100)-7 conjugates (red, green, or blue), on the other hand, showed little or no change in their absorbance upon heating over this temperature range. The absorbance of the corresponding physical mixtures of catalase and PAA also showed substantial change in absorbance over this temperature range (light pink, light green, and light blue lines). Thus, covalent conjugation to PAA improved the temperature of denaturation of catalase, as expected.

Protection Against Negatively Charged Inhibitor. The presence of anionic polymer shroud around the enzyme was expected to repel the anionic inhibitors and thereby protect enzyme activities. This expectation was tested using ascorbate, a known inhibitor of catalase.[29] Interestingly, Cat-PAA(100)-7 retained nearly 100% of its original activity in the presence of the ascorbate (270 μM) while the addition of ascorbate reduced catalase activity by 70% (Figure 7A, black solid line and black dashed lines, respectively).

The relative residual activities of the samples are compared in Figure 7B. Overall, the PAA shroud protected the enzyme against the anionic inhibitor to a significant extent. If electrostatic repulsion was the primary method by which

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PAA protected catalase from anionic ascorbate, it was expected that the cationic inhibitors would have the opposite effect. The positively charged inhibitor copper(II) was used to examine this hypothesis. In the presence of copper(II), Cat-PAA(100)-7 conjugate retained only 20% of its original activity while unmodified catalase retained ~50% activity (Figure 7B), which agrees well with our hypothesis. Thus, anionic PAA protected catalase from similarly charged inhibitors primarily due to electrostatic repulsion.

DISCUSSION

Protein−polymer conjugates are often prepared by site selective methods in which telechelic end groups of polymers are used as functional groups for covalent attachment to a specific site on the protein. This chemistry, however, does not allow for conjugation of more than one polymer chain per protein, either by multiple attachments on a protein or multiple sites on the polymer. Attachment of multiple polymer chains onto a protein through multiple sites on both protein and the polymer by nonspecific but controlled conjugation methods may result in products with different properties than those obtained from site-specific attachment or attachment at a few sites. Additionally, random, nonspecific conjugation chemistry is readily scalable, which will be cost-effective for practical applications. The synthetic polymer could play a key role in regulating the transport of small molecules into or out of the nanogel, and is expected to be biologically stable while providing a shroud of protection. In keeping with these ideas, we report here nonspecific, controlled, random conjugation via EDC chemistry that produces Cat-PAA conjugates that can be stored long-term, have high thermal stabilities, and are exceptionally resistant to proteases and ascorbate inhibition.

The synthesis pH appeared to have significant influence on activity/structure retention of the conjugates—pH 6.0 or 7.0 was essentially benign, while pH 5.0 was not. One reason for this could be that the enzyme structure was frozen to some degree at the synthesis pH by the covalent linkage of its lysine side chains with the carboxyl groups on the polymer. This deduction is supported by the fact that catalase's optimum pH for catalysis is around 7.0,[12] which corresponds to the conjugates with highest activity.

Complete conjugation of catalase with PAA was confirmed for all catalase:PAA mole ratios by agarose gel electrophoresis and SDS/PAGE. When compared to catalase and the catalase/PAA physical mixtures, conjugates showed increased mobility toward the cathode in the agarose gel because of the negative charge carried by the PAA chains attached to the protein. The physical mixtures showed similar electrophoretic mobilities to that of catalase because physical association between catalase and PAA was weak and the disappearance of the catalase bands after conjugation indicated complete conversion to Cat-PAA conjugates. Because the estimated average pore size of the agarose gel was 450 nm[34] and the conjugates moved substantially in the gels, we suspected that these were likely to be smaller than 450 nm in size. The TEM data and DLS data were in agreement with these inferences.

