Butyrylcholinesterase nanocapsule as a long circulating bioscavenger with reduced immune response

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Abstract

Butyrylcholinesterase (BChE) is the most promising bioscavenger candidate to treat or prevent organophosphate (OP) poisoning. However, the clinical application of BChE is limited by two obstacles: an inadequate circulation half-life and limited sources for production. Although several modification technologies including glycosylation and PEGylation have been developed to improve its pharmacokinetics, none of them have been able to outperform blood-derived native BChE. In this work, we designed a long-circulating bioscavenger nanogel by coating equine serum-derived BChE with a zwitterionic polymer gel layer. This zwitterionic gel coating protected BChE from denaturation and degradation under harsh conditions. Notably, the nanocapsule exhibited a long circulation half-life of ~ 45 h, a three-fold increase from the unmodified native version, enabling both therapeutic and prophylactic applications. In addition, the gel coating reduced the immunogenicity of equine BChE, unlocking the possibility to use non-human derived BChE as an OP bioscavenger in humans.

Key words: Nanogel, butyrylcholinesterase, organophosphate, circulation, protein delivery, and immune response

Introduction

Organophosphates (OPs) are a class of highly toxic synthetic compounds frequently used as pesticides. These compounds, such as dichlorvos and parathion, exert toxicity to insects and other animals, including humans, by inactivating acetylcholinesterase (AChE) in the peripheral and central nervous systems.[1, 2] This rapidly increases acetylcholine levels at cholinergic synapses and produces an acute cholinergic crisis, resulting in

neuromuscular paralysis throughout the body and death by respiratory failure.[3, 4] Though their use as pesticides has been regulated in many countries, OPs remain one of the most common causes of poisoning worldwide. The World Health Organization attributes 200,000 deaths per year in developing countries to OP poisoning.[5] Moreover, many of the most dangerous nerve agents developed as chemical weapons are potent OPs, including soman, sarin, VX and tabun.

Although current anticholinergic antidotes can prevent lethality from OP poisoning, they are unable to mitigate many common post-exposure effects including convulsions, incapacitation, performance deficits and permanent brain damage.[6] Bioscavenger enzymes are a promising alternative treatment, as they can bind to and neutralize OP compounds in the bloodstream before the toxin reaches its physiological target.[7] Plasma-derived butyrylcholinesterase (BChE) has been widely studied as a bioscavenger due to its rapid and irreversible binding to OPs. Exogenously administered BChE from equine or human serum has been shown to protect animals from OP poisoning as well as prevent post-exposure effects.[6, 8] Human plasma-derived BChE is considered a qualified bioscavenger candidate, having an acceptable circulation half-life *in vivo* to fulfill its mission without causing an immune response or adverse effects.[9] However, production difficulties have hindered the clinical application of human plasma-derived BChE. An extremely large amount of outdated human plasma is needed to generate even a single dose. It has been calculated that preparing a small stockpile of plasma-derived BChE (5000 doses) would require dedicating the entire annual U.S. supply of outdated plasma to its production, at a considerable cost.[10] Genetic engineering can produce recombinant human BChE (rh-BChE) at a lower cost, but rh-BChE has a much shorter circulation half-life than the native enzyme.[11] Alternatively, BChE derived from other animal sources such as equine serum may be used to supplement human BChE, though immunogenicity challenges must first be addressed.[12]

A technique that can effectively improve BChE pharmacokinetics (PK) as well as reduce its immunogenicity is clearly in demand to advance the application of enzyme-based bioscavengers. Glycosylation and PEGylation have been adopted to modify rh-BChE, but the resulting circulation half-life is still unsatisfactory.[13] PEGylation failed to improve the PK of plasma-derived native BChE.[14] Moreover, increasing evidence shows that many PEGylated protein products induce the production of anti-PEG antibodies after

administration, challenging the future of this technology.[15-17] To the best of our knowledge, no modified BChE has PK properties superior to its natural version.

