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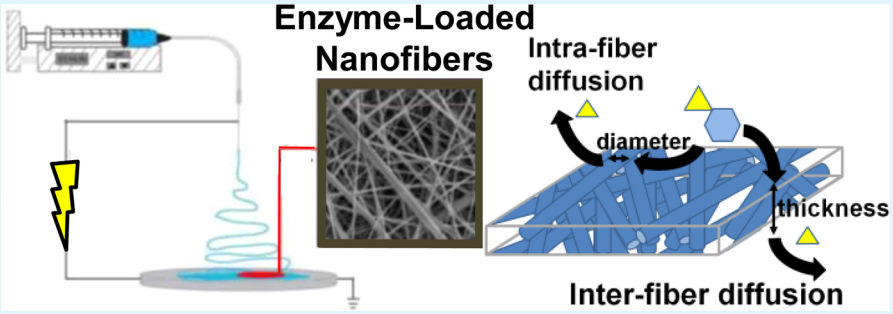
Cross-linked Polymer Nanofibers for Hyperthermophilic Enzyme Immobilization: Approaches to Improve Enzyme Performance

Christina Tang,[†] Carl D. Saquing,[†,‡] Stephen W. Morton,[†] Brittany N. Glatz,[†] Robert M. Kelly,[†] and Saad A. Khan*[,†]

[†]Department of Chemical and Biomolecular Engineering North Carolina State University, Raleigh, North Carolina 27695, United States

[‡]DuPont Central Research and Development, 200 Powder Mill Road, Wilmington, Delaware 19880-0304, United States

Supporting Information



ABSTRACT: We report an enzyme immobilization method effective at elevated temperatures (up to 105 °C) and sufficiently robust for hyperthermophilic enzymes. Using a model hyperthermophilic enzyme, α-galactosidase from *Thermotoga maritima*, immobilization within chemically cross-linked poly(vinyl alcohol) (PVA) nanofibers to provide high specific surface area is achieved by (1) electrospinning a blend of a PVA and enzyme and (2) chemically cross-linking the polymer to entrap the enzyme within a water insoluble PVA fiber. The resulting enzyme-loaded nanofibers are water-insoluble at elevated temperatures, and enzyme leaching is not observed, indicating that the cross-linking effectively immobilizes the enzyme within the fibers. Upon immobilization, the enzyme retains its hyperthermophilic nature and shows improved thermal stability indicated by a 5.5-fold increase in apparent half-life at 90 °C, but with a significant decrease in apparent activity. The loss in apparent activity is attributed to enzyme deactivation and mass transfer limitations. Improvements in the apparent activity can be achieved by incorporating a cryoprotectant during immobilization to prevent enzyme deactivation. For example, immobilization in the presence of trehalose improved the apparent activity by 10-fold. Minimizing the mat thickness to reduce interfiber diffusion was a simple and effective method to further improve the performance of the immobilized enzyme.

KEYWORDS: *enzyme immobilization, nanofibers, electrospinning, biocatalysts, mass transfer*

INTRODUCTION

Electrospinning is a simple technique used to generate nanofibrous membranes and nanofiber composites.[1−5] Briefly, a high voltage (1−30 kV) is applied to a polymer solution or melt to induce the formation of a liquid jet, which is continuously extended and deposited on a substrate as random, nonwoven nanofibers. The materials produced using this method have exceptional specific surface area and may be of use in a broad range of applications, including as solid supports for immobilization of catalysts and biocatalysts. For example, enzymes are highly efficient and selective biocatalysts. Practical application of enzymes is often limited by lack of enzyme stability, which can be addressed by utilizing enzymes with intrinsic thermostability, such as those from hyperthermophilic microorganisms that can be further stabilized through immobilization.[6−9] Additional advantages of immobilization include avoidance of product contamination and ease of recovery of the biocatalysts, which facilitates reuse. One of the main challenges of immobilization is that it often results in an apparent decrease in catalytic activity of the enzyme, which depends greatly on the structure (size and shape) of the support material.[10,11] Herein, we explore the use of electrospun nanofibers as support materials for enzyme immobilization with careful attention to potential enzyme deactivation and mass transfer limitations.

