A Simple Approach to a Vastly Improved Acetylcholinesterase Activity and Stability at Elevated Temperatures Using Magnetic Microbeads and Poly(N-(3-aminopropyl methacrylamide)) Hydrogel Supports

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Abstract

The thermal stabilization of enzymes is a critical factor in the development and reliability of enzyme-based processes and functional materials. Using a simple amine coupling approach for enzyme immobilization onto magnetic microbeads, followed by encasement of the beads in a hydrogel, we demonstrate that the thermal stability of the enzyme acetylcholinesterase can be increased dramatically. For example, when free and microbead-immobilized enzyme ("EM Conjugate") are incubated overnight in a dry state at 63 °C (140 °F), the catalytic efficiency (k_{cat}/K_m) of the latter is higher than the former by six orders of magnitude (a factor of 2.16×10^6). This effect arises mostly through a ~29,700-fold decrease in K_m experienced by the EM Conjugate, relative to that of the free enzyme. Encapsulation of the EM Conjugate in a hydrogel based on poly(N-(3-aminopropyl methacrylamide)), which contains a primary amine, affords the enzyme additional stability when incubated overnight at 63 °C in an aqueous state. For example, its catalytic efficiency is four orders of magnitude higher than that of both the free enzyme (a factor of 4.34×10^4) and that of the EM Conjugate alone (a factor of 1.78×10^4) after all are incubated overnight at 63 °C. The presence of the hydrogel also caused the Michaelis constant to decrease by 1.38×10^4 relative to that of the EM Conjugate, reaching a value of 2.18×10^{-3} M. Thus the hydrogel enables the AChE substrate binding site to retain a significant amount of its natural affinity for the substrate, after heating. This effect may occur via ion-pairing by the primary amines in the hydrogel polymer repeat unit, which are protonated and positively-charged at the assay pH. To the best of our knowledge, this simple method for enzyme thermal stabilization is novel and has not yet been investigated.

Keywords

acetylcholinesterase, thermal stability, Michaelis constant, hydrogel, microbeads

1. Introduction

Enzymes have for some time served in major applications in the pharmaceutical, food, detergent, leather, paper, and textile industries. More recently they have appeared as key components in biosensors and various types of functional materials. In this context, they have been successfully

immobilized onto a wide variety of surfaces using interactions such as reversible physical adsorption, ionic linkages, affinity binding, and irreversible but stable covalent bonding [1-3]. Commonly used macroscale supports have ranged from carboxymethyl-cellulose and ion exchange resins, to silica [4] and porous glass [5] to various ceramics such as aluminum oxide, metals such as titanium, and various types of synthetic polymers. Of particular interest are micro- and nanostructured/scaled immobilization surfaces such as micro- or nanoparticles [6-10], mesoporous silica [11-16], graphene nanodots [17], and quantum dots [18], since they can often boost the activity of the bound enzymes by several-fold [19].

However, even when immobilized, most enzymes are vulnerable to thermal denaturation at temperatures of a few tens of degrees centigrade above the optimum for their activity. At a given temperature, the stabilization ratio is the half-life of the immobilized form divided by that of the free form, and it is in general not higher than $\sim 10^4$ [20]. Thermal denaturation generally involves a reversible initial step or steps, followed by irreversible final steps that lead to aggregation or covalent changes [21]. The reversible initial step occurs at the melting point of the enzyme, which is often in the range 60-80 °C in an aqueous environment [22].

Herein, we report a simple, very effective approach for enzyme thermal stabilization that is based on magnetic microbead supports that can be encapsulated in a hydrogel. Both the fully hydrated and dried (xerogel) forms of the hydrogel were used as the encapsulant. This creates an environment for the enzyme that allows it to maintain maximum activity over time, at ambient and elevated temperatures. We used human recombinant acetylcholinesterase (AChE) (E.C. 3.1.1.7) as a model enzyme, since it has been well studied, and is vital in vertebrate biochemistry and very important in biosensor technology. AChE is a complex and relatively large enzyme (MW approx. 260,000) with a primary role in cholinergic neurotransmission. It functions as a tetramer with each subunit possessing an active site [23-25]. It is used as a key component in sensors for organophosphorus-based pesticides such as methyl parathion and paraoxon, and chemical warfare agents such as sarin, soman, and VX. Strategies for long-term stabilization of this enzyme are therefore relevant, especially those capable of stabilizing it at high temperatures that can occur in the natural environment, enabling device reliability and longevity. A relevant testing temperature would be near 60 °C (~ 140 °F). Temperature levels approaching this may occur in warmer regions of the world where highly toxic organophosphorus pesticides have been widely used, or in arid or desert regions subject to armed conflict.