In this work, we have developed a zwitterionic nanogel encapsulation technology to modify BChE from equine serum. Zwitterionic polymers continue to attract growing attention as an important class of super-hydrophilic biomaterials, demonstrating ultra-low levels of protein adsorption and exceptional biocompatibility.[18-20] Surfaces modified with zwitterionic polycarboxybetaine (PCB) have exhibited protein adsorption below 0.3 ng/cm² from 100% blood plasma or serum.[21] In a recent study, conjugating PCB to a protein increased its stability without sacrificing its bioactivity.[22] Moreover, PCB-based nanoparticles and nanogels were shown to have a very long *in vivo* circulation time and not to induce a measurable antibody response in rats after repeated intravenous (IV) injections.[23, 24] Based on these findings, we hypothesized that encapsulating BChE inside a PCB nanogel could enhance its stability and shield it from recognition by the immune system. In the meantime, the porous gel network allows OP toxins to diffuse in so that these toxins can be neutralized by enzyme binding sites, enabling it to act as a long circulating bioscavenger.

Materials and Methods

Materials and instruments

Butyrylcholinesterase (BChE) from equine serum, N-Acryloxysuccinimide (NAS), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), dimethyl sulfoxide (DMSO) anhydrous, bovine trypsin, bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), and paraoxon-ethyl were purchased from Sigma-Aldrich, and were used as received. Carboxybetaine acrylamide (CBAA) monomer was synthesized following a published method.[25] Synthesis of carboxybetaine acrylamide crosslinker (CBAAX) is described in the supporting information.

Dynamic light scattering and zeta potential were measured using a Malvern nano zetasizer in PBS 7.4 buffer. Transmission electron microscopy was performed using a Tecnai G2 F20 Supertwin TEM, with uranyl acetate as a negative staining agent. All bioassays were done using a BioTek Cytation 3 Multi-Mode Reader.

Protein encapsulation

For zwitterionic nanogel encapsulation, BChE was first modified to introduce acryloyl groups onto its surface. Taking the synthesis of n-BChE-50-0.2x as an example, the reaction was performed by dissolving 20 mg BChE into 10 ml 50-mM HEPES buffer (pH 8.5), followed by adding 100 μ l of NAS DMSO solution (20 mg/ml) dropwise. The reaction was stirred at 4 °C for two hours. The polymer encapsulation was done via *in situ* radical polymerization by adding 400 mg CBAA monomer, 80 mg of CBAAX crosslinker, 10 ml HEPES buffer, 15 mg APS and 60 μ l TEMED into the former reaction solution. After stirring for another 2 hours, the reaction mixture was concentrated and washed repeatedly with PBS 7.4 using 150 kDa molecular weight cutoff centrifugal filters. All other samples were prepared in the same way with adjusted NAS and crosslinker amounts. The protein residue activity was measured by a commercially available activity kit (Arbor assays, USA).

Samples used for *in vivo* tests were labeled with FITC. Native or encapsulated BChE was dissolved in 0.1 M sodium carbonate buffer (pH 9) at a protein concentration of 2 mg/ml. Then, 50 μ l of a 1mg/ml FITC DMSO solution was slowly added to each milliliter of the protein solution. The reaction was kept at 4 °C for 8 h in the dark. After reaction completion, the labeled samples were concentrated and washed repeatedly with PBS 7.4 using 100 kDa molecular weight cutoff centrifugal filters.

Measurements of thermal and protease resistance

For thermal stability tests, native and encapsulated protein samples (protein concentration 1 μ g/mL in 50-mM HEPES buffer, pH 8.5) were incubated at 55°C in a water bath. At different time points, each sample was taken out and quenched in an ice bath until measurement. After the final samples were taken out at 2 h, activities of all samples were measured using the butyrylcholinesterase fluorescent activity kit following the manufacturer's protocol. Values were recorded as percentage of each sample activity before heating.

For the protease resistance test, native and encapsulated BChE samples were co-incubated with bovine trypsin at 1:1 weight ratio at 37°C in 50-mM HEPES buffer, pH 8.5. After 0.5 h or 24 h, samples were taken out and measured using the butyrylcholinesterase fluorescent activity kit.