Enzyme immobilization via covalent attachment to the surface of nanofibers has been achieved. However, these processes generally utilize hydrophobic materials which often require modification to increase the biocompatibility of the surface and enzyme loading is limited.[12] Immobilization by

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encapsulation within electrospun nanofibers via coating enzyme loaded fibers with a water insoluble polymer using chemical vapor deposition,[13] coaxial electrospinning,[14,15] or emulsion electrospinning[16−20] has also been reported. Recently, coaxial electrospinning has also been used to encapsulate multienzyme systems.[15] However, coaxial electrospinning requires a complicated setup and control of multiple feed rates. Furthermore, coaxial and emulsion electrospinning involve the use of water insoluble materials that may cause conformational changes to the enzyme, resulting in deactivation due to poor biocompatibility. To avoid the use of hydrophobic materials with poor biocompatibility, we focus on immobilization by entrapment within chemically cross-linked biocompatible polymer nanofibers that can accommodate high enzyme loadings.[11,21] Entrapment is achieved by electrospinning a blend of water-soluble polymer and enzyme and subsequently cross-linking the polymer entrapping the enzyme in a water-insoluble fiber.

In related work, a handful of studies have begun to explore the use of chemically cross-linked PVA-based nanofibers for immobilization of noble metal nanoparticle catalysts[22−25] as well as biocatalysts[10,12,22,26,27] with promising results. The high specific surface area of the nanofibers offered improved performance when compared to film support materials.[10,22,27] For example, lipase immobilized in PVA nanofibers cross-linked with MDI in THF was 6 times more active than a film prepared in a similar manner.[27] Additionally, immobilization facilitates recovery and reuse of the catalyst.[10,22,26] Immobilized acetylcholinesterase retained more than 70% of the original activity after 10 reuses,[26] whereas the immobilized metal nanoparticles were reused up to three times without significant deterioration of catalytic performance.[26] Additionally, entrapment within PVA nanofibers improved biocatalyst stability at elevated temperatures (up to 40 °C).[11,26]

The main challenge in this approach has been a decrease in apparent catalytic activity upon immobilization,[10,11,26,27] in some cases by up to 2 orders of magnitude when compared to the free enzyme.[27] The cause of the loss in activity remains poorly understood. Enzyme deactivation could occur due to conformational changes during the immobilization process.[10−12] Mass transfer may limit the accessibility of the substrate to the active site, reducing the apparent activity of the immobilized enzyme.[10−12] Improvements in apparent catalytic activity upon immobilization may be possible if the cause of the loss of activity is determined.

In this study, we immobilized a model hyperthermophilic enzyme by entrapment within a chemically cross-linked polymer nanofiber by electrospinning a PVA/enzyme solution followed by chemically cross-linking the fibers with glutaraldehyde. By examining the effect of the reagents used in the cross-linking reaction, the importance of deactivation on the loss of activity upon immobilization relative to mass transfer limitations was established. On the basis of this understanding, we used additives as well as minimized mat thickness as simple and effective methods to improve the apparent activity of the immobilized enzyme.

EXPERIMENTAL SECTION

Materials. Poly(vinyl alcohol) (Mowiol 40−88, average molecular weight 205 000 g/mol, 88% hydrolyzed), hydrochloric acid, sodium chloride, *p*-nitrophenyl-α-D-galactopyranoside, trehalose dihydrate, acetone (ACS reagent grade) were obtained from Aldrich (St. Louis, MO). Sodium acetate and raffinose pentahydrate were obtained from Alfa Aesar (Ward Hill, MA). Sodium carbonate and glycerol (anhydrous) were from BDH (Poole, U.K.) and Fluka (Steinheim, Germany), respectively. Acetic acid and glutaraldehyde (50% aq) were received from Acros (Geel, Belgium). Growth media, other buffer reagents (Tris base, phosphate buffered saline), were obtained from Fisher Scientific and Sigma-Aldrich. All materials were used as received.

Protein Expression/Purification. α-Galactosidase from *Thermotoga maritima* was expressed in *E. coli* and purified as previously described.[28] Briefly, *E. coli* containing the α-galactosidase gene (MSB8, ORF TM1192) was cloned and inserted into pET24d (Novagen) and transformed into *E. coli* that was grown in laboratory scale fermentors (working volume ∼1.8 L) in Luria broth with kanamycin (50 mg/L) at 37 °C overnight. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were lysed and centrifuged to remove cell debris. For further purification the supernatant was filtered and heat treated at 70 °C for 20 min to denature most *E. coli* proteins, which were removed by centrifugation and sterile filtering. A portion of the resulting material was stored at 4 °C and used for further experiments (designated as heat treated), while the other portion was purified further using anion exchange chromatography and designated AEC purified. For each level of purification, protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Samples before and after purification were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis using precast polyacrylamide gels. Results from the purification are included in the Supporting Information (Table S.1, Figure S.1)

Immobilization. Immobilization of α-gal within electrospun nanofibers required two steps. First, an aqueous solution of PVA and α-gal was prepared and electrospun to physically entrap the enzyme within the solid fiber. Next, the as-spun α-gal loaded fibers produced by electrospinning were chemically cross-linked with glutaraldehyde. Because glutaraldehyde can also react with the amine groups of the enzyme chemical cross-linking of the polymer to render the fiber insoluble may also result in covalent binding of the enzyme to the polymer support. During the immobilization process, we do not observe any evidence of the formation of cross-linked enzyme aggregates.

