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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.

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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 ~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

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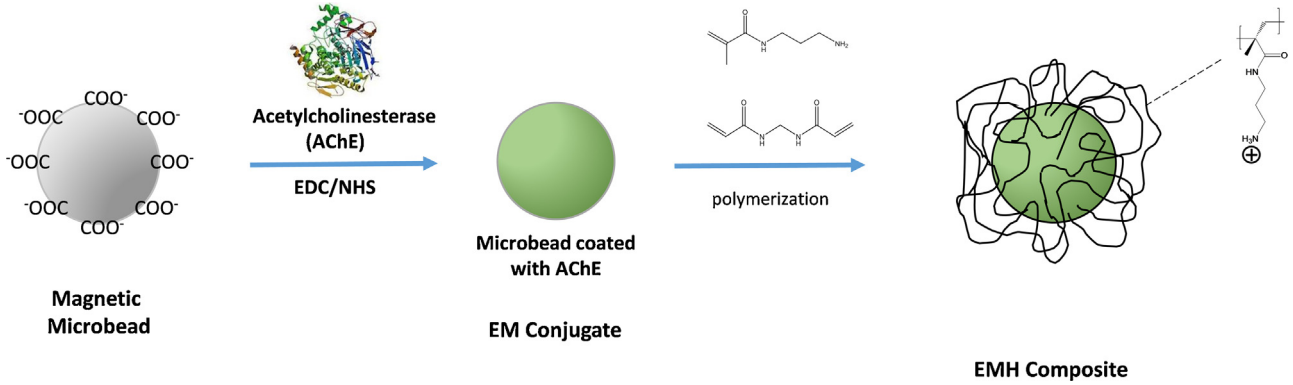

Fig. 1. Synthesis scheme for the EM Conjugate and EMH Composite.

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)) (Fig. 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 straight-forward, 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 Fig. 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 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) (Fig. 1). After the activated microbeads were isolated and washed with PB, 100 μl of a solution containing AChE (concentration 1.0 mg/ml in 0.01 M 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 1 h at RT, the solution was incubated overnight