Enzyme inhibition by OP

Model organophosphate toxin paraoxon-ethyl was used to test the binding between native BChE and BChE nanocapsules. This assay was done by mixing BChE protein or nanocapsule solutions (HEPES, pH 8.5) with paraoxon solutions at different concentrations. The mixture was incubated at 37°C for 30 min and residual activity was measured using the butyrylcholinesterase fluorescent activity kit.

Cell culture and cytotoxicity assay

Cytotoxicity was assessed through an MTT assay. NIH3T3 cells were seeded in a 96-well plate at a density of 2×10^4 cells/well and incubated overnight. The following morning, the culture medium in each well was replaced with 180 μ L of fresh medium. Proteins or nanogel samples (20 μ L in PBS) were then added to each well. After incubation for 24 h, the medium was replaced with 200 μ L fresh medium containing 20 μ L MTT (5 mg/mL in PBS) and incubated for another 4 h. Finally, all medium was removed and 150 μ L/well DMSO was added, followed by shaking for 15 min. The absorbance of each well was measured at 490 nm with pure DMSO as a blank. Non-treated cells were used as a control and the relative cell viability ($\text{mean} \pm \text{SD}$, $n=3$) was expressed as $\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}} \times 100\%$.

Pharmacokinetics and biodistribution study

The pharmacokinetics of native and encapsulated BChE was studied using Sprague Dawley rats (male, body weight 74~100 g) as an animal model. Each sample was tested in six rats to generate statistical significance. All animal experiments adhered to federal guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee. For pharmacokinetic studies, each FITC-labeled protein sample was administered to the rat via tail vein injection at a dose of 4 mg/kg body weight. Blood samples were collected from the tail vein at 5 min, 6 h, 24 h, 48 h and 72 h after the injection. The blood samples were put in heparinized vials, centrifuged, and the enzyme content in plasma was estimated by fluorescence intensity. The IV injections and bleeding procedure were repeated twice, with one week between each injection.

At 72 h after the second injection, three rats in each group were killed and the heart, liver, spleen, lung, kidney and blood were collected for biodistribution analysis. The collected organ tissues were homogenized using a tissue ruptor, followed by centrifugation at $3200 \times g$ for 30 min at room temperature. The fluorescence of particles in the tissues was

measured using a microplate reader and compared to a standard curve generated using FITC-labeled samples added to untreated homogenized tissues. Two weeks post the second injection, all other rats were killed, 5 mL of blood was drawn from each using cardiac punch, and serum was prepared for antibody detection assays.

Enzyme-linked immunosorbent assay (ELISA)

For ELISA experiments, 100 μ L antigen solution (10 μ g/mL of native BChE) prepared in 0.1 M sodium carbonate buffer, pH 9.5, was used to coat each well of the 96-well plates. During the coating procedure, plates were incubated at 4 °C overnight. After removing antigen solutions, the plates were washed five times using phosphate-buffered saline washing buffer and then filled with blocking buffer (1% BSA solution in 0.1 M Tris buffer, pH 8.0). After incubation at room temperature for 1 hour, blocking buffer was removed and all wells were washed another five times. Serial dilutions of rat sera in PBS containing 1% BSA were added to the plates (100 μ L/well), which were incubated for 1 h at 37 °C. The plates were then washed five times. Goat anti-rat IgM or IgG conjugated to horseradish peroxidase (HRP) (Bethyl labs, USA) was used as the secondary antibody for detection of IgM and IgG. After adding the secondary antibody, plates were incubated at room temperature for 1 hour, and then washed five times before the addition of 100 μ L/well HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB, Bethyl labs). The plates were shaken for 15 min and 100 μ L stop solution (0.2 M H₂SO₄) was added to each well. Absorbance at 450 nm (signal) and 570 nm (background) was recorded by a microplate reader. Pre-bleeding sera were used as negative control for all ELISA detections. The positive signal was defined as absorbance significantly larger than corresponding negative control.

Statistics

Student's t test was chosen to compare two small sets of quantitative data when data in each sample set were related, with $P < 0.05$ being considered as statistically significant.