Electrospinning. Aqueous PVA solutions were prepared by stirring mixtures of PVA and deionized water at 60 °C until homogeneous and stored at 4 °C until further use. In some cases, we varied the solvent of the electrospinning solution. In these cases, PVA was combined with the specified solvent (i.e., 200 mM raffinose, 1 M Trehalose, 1 M glycerol, 50 mM Tris buffer (pH 8.2), 58.3 mM sodium acetate buffer (pH 5.5), 10 mM phosphate buffered saline (pH 7.4), 0.1 wt % NaCl, or 1.0 wt % NaCl) and stirred at 60 °C until homogeneous, and stored at 4 °C. PVA and α-gal were combined in appropriate proportions at room temperature and stirred briefly. The amount of PVA used is reported as mass of PVA per mass of total solution, typically 7 wt % PVA based on previous work[21,29] (details of the effect of PVA concentration are presented in Figure S2 in the Supporting Information). The enzyme loaded is determined by the amount of enzyme in initial blend that is electrospun (mass of enzyme per mass of polymer).

$$\text{enzyme loading (\%)} = \frac{\text{mass of enzyme in the electrospinning solution}}{\text{mass of PVA in the electrospinning solution}}$$

No loss of enzyme is expected using nonsolvent cross-linking; therefore, the effective enzyme immobilized is the enzyme loading based on previous studies.[11]

In some cases, the pH of the solutions was adjusted by adding small amounts of HCl and stirring briefly. The pH was measured with a Horiba Twin B-213 pH meter. The zero-shear viscosity of the electrospinning solutions was measured at 25 °C using a TA Instruments AR-G2 rheometer using a 40 mm diameter, 2° cone and plate geometry.

To electrospin, we used a point-plate configuration where PVA/α-gal solution was loaded into a syringe fitted with a stainless steel needle

(0.508 mm I.D.) and attached to a power supply (Gamma High Voltage Research, D-ES-30PN/M692). A flow rate of 0.5 mL/h, collecting distance of 15 cm between the tip of the needle and the ground collector plate covered with foil and applied voltage (positive polarity) of 10−22 kV were used. Fibers were typically collected over 2 to 4 h. For a 4 h collection, the typical mat thickness was 50 μm (measured with a micrometer).

Cross-linking. Small sections (1″ squares) of electrospun material were cut and immersed in acetone containing 5 vol % glutaraldehyde and 0.12 vol % HCl at ambient conditions. After 2.5 h, the samples were removed and dried at ambient conditions, and stored at 4 °C. In some cases, after 2.5 h, the samples were removed and placed in Tris buffer at ambient conditions.

Fiber Characterization. We characterized the fibers before and after cross-linking. To examine the fiber morphology, samples were sputter coated with a ∼10 nm layer of gold and analyzed with scanning electron microscopy (SEM, FEI XL-30) at 5 kV.

Apparent Enzyme Activity. Apparent α-gal activity was measured using a previously reported assay monitoring the enzymatic release of *p*-nitrophenol (pNP) from *p*-nitrophenyl-α-D-galactopyranoside via the UV absorbance at 405 nm.[28] Briefly, free and immobilized enzymes were incubated in *p*-nitrophenyl-α-D-galactopyranoside in 58.3 mM sodium acetate buffer (pH 5.5) at concentrations between 0.2 and 5 mM (standard 4.43 mM) and temperatures between 37 and 120 °C (standard 75 °C). After some time (depending on the temperature and the mass of enzyme present), 1 M sodium carbonate was added to stop the reaction. The amount of pNP converted in the assay time was determined by measuring the UV absorbance of the reaction media at 405 nm using a Jasco V550 spectrophotometer. Water was used as a negative control to account for any pNP converted due to means other than enzymatic release. Apparent activities reported are in units of mol of pNP per minute under the specified assay conditions per microgram of enzyme present. The mass of immobilized enzyme was determined from the mass of membrane cross-linked and the enzyme loading used assuming homogeneous fibers and complete solvent evaporation. Using these assumptions, although reasonable, would result in an underestimation of the true enzyme activity. To better reflect the true enzyme activity, the quantification of active sites would be useful, but is outside the scope of this paper. Measurements of apparent activity were made at least in triplicate.