Commercially available fluorescent paramagnetic microbeads coated with carboxylated polystyrene were chosen as the physical supports for the enzyme. The hydrogel is based on poly(N-(3-aminopropyl methacrylamide)) (Figure 1). The primary amine of the polymer repeat unit contributes to the hydrophilicity of the material. Also, the amine is protonated at the pH used in the assays (pH 7.4). Since the AChE is above its isoelectric point (pH range 5.6 to 6.0) at pH 7.4, it has a net negative charge. Thus, the positively-charged amines can form ionic linkages with the AChE which can contribute to thermal stability. This type of hydrogel has a relatively high equilibrium water content, well over 50% by weight, which permits encapsulated enzyme to have good access to substrate in the solution phase. A “solution phase-like” environment is known to stabilize biomolecules, resulting in a high degree of function [26]. The coupling chemistry for enzyme attachment to the magnetic microbeads is straightforward, well-established, and is done in aqueous solution at near-neutral pH. It involves activation of the carboxyls with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), which allows covalent attachment of enzyme through the ϵ -amines of lysine residues. After enzyme attachment, the beads are washed and isolated, processes in which their magnetism is useful. The covalent attachment to the bead surface contributes to enzyme thermal and temporal stability. In our effort, we focused on

using microbeads having a diameter of 0.45 to 0.5 μm . From this point onwards, the enzyme-derivatized microbeads will be referred to as the “EM Conjugate”, and the hydrogel-encapsulated EM Conjugate will be referred to as the “EMH Composite”. The synthesis scheme for both is given in Figure 1.

A main focus of this study was to compare AChE kinetics in free solution vs. that of the EM Conjugate in free solution, and vs. that of the EMH Composite in its hydrated and xerogel forms. We investigated the effects of drying each material overnight at ambient (25 °C) and elevated (63 °C) temperatures prior to assay. The latter is above the denaturation temperature of the enzyme [4]. Koh and coworkers have investigated interesting related approaches involving enzyme-derivatized silica microspheres [27] and magnetic nanoparticles [28] embedded in poly(ethylene glycol) hydrogels, but do not report thermal stability data. To the best of our knowledge, the thermal stabilization approach described in our work herein has not previously been investigated.

2. Experimental

2.1 Preparation of EM Conjugate.

The EM Conjugate was formed using ethylcarbodiimide (EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) chemistry [29] to link enzyme primary amines with carboxylate on the magnetic microbead surface. EDC was purchased from ThermoFisher Scientific (Waltham, MA) and microbeads (SPHERO™ Fluorescent Carboxyl Magnetic Particles, Nile red, 1% w/v in aqueous solution, 0.47 μm diameter) were purchased from SpheroTech, Inc. (Lake Forest, IL). As purchased, the microbeads had a concentration of 1.0×10^8 microbeads/ml. First, the the carboxylic acid groups on the surface of the microbeads were activated. This was accomplished by removing a 50 μL aliquot of microbead solution, isolating the beads magnetically, removing the supernatant, and then washing the microbeads twice with 100 μL of a 0.1 M sodium phosphate buffer, pH 6.0 (PB). In this manner, 5.0×10^6 microbeads were prepared for EDC coupling with AChE. The microbeads were then resuspended in 50 μL PB, which was then introduced into 100 microliters of a solution of EDC and sulfo-N-hydroxysuccinimide (NHS) (50 mg/ml each; Thermo Fisher Scientific, Waltham, MA, USA) which was then incubated for 30 min at room temperature (RT) (Figure 1). After the activated microbeads were isolated and washed with PB, 100 μL of a solution containing AChE (concentration 1.0 mg/ml in 0.01M phosphate buffered saline, pH 7.4 (PBS), Sigma Chemical Co., St. Louis, MO, USA) was added and allowed to react with the microbeads at RT for 1 h. Continuous agitation of the microbead-enzyme mixture allowed formation of the EM Conjugate, and provided a homogeneous suspension with no microbead aggregation. After 1h at RT, the solution was incubated overnight at 4°C. The newly-formed EM Conjugate was subsequently washed twice with PBS then stored at 4°C until further use.