Results and Discussion

To coat the surface of BChE with a zwitterionic polymer gel, the protein was first chemically modified to introduce surface acryloyl groups, enabling carboxybetaine acrylamide (CBAA) monomer and crosslinker (CBAAX) to be grafted from the surface using free radical polymerization. As shown in Scheme 1, this process covered the

enzyme with a non-fouling PCB hydrogel layer. This gel capsule was designed to shield BChE from premature proteolysis and immune system recognition while simultaneously allowing OP toxins to diffuse in for neutralization by enzyme binding sites. By adjusting reaction feed ratios, both the number of polymer-protein conjugation sites and thickness of the hydrogel layer could be adjusted. The number of acryloyl groups anchored on the protein surface, which corresponds to the conjugation sites connecting the protein and polymer shell, was adjusted by changing the feed ratio of NAS and BChE. The physical properties of nanogel bioscavengers prepared under different conditions are listed in Table 1. Sample names are assigned as n-BChE-**a**-**b**x, where **a** denotes the molar feed ratio between NAS and protein in the acrylation step, and **b** designates the crosslinker content during polymerization. Dynamic light scattering measurements confirmed the formation of nanogels, as the encapsulated BChE increased in hydrodynamic size compared to the unmodified enzyme. The activity of the enzyme was well maintained at around 90% even when it was conjugated with up to 30 acryloyl groups per subunit. We tuned the crosslinker concentration to control the size of the nanogels, and found 10% and 20% crosslinker content to result in hydrodynamic nanocapsule sizes of ~20 nm and ~30 nm, respectively. The dry size of n-BChE-50-0.2x was measured by transmission electron microscopy (TEM), averaging 15.8 nm in size (Figure 1A). Encapsulating BChE in zwitterionic nanogels also neutralized the negative surface charge of the native protein (-19.1 mV), as measured by zeta potential. A thicker gel coat corresponded to a more neutral surface, indicating better protein surface coverage and protection.

Scheme 1. (A) Fabrication of BChE nanocapsules. The OP toxins can freely diffuse through the zwitterionic gel layer and (B) The zwitterionic gel layer protects the particle from opsonization and immune cell uptake.

Table 1 Characterization of BChE nanocapsules.

Sample ID	Surface vinyl group number ^a	Hydrodynamic size (nm) ^b	Zeta potential (mV)	Residue activity (%)
Native BChE	—	14.5±0.8	-19.1±2.9	100±4
n-BChE-5-0.2x	3.2	26.4±1.2	-1.9±0.2	92±8
n-BChE-50-0.2x	30.3	30.2±2.3	-5.2±0.5	88±6
n-BChE-200-0.2x	45.7	30.6±1.8	-4.9±0.3	53±11
n-BChE-50-0.1x	30.3	20.6±3.0	-10.5±1.4	89±7

a Measured by MALDI-TOF MS; data expressed as number per subunit.

b Measured by dynamic light scattering.

Figure 1. (A) TEM image of nanocapsules; (B) Relative activities of native BChE and nanocapsules incubated at 55 °C; (C) co-incubated with trypsin (* $P < 0.05$) and (D) BChE inhibition by paraoxon-ethyl.

Due to their structural complexity, protein therapeutics are often prone to instability

during storage, which causes great inconvenience in many applications. We performed thermal stress tests in this work to evaluate the thermal stability of enzymes prepared under different encapsulation conditions. As shown in Figure 1B, all nanocapsules retained better activity than native BChE when immersed in a 55°C water bath. This protection effect arises from a combination of two distinct mechanisms. For one, the CB monomer has substantial structural similarity to protein stabilizing agent glycine betaine. Encapsulating a protein in a PCB gel network is analogous to placing it into a highly concentrated protein stabilizing solution.[22, 24] The covalent conjugation sites between the protein and polymer gel network work alongside this stabilizing effect to provide additional protection against conformational changes,[26] which was confirmed by observing higher protein stability when a greater number of protein-polymer conjugation sites were present. The higher thermal stability extends the shelf life of this bioscavenger, and reduces the high cost of its production and use.