Kinetic Parameters. To determine the apparent kinetic parameters for the hydrolysis of pNP-α-D-galactopyranoside small sections of electrospun material were cut into small pieces of equal mass (∼10 mg) and cross-linking. The initial rate of hydrolysis was determined from the spectrophotometric assay using initial concentrations between 0.2 and 1 mM in 58.3 mM sodium acetate buffer, pH 5.5. The kinetic parameters were determined from Lineweaver−Burk plots of initial rates of hydrolysis measured at various substrate concentrations similar to previous reports.[30]

Thermal Stability. The apparent thermal stability of the free and immobilized α-gal were measured by incubating α-gal in 58.3 mM sodium acetate buffer, pH 5.5 at 90 °C for predetermined amounts of time and then measuring the residual activity at 90 °C. Assuming enzyme inactivation occurred in a single-step process,[31] the first-order deactivation rate constant (k_1) at 90 °C, pH 5.5 was estimated from the slope of a semilog plot of fractional activity versus time and used to determine the half-life ($t_{1/2} = \ln(2)/k_1$). Immobilized α-gal samples for this experiment were prepared by cutting small sections of electrospun material into pieces of equal mass (∼3 mg) and cross-linking.

Effect of Cross-linking Reaction Reagents on the Free Enzyme. To determine the effect of acetone, GA, and HCl on the enzyme, a small amount of α-gal was placed in a vial and then exposed to a small amount of acetone, acetone with GA, acetone with HCl, or acetone with GA and HCl (∼300 μL), which evaporated over several hours. The amounts of GA and HCl were the same as used in the cross-linking reaction, 5 vol % and 0.12 vol %, respectively. The activity after exposure to acetone, or acetone with GA and/or HCl was measured under standard conditions at 75 °C.

Enzyme Leaching. To evaluate enzyme leaching during the activity assay, samples were removed from the acetone cross-linking reaction media and placed directly in Tris buffer. Samples were then removed from Tris after increasing amounts of time (from 15 min to 48 h) and allowed to dry briefly at ambient conditions before storing at 4 °C. Residual activity of the washed samples was measured under standard conditions at 75 °C.

RESULTS AND DISCUSSION

Enzyme Immobilization. We electrospun blends of α-gal and PVA using 7 wt % PVA to produce uniform enzyme-loaded nanofibers (Figure 1a) and chemically cross-linked the PVA to immobilize the enzyme within a water-insoluble fiber. Maintaining the structure of the fiber is critical to capitalize on the high specific surface area of the electrospun nanofibers. The integrity of the fibers was maintained after cross-linking (Figure 1b) and the cross-linked fibers maintained their structure after soaking in water overnight (Figure 1c). When immobilizing hyperthermophilic enzymes, we must also consider the effect of elevated temperatures on the structure of the fibers; therefore, we examined the fibers after exposure to aqueous media for approximately 48 h at 75 °C (Figure 1d). Because no significant change in fiber structure was evident from the SEM micrographs, we ascertained that the chemical cross-linking with GA yielded a sufficiently robust polymer fiber for immobilization of a hyperthermophilic enzyme. This may offer improvements upon previous work, as Wang and Hsieh reported that cross-linking in ethanol appeared to affect the packing of the fibers due to swelling of PVA in ethanol,[11] and Wu et al. reported that vapor phase cross-linking caused fiber fusing and increased fiber packing, which resulted in decreased surface area and may have contributed to reduced catalytic activity.[10]

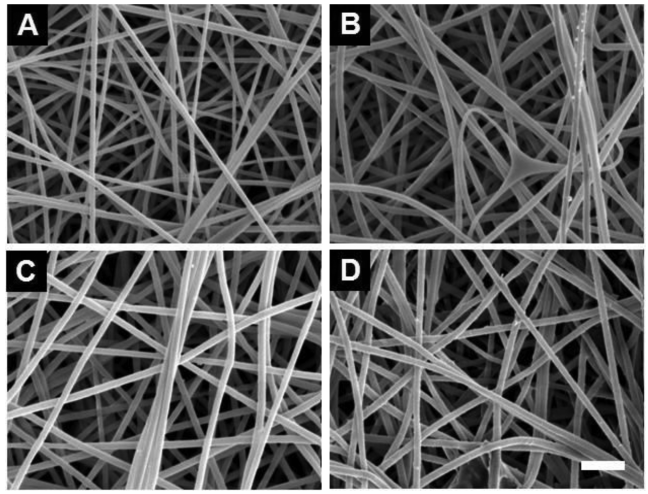

Figure 1. SEM micrographs of (A) electrospun 7 wt % PVA, 0.1% α-gal fibers, (B) electrospun 7 wt % PVA, 0.1% α-gal fibers after cross-linking with glutaraldehyde, (C) cross-linked 7 wt % PVA, 0.1% α-gal fibers after soaking in deionized water at room temperature overnight, and (D) cross-linked 7 wt % PVA, 0.1% α-gal fibers after activity assay at 75 °C. Scale bar represents 2 μm.