Covalent conjugation consumed reactive primary amines, as determined from the TNBS assay, and nearly 60−70% of the primary amines present in the enzyme were not accessible in the conjugate. Thus, there has been extensive linking between the enzyme and the polymer, which resulted in nanogels seen in the TEM and supported by DLS data. All conjugates were completely soluble without any precipitation for 10 weeks or longer, and after storing at 8 °C for 10 weeks (Cat-PAA(500)-7 stored at pH 7.0) retained 60−90% of initial activity, while unmodified catalase completely lost its activity under the same conditions after only 2 weeks. The enhanced stability of the conjugates may be attributed to the PAA's ability to protect the protein from bacteria and protease digestion, and inhibition of the dissociation of its multimeric state. These are the first examples of introducing extended stability and resistance to protease digestions of catalase by conjugation with PAA.

The rate of protease digestion of the protein in these nanogels was expected to decrease in the presence of PAA, which creates a protective cross-linked network and serves as a physical barrier around the protein, slowing the rate of diffusion of proteases or bacteria toward the entrapped enzyme.[35] We chose two representative examples to test these ideas and exposed catalase, Cat-PAA(500)-7, and the corresponding catalase/PAA physical mixture (1:500, pH 7.0) to trypsin and chymotrypsin for 90 min. Thus, there have been considerable improvements in resistance for digestion of the conjugate by both protease enzymes, trypsin, and chymotrypsin. These studies indicate that wrapping the enzyme with the synthetic polymer endowed greater resistance to degradation by proteases. This could be vital for biological applications of enzyme conjugates, where the samples are often exposed to high levels of proteases. The diffusion of trypsin or chymotrypsin through the PAA gel is perhaps too slow to effectively inactivate the protein, due to the sterically crowded substrate, independent of the pI of the protease.

Proteins have limited thermal stability, which is a major concern that limits their utility. We previously showed that conjugation to PAA stabilized hemoglobin against steam sterilization, but hemoglobin−PAA conjugate denatured around the same temperature as the unmodified protein.[4] Since a larger number of lysine residues are present on catalase (108) than hemoglobin (48), we expected a greater number of linkages between the protein surface and the polymer, which would result in a substantial decrease in the conformational entropy of the protein's denatured state. This, in turn, should enhance the thermal stability of the Cat−PAA conjugates well beyond that of the corresponding hemoglobin−PAA conjugate. Cat-PAA(100)-7, Cat-PAA(500)-7, and Cat-PAA(1000)-7 were chosen as representative examples, and did not show any signs of denaturation upon heating to 95 °C. The corresponding physical mixtures indicated some signs of denaturation, while catalase was completely denatured around 68 °C. Thus, the thermal stability of the protein was enhanced to a significant extent by the polymer matrix, which could be partly attributed to the inhibition of the dissociation of the multimeric enzyme due to the surrounding polymer scaffold.

Finally, the strong negative charge of PAA in the nanogel (pH 7.0) was expected to repel negatively charged inhibitors, a novel property of the conjugate that effectively keeps negatively charged species from interacting with catalase by repulsive electrostatic forces. This idea was tested by monitoring the ability of ascorbate to inhibit catalytic activity of Cat-PAA(100)-7. Ascorbate normally binds to catalase after a peroxide enters the active site and reduces the heme iron from Fe(V) to Fe(IV).[36] The repulsive charge interaction between the negatively charged inhibitor and the negatively charged PAA was strong enough that ascorbate did not substantially inhibit the activity of the conjugate. The activity of unprotected catalase, however, was reduced by nearly 70%. On the other hand, inhibition by Cu(II) was not impeded and the conjugate

was inhibited more than the unmodified enzyme. This simple concept of attaching a charged polymer to any protein at multiple sites can be used to alter its sensitivity toward particular inhibitors or to alter the selectivity toward specific substrates. This provides a powerful tool to control the catalytic activities of polymer-conjugated enzymes. To the best of our knowledge, no such reports are available in the literature. When tested further, current approach could significantly enhance the ways in which a protein's properties can be manipulated in a rational, predictable manner by bioconjugation methodologies.