The *in vivo* stability of a therapeutic protein is not only challenged by its own structural vulnerability, but also by various proteases from organs and tissues. We chose trypsin as a model protease to evaluate the proteolytic stability of native and encapsulated BChE preparations. As shown in Figure 1C, after co-incubation with trypsin for 30 min, all samples showed an initial activity drop as some BChE was enzymatically degraded. The native protein lost half of its activity, while the nanocapsules maintained ~65% and ~80% of their original activities depending on the nanogel thickness. Notably, while native BChE lost all activity after 24 h of incubation (suggesting complete digestion), the sample encapsulated in a thicker polymer layer still retained ~60% of its activity. This protection from proteolysis is a major benefit of the non-fouling properties of the gel. The highly hydrated zwitterionic gel sterically hinders contact between BChE and proteases. Such proteolytic resistance contributes to long-term enzyme efficacy *in vivo*. For the following experiments, we chose n-BChE-50-0.2x as the representative nanogel-encapsulated bioscavenger due to its balance between high residual activity and stability.

Improved stability to environmental changes and proteolytic degradation is commonly gained at the expense of bioactivity. However, BChE nanocapsules avoided this compromise, exhibiting high stability with minimal sacrifice to activity. To determine the *in vitro* OP scavenging ability of these nanocapsules, we assayed their activity following

incubation with the model OP compound paraoxon, the active ingredient of insecticide parathion. The inhibition profiles of native BChE and nanogel-encapsulated BChE were virtually indistinguishable, with respective EC₅₀ values of 140 nM and 125 nM (Figure 1D).

As the nanogel-encapsulated BChE demonstrated enhanced stability and maintained OP neutralization ability *in vitro*, we tested its cytotoxicity before further *in vivo* evaluation. Native BChE showed no cytotoxicity to NIH 3T3 cells as expected, since it is derived from mammalian blood (Figure 2). Critically, the zwitterionic nanogel-encapsulated enzymes remained completely nontoxic, and cells retained 100% viability after incubation with BChE nanogels at very high concentrations, which can be attributed to the superior biocompatibility of PCB.

Figure 2. Cytotoxicity testing results from a MTT assay. The values represent percentage cell viability (means $\pm$ SD, n=3).

Improving circulation half-life is the core objective of BChE modification technologies. Serum-derived native BChE is highly glycosylated, enabling it to circulate significantly longer than non-glycosylated ‘naked’ recombinant BChE. Thus, glycosylating rhBChE has been considered a key strategy to boost its circulation time closer to that of the native enzyme.[9] PEGylation has also been applied to modify non-glycosylated rhBChE, and resulted in a similar half-life to glycosylated rhBChE in a mouse model[27]—neither

modification could outperform serum-derived native BChE. In addition, Saxena *et al.* PEGylated native glycosylated BChE, but no improvement in circulation time was observed.[14] In this work, we studied the PK behavior of equine serum-derived BChE before and after zwitterionic nanogel encapsulation through repeated *i.v.* injections of FITC-labeled samples in healthy Sprague–Dawley rats. As shown in Figure 3A, all circulation curves fit a two compartment model. PK parameters are listed in Table 2. After the initial injection, unmodified native BChE exhibited an elimination half-life ($t_{1/2\beta}$) of 15.6 h, in general agreement with values reported in the literature.[12] Remarkably, the circulation time of BChE nanocapsules demonstrated a threefold improvement over the native enzyme, with a $t_{1/2\beta}$ of 45 h. Moreover, the AUC_{∞} (area under curve) of the nanocapsules was 6.7 times that of native BChE, indicating significantly higher systemic availability. Zwitterionic polymers have a unique ability to extend the circulation time of nanoparticles beyond that of conventional biomaterials like PEG.[23, 24] The “stealth” characteristics of zwitterionic PCB and the comprehensive surface coverage of nanogel encapsulation appear to work synergistically to help these bioscavengers avoid reticuloendothelial system (RES) clearance and thus circulate for a significantly longer time.

Figure 3. Blood circulation profiles of native BChE vs. nanocapsule. Circulation curves after the first *i.v.* administration (A) and second administration (B).