We also considered the robustness of this immobilization method with regards to enzyme leaching. The fibers were placed directly into Tris buffer after cross-linking. After various amounts of time (from 15 min to 48 h), the fibers were removed and the residual activity remaining in the washed

fibers was measured (Figure S.4, Supporting Information). Importantly, despite wash times up to 48 h, there was no decrease in apparent activity of the washed fibers indicating the enzyme is effectively immobilized within the fibers which are sufficiently robust to capitalize on the unique properties of hyperthermophilic enzymes (ability to function at temperatures up to 105 °C).

Immobilized Enzyme Characterization. Next, we focus on characterizing the properties of the immobilized enzyme. We examined the specific activity of the free and immobilized α-gal as a function of temperature. Figure 2A shows the specific activity for free and immobilized enzymes normalized to the respective maximum specific activity. Both the free and immobilized enzymes were optimally active at ~95 °C, as expected,[28] above which the activity for both the free and immobilized enzymes decreased. The hyperthermophilic nature of the α-gal was retained after immobilization, as the specific activity of the immobilized enzyme at 95 °C was 1 order of magnitude higher than the specific activity at 37 °C. The fractional activity (relative to free enzyme) for each level of enzyme purification is included in Table 2 of the Supporting Information. We note that the activity was not significantly affected by the purification level (Table S.2, Supporting Information). Due to ease of preparation, the heat treated enzyme preparation was used for further experiments. We also examined the thermal stability of the immobilized α-gal using the apparent half-life at 90 °C as a metric (Figure 2B). At 90 °C, the half-life of the immobilized enzyme was about 110 min, compared to 20 min for the free enzyme at the same conditions, comparable to previous reports[32−34] and may be attributed to covalent interactions between enzyme molecules and the enzyme and polymer which prevents subunit dissociation of the α-gal dimer and enhancing rigidity of the enzyme, respectively.[28,35−37]

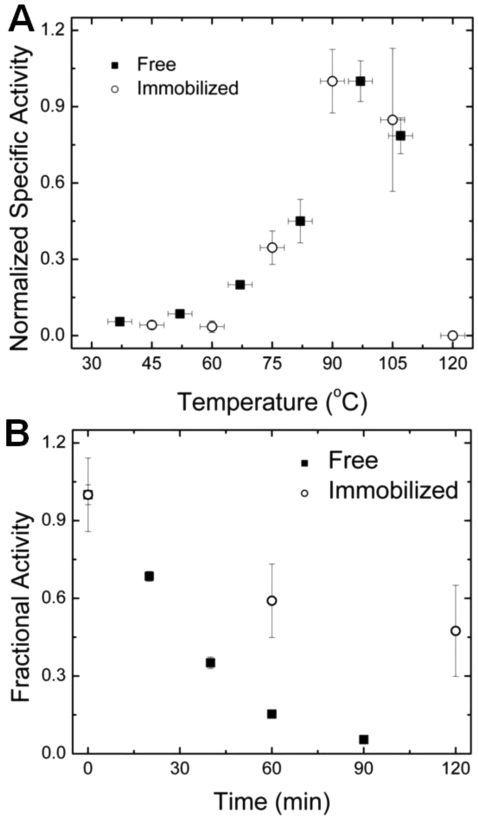

Figure 2. (A) Apparent specific activity of free and immobilized α-gal (AEC purified) as a function of temperature normalized to the maximum specific activity of the free and immobilized enzyme, respectively. (B) Thermal stability of free and immobilized α-gal at 90 °C and pH 5.5. Free enzyme indicated the enzyme in solution recovered after purification and "immobilized" is the enzyme entrapped within the cross-linked fiber.