2.2 Characterization of EM Conjugate.

The EM Conjugate was characterized using FTIR spectroscopy, using a Bruker Vertex 70 FTIR Spectrometer in transmission mode. Underivatized microbeads were also characterized for comparison. Before testing, the EM Conjugate sample was repeatedly washed with deionized water to remove buffer salts. It was then placed onto a ZnSe support, then dried under nitrogen at 40 °C overnight to form an adherent thin film, which was subjected to FTIR analysis. The spectrometer sampling chamber was thoroughly purged with dry nitrogen before sample interrogation.

2.3 Preparation of EMH Composite.

The EM Conjugate was encapsulated in the hydrogel matrix to form the EMH Composite. The hydrogel was based on N-(3-aminopropyl) methacrylamide (APMA) and N, N' methylene bisacrylamide (MBA) (Figure 1). Solutions of APMA in PBS (200 μ l, 300 mg/ml, Polysciences, Inc., Warrington, PA, USA), MBA in PBS (200 μ l, 40 mg/ml, Sigma Chemical Co, St Louis) and EM Conjugate in PBS (10 μ l, 9.6×10^8 microbeads/ml) were mixed, followed by the addition of sodium persulfate (11 μ l, 0.2 μ g/ml, Sigma Chemical Co.) and TEMED (2 μ l, 7.7 μ l/ml, BioRad, Hercules CA) to initiate polymerization. The polymerization/EM Conjugate solution was introduced into a 96-well flat-bottom microtiter plate for subsequent confocal imaging and kinetic measurements, as 50 microliter per well aliquots. The plate was placed in an inert atmosphere (N_2) for 30 min at RT for polymerization, then removed and stored at 4°C (Figure 1). Gelation was evident within minutes but additional time (overnight incubation) was allowed to thoroughly complete the polymerization process, forming the EMH Composite. The samples were kept in their hydrated form (swollen) at 4°C until testing. This was done by introducing 200 μ L PBS into each well before storage. This PBS layer also allowed diffusional removal of unreacted monomer and initiator by-products from the hydrogel, and was replaced twice during storage. The final layer was discarded immediately before enzyme assay.

2.4 Characterization of EMH Composite.

Thermogravimetric analysis (TGA) of the AChE-microbead hydrogel was performed using a TA Instruments Model 2910 Thermogravimetric Analyzer, temperature ramp rate 5°C/minute. Hydrogel samples were analyzed using a Nikon TE2000 Inverted Confocal Microscope with excitation and emission wavelengths set at 550 nm and 635 nm, respectively. Laser power and gain settings were set at 10% and 70% respectively. Images were acquired from the center of each well to minimize meniscus effects on sample thickness.

2.5 Enzyme Activity Assay.

In a series of experiments using a 96-well plate format, the EMH Composite, EM Conjugate, and AChE in solution at equivalent concentrations were exposed to acetylthiolcholine substrate with Ellman's reagent (Sigma Chemical Co., St. Louis), and the reaction kinetics was followed colorimetrically.

Enzyme incubation conditions - For AChE alone and EM Conjugate alone, 10 μ l of each solution (compositions given below) were placed in each well, then air dried overnight. For the EMH Composite, the samples were tested swollen and dry (as xerogels). Xerogel formation occurred when hydrogel samples having a 10 μ l volume were exposed to convective air flow at RT overnight.

Amount of enzyme per well – For the EM Conjugate and the EMH Composite a total of 1.18×10^6 microbeads were used per well. If it is assumed that each bead is covered with a monolayer of enzyme, the resulting number of enzyme molecules per well is 4.64×10^{10} (see discussion below). For the assay of free enzyme, this number of enzyme molecules was added to each well, which corresponds to 2 μ l of a 1 μ g/mL solution of AChE being added to each well.

