In Vitro Characterization of Cationic Copolymer-Complexed Recombinant Human
Butyrylcholinesterase

Carey Pope¹, Chibuzor Uchea¹, Nicholas Flynn², Kirstin Poindexter¹, Liyi Geng⁴, W.
Stephen Brimijoin⁴, Steve Hartson³, Ashish Ranjan¹, Joshua D. Ramsey² and
Jing Liu^{1*}

¹Department of Physiological Sciences

²School of Chemical Engineering

³Department of Biochemistry and Molecular Biology

Oklahoma State University

Stillwater, OK 74078

and

⁴Department of Pharmacology and Experimental Therapeutics

Mayo Clinic

Rochester, MN

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*Send correspondence to Dr. Jing Liu, Center for Veterinary Health Sciences, 264
McElroy Hall, Oklahoma State University, Stillwater, OK, 74078; jing.pope@okstate.edu

Abstract

Effective use of exogenous human BChE as a bioscavenger for organophosphorus toxicants (OPs) is hindered by its limited availability and rapid clearance. Complexes made from recombinant human BChE (rhBChE) and copolymers may be useful in addressing these problems. We used *in vitro* approaches to compare enzyme activity, sensitivity to inhibition, stability and bioscavenging capacity of free enzyme and copolymer-rhBChE complexes (C-BCs) based on one of nine different copolymers, from combinations of three molecular weights (MW) of poly-L-lysine (PLL; high MW, 30-70 kDa; medium MW, 15-30 kDa; low MW, 4-15 kDa) and three grafting ratios of poly(ethylene glycol) (PEG; 2:1, 10:1, 20:1). Retarded protein migration into acrylamide gels stained for BChE activity was noted with all copolymers as the copolymer-to-protein ratio was increased. BChE activity of C-BCs was lower relative to free enzyme, with the 2:1 grafting ratio showing generally greater reduction. Free enzyme and C-BCs showed relatively similar *in vitro* sensitivity to inhibition by paraoxon, but use of the 20:1 grafting ratio led to lower potencies. Through these screening assays we selected three C-BCs (high, medium and low MW; 10:1 grafting) for further characterizations. BChE activity was higher in C-BCs made with the medium and low compared to high MW-based copolymer. C-BCs generally showed higher stability than free enzyme when maintained for long periods at 37°C or following incubation with chymotrypsin. Free enzyme and C-BCs were similarly effective at inactivating paraoxon *in vitro*. While these results are promising for further development, additional studies are needed to evaluate *in vivo* performance.

Key words: bioscavenger, organophosphate, nerve agent, paraoxon

1. Introduction

Organophosphorus anticholinesterases (OPs) are among the most toxic of synthetic chemicals [1]. Their pronounced acute toxicity derives from potent inhibitory effects on the enzyme acetylcholinesterase (AChE). OPs bind covalently to the active site serine residue (Ser203) of AChE, blocking the catalytic hydrolysis of the neurotransmitter acetylcholine [2]. Extensive AChE inhibition leads to accumulation of acetylcholine at cholinergic synapses and resulting prolonged stimulation of cholinergic receptors in a variety of tissues. This mode of action led to widespread use of OPs as insecticides and, more notoriously, their misuse as weapons of chemical warfare and terrorism [3].

Systemic administration of exogenous bioscavenger proteins (i.e., circulating proteins that can bind to toxicants before they distribute to target tissues) was first shown to block OP toxicity over two decades ago [4,5]. Bioscavengers can prevent tissue AChE inhibition and block the expression of some of the most debilitating consequences of OP intoxication, i.e., respiratory depression, seizures, and neuropathology [6,7]. Several types of OP bioscavengers have been evaluated, with human butyrylcholinesterase (hBChE) and paraoxonase (PON1) being the leading stoichiometric and catalytic bioscavengers, respectively [8,9].

Butyrylcholinesterase is the “sister” enzyme of AChE, distributed throughout the body but in highest concentrations in the liver and plasma [10]. Purified hBChE has been shown to be a stable, effective, and safe bioscavenger against the most toxic OP nerve agents in animal models [11]. Intravenous administration of BChE has been used in humans since the 1950’s to treat OP intoxications, succinylcholine toxicity, and more

1 recently in Phase I clinical trials (reviewed in [10]). A major limitation in its application
2 however is the need for a large amount of purified protein, due in part to its relatively
3 rapid clearance from the circulation. Parenterally administered hBChE has a mean
4 retention time of about 50-100 hours in multiple species [8,11,12,13,14,15] although it
5 may have longer residence in humans [16]. Production of sufficient amounts of purified
6 hBChE is also challenging due to the limited supply of human plasma, the relatively
7 limited yield of enzyme from plasma, and significant expense, all of which have
8 hindered the application of hBChE as prophylactic agent against OP intoxications [10].