Estimates of the kinetic parameters indicated that immobilization did not significantly affect the apparent K_m; however, v_{max} was significantly lower upon immobilization (Table S.3, Figure S.3, Supporting Information). Upon immobilization, the apparent v_{max} (measured in moles of pNP per minute) fell from on the order of 10^{-7} to 10^{-11}, similar to previous reports of enzyme immobilized using polymer supports (see refs 30 and 38−40). We also noted an increase in activation energy by a factor of approximately 2.5 of the immobilized enzyme when compared to the free enzyme. Decreases in v_{max} upon immobilization may be attributed to diffusion limitations or structural changes in the enzyme.[41,42] Increases in activation energy of enzymes may indicate structural modifications in the enzyme introduced upon immobilization[43,44] or diffusion limitations.[45,46] Further experiments are required to better understand the reasons for the apparent decrease in activity.

Enzyme Deactivation. To assess possible enzyme deactivation due to exposure to the chemical cross-linking reaction, we exposed the free enzyme to acetone as well as acetone with GA and/or HCl for 3 h as that was approximately the time required to render PVA fibers insoluble. The activity of free enzyme was not affected by exposure to acetone for this period of time (Figure 3), compared to a 15% loss reported when lipase was exposed to ethanol for 1 h.[11] When exposed to the acidic conditions used in cross-linking (acetone and HCl), the activity decreased by ~50%, suggesting the acidic conditions of the cross-linking reaction may affect the activity of the immobilized enzyme. Exposure to glutaraldehyde in acetone also affected the activity of the enzyme by ~70%. The combination of GA and HCl in acetone had the largest effect on the activity of the treated enzyme. The activity of the free enzyme was less than 25% of free enzyme not exposed to the combination of acetone with GA and HCl. Although the reagents used in the cross-linking reaction are detrimental to catalytic activity of the enzyme, this loss cannot account for the significantly lower (cf. orders of magnitude) activity upon immobilization that is observed.

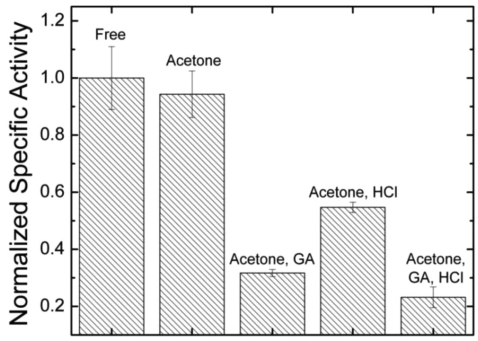

Figure 3. Effect of acetone, glutaraldehyde, and HCl on the free enzyme measured at 75 °C.

Mass Transfer Limitations. Next we considered diffusion limitations as mass transfer of the substrate to and product

from the immobilized enzyme may also affect the apparent activity of the immobilized enzyme.[36,46] In this system, we must consider both intrafiber diffusion (within a single fiber) and interfiber diffusion (between multiple layers of fibers). Since we assume the fiber is a homogeneous blend of polymer and enzyme, as we increase the enzyme loading, the enzyme concentration at a given r within the fiber increases. As the enzyme concentration increases at a given r, the intrafiber diffusion (within a single fiber) should decrease. Varying the electrospinning time to control the mat thickness, we could explore the effect of diffusion between multiple layers of fibers (interfiber diffusion).

Figure 4A shows the effect of enzyme loading on apparent activity of the immobilized enzyme. In the absence of internal

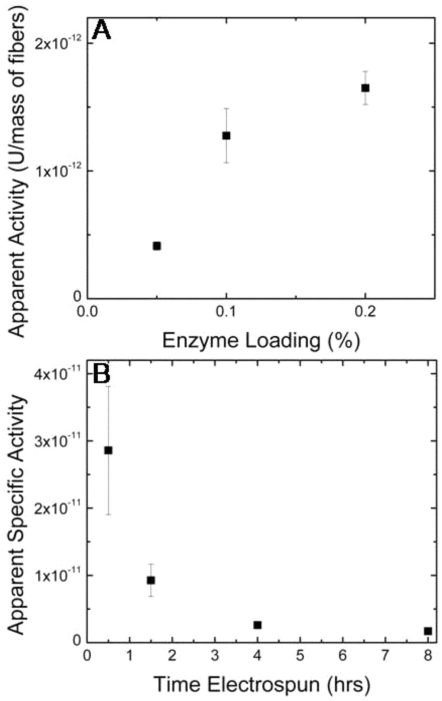

Figure 4. (A) Effect of enzyme loading on apparent activity of immobilized α-gal. (B) Effect of mat thickness on apparent activity of immobilized α-gal.

diffusion limitations within a fiber and assuming a homogeneous fiber, we would expect a linear increase in apparent activity with increasing enzyme loading.[47,48] However, increasing the enzyme loading by 4-fold (from 0.05 to 0.2%) resulted in a less than 2-fold increase in apparent activity. This suggests that the diffusion of the substrate to and diffusion of product from the immobilized enzyme within the fiber contributes to the lower apparent activity of the enzyme upon immobilization. Because we examined enzyme loadings up only to 0.2% (per weight of polymer), we assume that protein−protein interactions, which may alter enzyme conformation, denaturation, or blockage of the active site,[12] remain negligible as, at these enzyme loadings, interactions between the protein and polymer are considerably more likely.[12] Similar effects have been previously reported for immobilization of catalysts using electrospun polymer fiber supports.[49,50] Further discussion of the apparent diffusivity of the cross-linked fibers is included in the Supporting Information.