Activity assay - A series of experiments was performed employing 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent, Sigma Chemical Co.). Resultant product formation of 2-nitro-5-thiobenzoate (TNB) was measured and quantified spectrophotometrically at 412 nm. To each well, 60 μ l of PBS, pH 7.4 and 30 μ l of DTNB (5 mM) were added and mixed. Immediately prior to the kinetic measurements, acetylthiolcholine (10 μ l; 0.064 to 1000 μ g/ml plus blank) was applied to the wells. Absorbance measurements at 412 nm were taken over 30 min in 30 sec intervals using a Tecan Infinite M1000 Spectrophotometer (San Jose, CA). As the acetylthiolcholine is hydrolyzed by the AChE,

thiocholine is produced, which displaces TNB from DTNB. We constructed a standard curve for TNB at pH 7.4 which allowed us to correlate absorbance at 412 nm with concentration (Figure 5a). Kinetic parameters were calculated using SigmaPlot enzyme kinetics software (Systat Software, San Jose CA). The software uses a Lineweaver-Burk linearization of the substrate concentration and initial rate data. It then returns values of K_m and V_{max} . The activity of the free enzyme, the EM Conjugate, and the EMH Composite was measured after incubation at 25 °C and 63 °C.

3. Results and Discussion

A first task was to estimate how many AChE molecules were present on each EM Conjugate microbead, and per unit volume of EMH Composite. The molecular diameter of the enzyme is about 5 to 6 nm, which is roughly one percent of the diameter of the microbeads. If the enzyme is modeled as a hard sphere of diameter 5 nm, a simple calculation indicates that a theoretical maximum coverage of the microbead surface (assuming a monolayer of enzyme at maximum density) is about 39,250 molecules, if excess enzyme is used in the EM Conjugate formation step, which is the case. For the EMH Composite (fully hydrated) we used a bead loading of 1.18×10^7 beads per cm^3 composite. This corresponds to 4.64×10^{11} AChE molecules per cm^3 . For comparison, if it is assumed that an aqueous solution of AChE at a concentration of 0.1 mg/ml (2.315×10^{14} enzyme molecules per cm^3) is near the upper solubility limit, then the EMH Composite contains an amount of AChE that is ~500-fold lower than that of the concentration at its upper solubility limit. For the xerogel state of the EMH Composite, the enzyme concentration is about ten times higher, as is demonstrated by the thermogravimetric studies below.

To verify the presence of the AChE on the EM Conjugate, we performed FTIR studies on them and compared the resulting spectra with that of the underivatized microbeads. The comparison was done across the wavenumber range 1800 to 1400 cm^{-1} . The FTIR spectrum for the EM Conjugate (Figure 2) shows the expected Amide I and Amide II bands, which are characteristic of protein. The Amide I band group is in the range 1699 cm^{-1} to 1630 cm^{-1} . The strongest band is at 1654 cm^{-1} , and is associated with AChE α -helices (C=O stretch) [29], [30]. Second and third slightly weaker bands appear at 1699 cm^{-1} and 1630 cm^{-1} , which are associated with AChE β -pleated sheets. The Amide II bands appear in the range 1560 to 1523 cm^{-1} and correspond to N-H and C-N vibrations [31]. At the outlying ends of the spectrum, the band at 1716 cm^{-1} may correspond to the aspartate carboxyl stretch [32], and those at 1488 and 1454 cm^{-1} may correspond to polystyrene C=C stretches [33].

The Amide I, Amide II, and aspartate bands are not present for the underivatized polystyrene-coated magnetic microbeads themselves, which instead only show bands characteristic of plain polystyrene. These appear in the range 1600 to 1452 cm^{-1} . They are characteristic of various aromatic C=C stretches [33]. The pronounced spectral differences between the enzyme-supporting EM Conjugate and the underivatized microbeads provide strong physical evidence for the presence of the enzyme on the beads.

Thermogravimetric analysis (TGA) of the EMH Composite shows a relatively high equilibrium H_2O content (~86%). Figure 2 shows the weight distribution of each component. As is seen in Figure 3A, the majority of the hydrogel consists of free water (85.7%), with the hydrogel polymer and the EM Conjugate comprising about 10% and 4% of the mass, respectively. The relatively high water content arises mainly from the osmotic force contributed by the primary amine of the polymer repeat unit, which is protonated and thus positively-charged at neutral pH. The mass composition of the xerogel (dry hydrogel), formed by drying the hydrogel overnight, is shown in Figure 3B. Here, 67% of the mass