CONCLUSIONS

The current approach of using the lysine residues on catalase for conjugation with the COOH groups of PAA by EDC chemistry is a mild, robust, and efficient synthetic protocol to prepare stable and active Cat−PAA conjugates. The formation of water-soluble nanogels was supported by agarose gel electrophoresis and TEM studies. Quantitative conjugation was achieved, at three different pH (5.0, 6.0, and 7.0) conditions, while using three vastly different mole ratios of enzyme to polymer, without perturbation of the enzyme catalytic site or access to the substrate. Retention of activity is essential for practical applications, and extensive chemical modification is often deleterious. In our case, even conjugates with 1000-fold excess PAA retained 75−100% of the activity of unmodified enzyme with the exception of Cat-PAA(100)-5. More importantly, these Cat−PAA conjugates remained soluble and retained 25−90% activity when stored for 10 weeks at 8 °C, while catalase denatured completely and precipitated within just 2 weeks. The physical barrier of the polymer network around the enzyme could also be important in preventing the dissociation of the multimeric enzyme and maintaining its activity, and several factors are presented in this regard.

The long-term stability of the conjugates is strongly dependent on the synthesis pH and enzyme to polymer mole ratio. Conjugates synthesized at pH 5.0 and low polymer mole fractions resulted in distortion of enzyme structure and some loss in storage stability. This strategy of improving enzyme stability with the polymer matrix was further confirmed by thermal denaturation studies, where the conjugates did not show any signs of denaturation after heating to 95 °C. This may be attributed to the large number of lysine residues (108) on catalase, which were sufficient to induce a suitable number of cross-links between the protein and the polymer such that thermal denaturation was inhibited to a significant extent. Thus, the number of potential sites for conjugation plays an important role in stabilizing the enzyme against heat. Consistent with these conclusions regarding the importance of the gel network around the enzyme, Cat-PAA(500)-7 was 2.5-fold more resistant to trypsin digestion than unmodified catalase. The diffusion of the large protease into the gel network was expected to be slow, and protease digestion could thus be a diagnostic tool to predict stability of the conjugate. Furthermore, this negatively charged gel network of PAA repelled anionic ascorbate and protected the encased catalase against inhibition. Thus, (1) enzyme inhibition by particular inhibitors can be controlled in a predictable and rational manner, and (2) the polyelectrolyte gel matrix provides an electrostatic control over the interactions of small ions with the enzyme. Based on these results, one could predict that positively charged substrates should react faster with the PAA-encased enzymes, as they could be favorably sequestered by the anionic gel matrix. We tested this hypothesis with Cu(II) and it inhibited the conjugate more than the unmodified enzyme supporting the idea that charge could be important here.

In closing, random conjugation of catalase to PAA resulted in nanogels, which showed excellent thermal stability, improved storage stability, enhanced resistance to protease digestion, possible retention of its multimeric state, and high activity retention when compared to catalase. All of the conjugates demonstrated some residual activity after 10 weeks of storage at 8 °C. The charged groups of the polyelectrolyte shroud could be exploited to control the interactions of small ions, inhibitors, and charged substrates with enzymes. Finally, we conclude that the strategy demonstrated here brings us one step closer to designing enzymes that could be "Stable-on-the-Table".

MATERIALS AND METHODS

Materials. Catalase (bovine liver) and trypsin (bovine pancreas) and chymotrypsin (bovine pancreas) were purchased from Worthington Biochemicals (Lakewood, NJ). 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) was purchased from TCI America (Portland, OR). Poly(acrylic acid) (PAA, 45 wt %, MW 8000), L-ascorbic acid, glycine, picrylsulfonic acid solution (5%(w/v) in methanol), copper(II) nitrate trihydrate, and buffer solids were purchased from Sigma (St. Louis, MO). Dialysis membrane (25 kDa MWCO) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Biology grade agarose was purchased from Hoefer Inc. (Holliston, MA). Hydrogen peroxide (H_2O_2 30%) was purchased from Fischer Scientific (Waltham, MA).