Figure 4B shows the apparent specific activity of the immobilized enzyme activity as a function of electrospinning time (directly proportional to mat thickness[51,52]). Surprisingly, the apparent specific activity decreased as electrospinning time increased. Decreasing the electrospinning time from 8 to 0.5 h resulted in an approximate 17-fold increase in apparent specific α-gal activity, which indicates that interfiber diffusion may affect the apparent immobilized α-gal activity. The decrease in apparent specific activity with increasing thickness indicates that the additional enzyme in the additional layers of nanofibers do not contribute to the measured apparent activity and only enzyme immobilized in the outermost fibers contribute to the apparent activity. Previous work with lipase immobilized within chemically cross-linked PVA nanofibers indicates that mass transfer into the fibrous membrane may reduce the apparent activity of the immobilized enzyme, as Xie and Hsieh note that the apparent activity for membranes attached to a solid support (foil) is lower than that for detached membrane, as the foil limits the accessibility of some layers of fibers.[27] Therefore, the performance of the immobilized enzyme may be greatly improved by minimizing the number of layers of fibers in order to minimize interfiber diffusion. Further, we may be underestimating the specific activity of the enzyme that does contribute to the measured apparent activity by assuming all of the enzyme loaded in the fibers contributes to the apparent activity.

It is important to note that despite the recent attention nanofibrous supports have received in enzyme immobilization due to their high specific surface area,[53,54] both intrafiber and interfiber mass transfer limitations can affect the performance of the immobilized enzyme. Interfiber diffusion can be addressed simply by minimizing the thickness of the mat. This result can be applied broadly to improve enzyme performance using nanofibrous supports with any immobilization strategy (i.e., surface attachment, encapsulation, and entrapment) as well as to the immobilization of other catalysts such as noble metal nanoparticles.

Enhanced Immobilized Enzyme Performance. On the basis of the understanding of the causes of the apparent loss in activity upon immobilization, we next consider methods to improve the apparent activity of the immobilized enzyme. To prevent enzyme deactivation, we considered the use of buffers (sodium acetate buffer (SAB, pH 5.5), phosphate buffered saline (PBS, pH 7.4), and Tris (pH 8.2) as solvents for electrospinning to improve the microenvironment of the immobilized enzyme.[49,50,55] We observed a 5-fold improvement in activity when using Tris buffer, but not when using PBS at a similar pH or SAB at pH 5.5 (optimal for enzyme activity), despite the similar concentrations (~10 mM) of the buffers (Figure 5). Because improvement in activity is seen only with Tris and not the other buffers used, improved activity when using Tris is in part due to the pH rather than solely the presence of buffer salts. We further investigated the effect of pH at, above, and below the isoelectric point of α-gal (pI 5.3).[28] Combinations of Tris and HCl were used to achieve the desired solution pHs.

As shown in Figure 5, at pH 5.2 the apparent retained activity of the immobilized enzyme is similar to when using water as the solvent (pH ~5.5), at pH 7.8 obtained using Tris as the solvent, the apparent activity increased by 5-fold, and at pH 2.1 there is a 4-fold increase in apparent activity when compared to using deionized water as the solvent. Interestingly, the retained activity of the immobilized α-gal was higher at pH values above

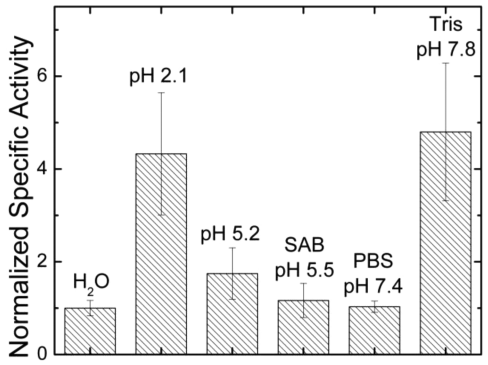

Figure 5. Effect of pH and buffers used for electrospinning on apparent activity of immobilized α-gal. Apparent activity is normalized to the activity measured using deionized water as the solvent for the electrospinning solution with no pH adjustment.