consists of the hydrogel polymer, and bound water and the EM Conjugate comprise 21% and 11% of the remaining mass, respectively. The bound water generally evaporates in the temperature range 100 °C to 200 °C. It likely plays a role in the thermal and temporal stabilization of the AChE. Figure 3C is a representative TGA trace of the swollen hydrogel, showing evaporation of bulk water at temperatures of up to 100 °C, and thermal degradation of the hydrogel polymer in the range 300 °C to 450 °C. The non-degrading residue C is composed of the EM Conjugate, which consist mainly of magnetic microbeads, and buffer salts. It is reasonable to assume that the EM Conjugate loading could be increased from ~4% to much higher levels, possibly as much as 20%, while maintaining sufficient diffusion pathways for substrate entrance and product release. The high water content of the EMH Composite is desirable, since it allows rapid diffusion of substrate and product into and out of the hydrogel matrix.

Fluorescence confocal images of the EMH Composite provided knowledge of the average distribution of the EM Conjugate after hydrogel formation. Images acquired showed no meniscus effect with clear visible fluorescence signatures of the EM Conjugate. Composite images of the control (Figure 4, left, hydrogel without EM Conjugate) and EMH Composite (right) were recorded. A high resolution 3D projection of the 40 µm-thick centroid from the wells was also obtained. The overall microbead distribution was observed to have a ratio of 60:40, with the majority of the beads residing below the centroid. Gravitational settling of the EM Conjugate occurred during the polymerization process. If desired, the distribution of the EM Conjugate can be magnetically controlled and used to pattern sensor arrays. Also, increasing the polymerization rate by photochemical means, for example, will prevent gravitational settling of the EM Conjugate.

Enzyme kinetic experiments were conducted using free AChE in solution, EM Conjugate, and EMH Composite. Each material was assayed at 25 °C after incubation in an aqueous state, and incubation in a dried state. Two incubation temperatures were used, 25°C and 63°C, each for a 24 hour period. Thus, twelve separate experiments were performed for each substrate concentration used. Representative plots for the first 30 minutes of each assay are depicted in Figures 5a-d with absorbance measurements at 412 nm for detection of TNB product formation. Substrate concentration was 1 mg/mL for the plots shown. Absorbance measurements were converted to micromoles TNB released, using the standard curve shown in Figure 5a. This provided initial rates of enzyme activity ($V_{\max}$), which were later used to calculate the turnover numbers k_{cat} . Variation of substrate concentrations allowed calculation of Michaelis constants for each material.

Figure 5b shows the initial rate behavior of the free AChE. Clearly, at incubation and assay temperatures of 25 °C the enzyme shows maximal activity and exhausts its substrate within the first few minutes. When it is dried and incubated at 25 °C, or hydrated/dried and incubated at 63 °C, its activity decreases dramatically. The results for the EM Conjugate are given in Figure 5c. Interestingly, when the enzyme is incubated in the dry state, at either the low or high temperature, it maintains a high level of activity when assayed at 25 °C. When the enzyme was incubated at 63 °C in the aqueous state, it showed a nearly complete loss of activity. Figure 5d gives the results for the EMH Composite. The initial rates are somewhat slower for it than for the EM Conjugate and free enzyme at 25 °C, likely because of substrate diffusion barrier effects by the hydrogel polymer. The initial rates for the xerogel, at both temperatures, are higher than that for the swollen hydrogel. This suggests that the xerogel, when exposed to assay solution, draws substrate in relatively rapidly because of osmotic effects arising from the protonated primary amine of the hydrogel polymer.

For the EM Conjugate, incubation in the dry state may allow the enzyme molecules to self-organize on the surface of the microbeads. This may occur readily since it is present in a tetrameric form

that has a distinct geometry. Also, AChE is known to possess a large subpopulation having a hydroxylysine-rich collagen-based “tail” region, located far from the active centers [34, 35]. The EDC coupling chemistry used to immobilize the enzyme onto the microbeads may heavily target these hydroxylysine residues, anchoring the tail and allowing the tetrameric active center-containing region to remain near the solution interface. The AChE may self-organize on the surface of the microbeads via interactions between the triple-helix collagen tails of neighboring enzyme molecules, as well as those between the tetrameric regions supporting the active centers. Also, it is known that the AChE active center resides in a cleft, or gorge, that is ~20 Angstroms below the surface of the protein. This region is known to not possess any lysine residues. Both of these structural features may assist the enzyme in retaining activity after immobilization. The lack of lysine residues prevents the active center from acting as the attachment site during the EDC coupling process, which would cause a decrease in activity [36, 37].