Instruments. Absorption spectra were recorded on a HP8453 UV−visible spectrophotometer (Agilent Technologies, Santa Clara, CA). Catalase concentration was determined using the extinction coefficient at 280 nm, 420 000 M^{-1} cm^{-1}. All data was plotted using Kaleidagraph v 4.1.3.

Synthesis. The protein−polymer conjugates were synthesized using EDC[4] as the coupling agent, and carried out as reported previously with minor modifications. Synthesis procedure and purification can be found in Supporting Information Section S1.1. Reaction conditions are summarized in Supporting Information Table S1.0. Before the assay, all samples were centrifuged to remove any precipitated protein.

Characterization. Agarose gels and SDS gels were run to evaluate the presence of unreacted catalase after conjugation. The gels were prepared by standard procedures, detailed in Supporting Information Sections S1.2 and S1.3, respectively. Agarose gels were run in a horizontal gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc., Grand Island, NY). Sodium dodecyl sulfate (SDS) gels were run in a Bio-Rad Mini-Protean electrophoresis apparatus. Undialyzed samples were loaded into the gels (10 μM catalase).

Transmission electron microscopy (TEM) was used to determine sample morphologies. Tecnai T12 instrument operating at an accelerating voltage of 120 kV was used to obtain images. Catalase concentrations were 0.01 μM, and other parameters can be found in Section S1.4.

Nanogel sizes were confirmed using Precision Detectors CoolBatch+ dynamic light scattering (DLS) apparatus (Varian Inc., Santa Clara, CA). Standard procedures detailed in Section S1.5.

Retention of secondary structure was found by measuring the circular dichroism (CD) spectra with a JASCO model J715 spectropolarimeter (Easton, MD). Details on standard instru-

ment parameters can be found in Supporting Information Section S1.6.

To determine the enzymatic activity of undialyzed samples, the decomposition of hydrogen peroxide (H_2O_2) was followed from reported methods[37] with a 96-well plate reader (Flex Station 3 by Molecular Devices, LLC). Concentrations and conditions for the assay are recorded in Supporting Information Section S1.7.

To evaluate the extent of modification of primary amines, trinitrobenzenesulfonic acid (TNBS) assay was used from reported methods.[32,38]

Evaluation of Enzyme Properties. The resistance of catalase to protease digestion via trypsin was determined from reported methods, with minor modifications found in Supporting Information Section S1.8.[39] The catalytic activities of samples were measured as in Supporting Information Section S1.7. Samples were stored for 10 weeks at 8 °C in 20 mM Na_2HPO_4 buffer of the same pH as synthesis (5.0, 6.0, or 7.0).

Enhanced thermal stabilities of the conjugates were evaluated by taking the absorption spectra at 280 nm with increasing temperature at 5 s intervals. Each sample (1 μM) was gradually heated from 25 to 90 °C over 20 min with stirring. Temperature of the sample cell was measured directly by using a digital thermometer.

The interaction between negatively charged Cat−PAA conjugate and a charged inhibitor was established by measuring enzymatic activity in the presence of ascorbate or copper(II). Molar ratios and effective inhibiting conditions were taken from previously reported methods.[40,30] Specific reaction conditions are detailed in Supporting Information Section S1.9. Peroxidase activity of each sample was measured in triplicate as in Supporting Information Section S1.7.

■ ASSOCIATED CONTENT

Supporting Information

Experimental conditions for the synthesis of the conjugates and characterization, as well as agarose gel, TEM, and DLS, Soret CD, percent retention of protein structure, and initial and long-term kinetic traces for activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Poly(acrylic acid), PAA; 1-Ethyl-3-(3-(dimethylamino)propyl)-carbodiimide, EDC; Catalase-PAA conjugate, Cat-PAA

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