and below the isoelectric point; improved performance under highly acidic conditions is particularly surprising. Because our results indicate that the effect of enzyme deactivation (due to cross-linking and acidic pH) decreases activity is small relative to mass transfer, the improved activity at pH 2.1 compared to 5.2 (optimal for enzyme) is more likely a result of reduced mass transfer. In previous work, we observed that pH affected the protein distribution within the fiber. At the isoelectric point, a coaxial fiber with protein in the core was obtained while protein surface enrichment occurred at pHs above and below the pI.[21] Therefore, we attribute increased apparent activity to reduced intrafiber diffusion due to surface enrichment of the enzyme during processing.

We also considered additives[56] such as glycerol trehalose, known to improve protein stability, as well as a substrate,[57,58] raffinose, to protect the enzyme from deactivation during immobilization. Using concentrations comparable to these previous studies, the addition of 1 M glycerol in the electrospinning solution resulted in an approximate 2-fold increase in apparent activity when compared to α-gal immobilized in the absence of any additive (Figure 6) similar to previous reports.[59] The incorporation of the substrate raffinose (200 mM) in the electrospinning solution improved the apparent activity of the immobilized enzyme by approximately 3-fold (Figure 6). Of the additives, trehalose (1 M) had the biggest impact on improving immobilized enzyme activity as we observe a greater than 10-fold

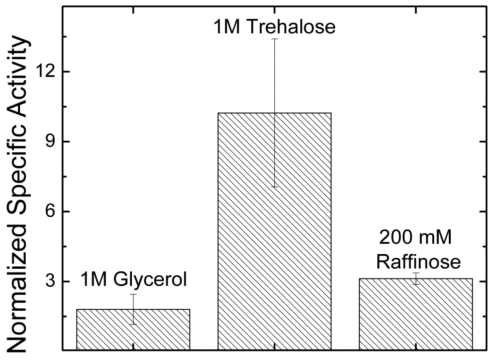

Figure 6. Effect of glycerol, trehalose, and raffinose incorporated into the electrospinning solution on apparent performance of immobilized enzyme. Apparent activity is normalized to the activity measured using deionized water as the solvent for the electrospinning solution.

improvement in the apparent activity of the immobilized enzyme (Figure 6). Similar enhancement in activity with trehalose has been reported with a protease[60] and comparable to previous studies that have found trehalose to be a superior stabilizer to glycerol and other sugars such as raffinose.[61]

Results from this section suggest that significant improvements (over 50-fold) in the performance of the immobilized enzyme in terms of apparent activity can be achieved by minimizing the fiber mat thickness as well as by incorporating trehalose during immobilization. However, further improvements are desired. Future work will be aimed at alternative cross-linking strategies that minimize nonspecific interactions with the enzyme while allowing for efficient diffusion of the reactants and products.

CONCLUSIONS

We present a method to immobilize hyperthermophilic enzymes within chemically cross-linked polymer nanofibers that is robust and effective at elevated temperatures. The cross-linked fibers retain their structure in aqueous media for over 48 h at 75 °C. Over this time, we have no indication of enzyme leaching from the support, indicating effective immobilization. Importantly, apparent activity is detected after immobilization and the hyperthermophilic nature is retained as the maximum activity occurs at ∼90−95 °C, and the activity at 90 °C is 1 order magnitude higher than at 37 °C. Notably, there is an apparent 5.5-fold increase in thermal stability of the enzyme at 90 °C (pH 5.5) upon immobilization. However, there is a trade-off between improved stability and apparent activity as we also observe a significant decrease in apparent activity upon immobilization. We therefore examined possible reasons for the apparent decrease in activity, including the effect of reagents used in the cross-linking reaction, as well as mass transfer limitations. Loss in activity due to exposure to these reagents can only account for a small fraction of the decrease in activity, while both intrafiber and interfiber diffusion significantly limit the rate of reaction. On the basis of the understanding of the causes of the decrease in apparent activity, we improved the performance of the immobilized enzyme using a cryoprotectant during the immobilization process to prevent enzyme deactivation and minimizing the thickness of the mat to reduce interfiber diffusion limitations.

ASSOCIATED CONTENT

Supporting Information

α-Gal purification results, resulting fiber structures as a function of PVA concentration, catalytic properties (K_m and V_{max}), Lineweaver−Burk plots, and order of magnitude for observed rates for Weisz−Prater analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*S. A. Khan. E-mail: khan@eos.ncsu.edu. Phone: 919-515-4519.

Notes

The authors declare no competing financial interest.

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