Figure 6 shows the values of the Michaelis constants K_m for each material. It represents the substrate concentration that results in a reaction velocity that is one-half of the maximum. It also is a measure of the active center affinity for substrate, with lower values reflecting higher affinity. For the AChE free in solution, the value for K_m obtained by us correlates closely with those found by other groups (see below). Three very significant findings are seen in Figure 6. First, for 25 °C aqueous and dry incubation the K_m values for the EM Conjugate and free enzyme are roughly equal. This suggests that the EDC coupling process did not cause the active centers to orient themselves in a manner that decreased substrate affinity. Also, it is of great interest that at elevated temperatures the EM Conjugate allowed the enzyme active center to retain a very low K_m after incubation at 63 °C in the dry state. The ratio to that of the free enzyme is ~29,670. The four order-of-magnitude increase in substrate affinity may arise from the EDC coupling process and its ability to organize the enzyme layer on the microbead surface. The fact that this huge difference between the two materials was not observed for the aqueous incubation indicates that water plays a major role in the AChE thermal denaturation process, whether it is immobilized onto the EM Conjugate or free in solution. However, it is intriguing that the EMH Composite maintained very low K_m values during *aqueous* incubation at 63 °C, factors of ~29,450 vs. that of free enzyme and of ~13,810 vs. that of the EM Conjugate. This suggests that three possible mechanisms are mediated by the hydrogel amine repeat units, which are positively charged – 1) they form ion-pairs with residues in the acetylcholine binding site “anionic locus”, which is known to contain negative charges (Figure 7) [25, 38], and this stabilizes the site, and/or 2) they form ion-pairs with other negatively-charged binding sites such as the peripheral anionic site [25], which cause allosteric effects that stabilize the active center and/or 3) they form ion-pairs with negatively-charged amino acid residues that are distributed generally over the enzyme surface, and stabilize the active center. AChE is above its isoelectric point at the assay pH, so will have an overall net negative charge. In a third mechanism, the hydrogel polymer is able to structure a large amount of the resident water through hydrogen bonding and electrostatic forces. This prevents the water from participating in AChE thermal denaturation processes, thus allowing the substrate binding site to maintain its natural conformation. We performed thermogravimetric studies on the EMH Composite that, in fact, confirmed that a substantial amount of structured water is present in the composite (see above). For incubation dry at 25 °C, aqueous at 25 °C, and dry at 63 °C, the EMH Composite showed K_m values that were generally about one magnitude higher than that of the EM Conjugate. This probably occurs because the hydrogel polymer chains hinder diffusion of substrate to the enzyme active center.

For the EM Conjugate, the K_m value of 1.15×10^{-4} M is close to the value reported by Yu et al [39] (0.67×10^{-4} M) for AChE immobilized onto amine-derivatized carbon nanotubes. For free enzyme, other groups have found K_m values that are near ours (1.75×10^{-4} M; 7.39×10^{-5} M; 2.06×10^{-4} M, [24, 40, 41]). Figure 8 shows the values of the turnover number k_{cat} for each material. It is a measure of the catalytic power of the active site, in this case involving the triad containing histidine, serine, and glutamate [25].

After aqueous incubation at 25 °C the EM Conjugate displays a k_{cat} that is nearly equal to that of the free enzyme ($\sim 2 \times 10^{-4}$ 1/s) and very near to those reported in the literature for free enzyme. Most notably, after dry incubation at 25 °C and 63 °C the EM Conjugate k_{cat} is 92- and 69-fold higher than that of the free enzyme, respectively. In relation to our suggestion regarding the enhanced K_m values shown by the EM Conjugate, the AChE may be oriented on the microbeads in a manner that allows conformations that favor optimal positioning of the catalytic His-Ser-Glu members. Overall, with the exception of the aqueous incubation at 63 °C, the EMH Composite showed k_{cat} values that were generally about one magnitude lower than that of the EM Conjugate, probably because the hydrogel polymer chains hinder substrate diffusion, as pointed out above, or also because of product inhibition effects.