For prophylactic applications, multiple doses of bioscavenger enzymes may be needed to prolong protection against OP toxins. A second injection of encapsulated or unmodified

BChE was thus given one week after the initial administration of each version, and PK profiles were evaluated as before. As shown in Figure 3B, an obvious accelerated blood clearance (ABC) phenomenon was noted in the group treated with unmodified BChE, with $t_{1/2\beta}$ decreasing to 12 h, AUC_{∞} values dropping by a third and MRT (mean residence time) decreasing by half. This significant decrease is mainly due to the immunogenicity of equine proteins in rats. A similar phenomenon was reported for human plasma derived BChE administered to mice.[14] In contrast, no significant difference in the PK profile was observed between the two injections of BChE nanocapsules, indicating the encapsulated bioscavengers are likely to maintain their superior PK performance through repeated doses.

Table 2 PK parameters after repeated injections

Parameters	Native BChE		n-BChE-50-0.2x	
	Dose 1	Dose 2	Dose 1	Dose 2
$t_{1/2\beta}$ (h)	15.6±1.2	12.0±0.4	45.0±4.8	48.5±6.2
AUC_{∞} (µg/mL × h)	344±22	247±9	2306±117	2331±189
AUC_{0-t} (µg/mL × h)	334±19	246±6	1571±86	1550±103
CL (mL/h)	3.5±0.4	4.8±0.3	0.5±0.1	0.5±0.1
MRT (h)	16.0±1.4	7.5±0.5	62.8±5.3	65.9±7.1

We explored the immunogenicity of this platform in further detail by collecting serum two weeks after the second injection and testing it for anti-BChE antibodies using direct ELISA. As shown in Figure 4, high titers of both IgM (titers 1:6400) and IgG (titers 1:12800) were found in the group treated with native equine BChE. Glycosylated equine serum BChE is expected to be highly immunogenic for rats, due to differences in glycosylation and protein sequences. Likewise, unmodified BChE derived from other species cannot be directly used in humans, as the resulting immune response would certainly reduce its efficacy and potentially induce life-threatening anaphylactic reactions. In contrast, no IgG and negligible IgM were detected in rats treated with BChE

nanocapsules, corroborating that the PCB nanogels effectively rendered the protein “invisible” to the immune system and thus mitigated antibody induction.

Figure 4. Detection of antibodies by direct ELISA: (A) Anti-BChE IgM and (B) Anti-BChE IgG. Serum from each rat is plotted separately.

Figure 5. Biodistribution of native BChE vs. nanocapsule. Each value is averaged from three rats. SDs are shown as error bars.

To examine the biodistribution of BChE nanocapsules, we gathered blood and tissue samples from treated rats 72 h after the second injection for analysis. These data are presented in Figure 5 as recovered fluorescence per gram of tissue. BChE nanocapsules sustained a high serum concentration, whereas unmodified BChE was completely cleared from blood circulation. Similar to most long circulating nanoparticles, accumulation of nanocapsules was mainly found in the liver and spleen.[23, 28] We observed lower levels of organ accumulation by unmodified BChE compared with the nanocapsules three days

after injection. This can be attributed to their differing levels of proteolytic stability. As observed from our *in vitro* tests, unprotected BChE was readily degraded by proteases after organ accumulation, but nanogel encapsulation partially shields BChE from protease recognition, delaying this metabolic process. The BChE nanocapsules in this work were prepared using amide-containing crosslinkers, which are expected to degrade through slow hydrolysis. If faster degradation is needed, ester or disulfide-based crosslinkers could be substituted to facilitate metabolism.

Conclusions

In summary, toxin-neutralizing enzyme BChE, derived from equine serum, was encapsulated in zwitterionic poly(carboxybetaine) nanogels. Encapsulated BChE exhibited greater proteolytic and thermal stability than its native precursor without sacrificing its ability to neutralize OP toxins. These nanocapsules demonstrated a superior circulation half-life *in vivo*, outperforming the glycosylated native BChE by three times. Furthermore, repeated injections of bioscavenger nanocapsules did not induce antibody production or lose efficacy. This powerful strategy unlocks the possibility to use animal tissue derived BChE as supplement or replacement for the inadequate human native BChE. Enzyme bioscavengers encapsulated in zwitterionic nanogels are a promising strategy to be used in both clinical and military settings.

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