Figure 9 gives the values of the overall catalytic efficiency k_{cat}/K_m for each material. For aqueous incubation at 25 °C, the EM Conjugate shows a value that is very similar to that of the free enzyme, 1.58×10^8 vs. 1.27×10^8 M. These values are only one order of magnitude lower than the accepted theoretical limit for enzymes in general (10^9 1/sec · M), which is a testament to the unusually high efficiency of AChE. For dry incubation at 25 °C and 63 °C, the EM Conjugate shows an efficiency that is two orders of magnitude higher than that of the EMH Composite, and a remarkable 2.16×10^6 -fold higher efficiency than that of the free enzyme, respectively. This six-order-of-magnitude increase is perhaps the key finding of the work presented herein. For aqueous incubation at 63 °C, the EMH Composite performs best, showing an efficiency that is $\sim 4.3 \times 10^4$ -fold higher than that of the free enzyme, and $\sim 1.7 \times 10^4$ higher than that of the EM Conjugate. The vast majority of this increase arises from the very low K_m value displayed by the EMH Composite, compared to that of the other two materials.

The results we obtained are consistent with previous studies of thermal inactivation of free and immobilized AChE and butylcholinesterase at temperatures above 50 °C, with the enzyme undergoing conformational changes in phases leading to complete loss in activity [5, 42]. At ambient (25 °C) temperatures, the better performance of the EM Conjugate vs. the free enzyme is noteworthy. From the classical point of view, enzyme immobilization on macroscale surfaces generally leads to a $\sim 50\%$ reduction in activity. Micro- and nanoscale or nanostructured surfaces, though, in general are known to preserve and/or enhance enzyme activity, but the degree of enhancement generally is not larger than one order of magnitude [19, 36].

In general, the findings of this work regarding the EM Conjugate and EMH Composite should be extendable to other enzymes. For example, lysine-rich “tail” regions could be added to virtually any enzyme via protein engineering methods, allowing EDC-mediated tail attachment to microbeads and EM Conjugate formation. Also, any enzyme with negatively-charged cation binding sites, or one that can be utilized at a pH above its isoelectric point, may experience the same manner of thermal stabilization by the poly(N-(3-aminopropyl methacrylamide)) hydrogel used in our work.

4. Conclusions

Using a simple amine coupling approach for enzyme immobilization onto magnetic microbeads, followed by encasement of the beads in a hydrogel, we demonstrate that the thermal stability of the AChE can be increased greatly. After incubation at 63°C for 24 hrs, the k_{cat}/K_m of the dried EM Conjugate was 6 orders of magnitude higher than that of the free enzyme, while that of the aqueous EMH Composite was 4 orders of magnitude higher. The effect generally arises through a very large decrease in K_m , and a relatively small increase in k_{cat} . The hydrogel, having a repeat unit that contains a positively-charged primary amine, may act to stabilize the enzyme through ion-pairing. To the best of our knowledge, this simple approach is novel and it is likely that this method for thermal stabilization can be extended to many other enzymes.

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Figure Legends

1. Synthesis scheme for the EM Conjugate and EMH Composite.
2. FTIR spectra of the EM Conjugate (A) and polystyrene-coated microspheres alone (B).
3. A) Mass distribution of the hydrated EMH Composite; B) as xerogel; C) thermogravimetric analysis of the hydrated EMH Composite.
4. Fluorescent confocal image of hydrogel with and without EMH Composite.
5. Standard curve for conversion of absorbance to micromoles TNB (A), and representative kinetic assay results for (B) AChE, (C) EM Conjugate, and (D) EMH Composite.
6. K_m values for AChE, EM Conjugate, and EMH Composite at 25° and 63°C.
7. Ion-pairing schemes that may contribute to thermal stabilization by the poly(N-(3-aminopropyl methacrylamide)) hydrogel.
8. Turnover number (k_{cat}) values for AChE, EM Conjugate, and EMH Composite at 25° and 63°C.
9. Catalytic efficiency (k_{cat}/K_m) values for AChE, EM Conjugate, and EMH Composite at 25° and 63°C.

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k_{cat}/K_m is 4.34×10^4 times higher than that of free enzyme when both are in aqueous solution and stored at 63 °C

k_{cat}/K_m is 2.16×10^6 times higher than that of free enzyme when both are dried and stored at 63 °C

Figure 1

Figure 2

Figure 3

A)

B)

C)

Figure 4

Figure 5a

Figure 5b

Figure 5c

Figure 5d

Figure 6

Figure 7

Figure 8

Figure 9