9 Recombinant hBChE (rhBChE), because it can be expressed in high levels using cells,
10 animals and even plants, remains a promising alternative for the hBChE. The human
11 enzyme exists primarily in tetrameric form, while recombinant enzymes are often
12 monomers or dimers. Some recombinant BChE enzymes have a very short circulatory
13 residence time, e.g., human BChE expressed in CHO cells (predominantly monomers
14 and dimers) has a half-life of only about two minutes. In contrast, these same
15 investigators reported that the recombinant tetramer (produced by inclusion of
16 polyproline) led to much longer circulation (about 16 hours [17]) . Chilikuri and
17 coworkers [18] reported that a rhBChE tetramer produced in HEK 293 cells had a mean
18 circulatory residence time in mice of about 18 hours. Thus even the tetramer
19 recombinant enzymes circulate with reduced time compared to the human serum
20 enzyme. The shorter circulation time, instability, and potential immunogenicity
21 compared to the serum enzyme pose obstacles for use of recombinant BChE as a
22 bioscavenger [10].

One approach that may address the problem of clearance is the use of polymers to protect the recombinant enzyme and thereby extend its circulation time [19-24]. Recombinant BChE complexed with poly(ethylene glycol) (PEG) increased mean retention time in mice from 18 hours to 36 hours and the PEG-enzyme complex was more resistant than the unmodified enzyme to inactivation *in vitro* by the protease chymotrypsin [18]. Increased circulation residence for catalytic bioscavengers has also been shown with PEG-modified enzyme [25]. A grafted copolymer of poly-L-lysine (PLL) and PEG (PLL-g-PEO) was used by Gaydess and coworkers [26] to form a copolymer-BChE complex (C-BC) using native human serum BChE or horse serum BChE. This C-BC was shown to enter the brain within 2 hours after parenteral administration, while no accumulation of BChE in brain was noted with administration of the free enzyme. These findings suggested that polymer or copolymer complexes with BChE can lead to different pharmacokinetic properties compared to the free enzyme, and that a C-BC might be useful as a bioscavenger.

We report the relative *in vitro* enzyme activity, sensitivity to the OP inhibitor paraoxon, stability (to prolonged incubation at 37°C and inactivation by chymotrypsin), and bioscavenging potency against paraoxon in free and copolymer-complexed rhBChE. The *in vitro* findings described herein provide information on the design and characteristics of copolymer-based complexes containing rhBChE that may be useful in the further development of a bioscavenger.

2. Materials and Methods

2.1. Chemicals and Materials

Methoxypoly(ethylene glycol), functionalized with n-hydroxysuccinimide ester was purchased from Creative PEGworks (Chapel Hill, NC). Glutaraldehyde (50%) was purchased from Fisher Scientific (Pittsburg, PA). Zeba Spin Desalting Columns (7 kDa cut-off) were purchased from Pierce Chemicals (Dallas, TX). Pre-cast nondenaturing polyacrylamide gradient gels (4-20%) were purchased from Bio-Rad (Hercules, CA). Paraoxon (O,O'-diethyl-*p*-nitrophenyl phosphate, >98% purity) was purchased from ChemService (West Chester, PA). Tritium-labeled acetylcholine iodide, specific activity 80.1 mCi/mmol, was purchased from PerkinElmer (Boston, MA). Poly-L-lysine, pig liver carboxylesterase, α -chymotrypsin from bovine pancreas (Type II) and all other reagents/chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Recombinant Human Butyrylcholinesterase (rhBChE) Purification

A total of twenty C57BL/6 mice were injected through the tail vein with 10^{13} particles of AAV8-VIP viral vector encoding human BChE. After four weeks (to reach maximum levels of expressed transgene), the mice were euthanized by overdose with pentobarbital and blood was collected by cardiac puncture. After centrifugation (10 min, 1,000 x g) serum was collected and dialyzed overnight at 4°C against 20 mM sodium acetate, 1mM EDTA. The dialyzed serum was centrifuged at 10,000 x g for 10 min and the supernatants were incubated overnight at 4°C with Q Sepharose equilibrated with 20 mM sodium acetate, 1 mM EDTA pH 4.0. The Sepharose beads were then washed with the same buffer until the OD₂₈₀ of the solution dropped below 0.04. Protein was then eluted with 500 mM NaCl in 20 mM sodium acetate, 1 mM EDTA, pH 4.0. The two eluents with the highest BChE activity were combined and desalted by spin desalting columns. The eluent was adjusted to pH 7-8 with 1 M Tris-Cl pH 8.5 and incubated at

4°C with procainamide Sepharose 4B (generously supplied by Dr. Oksana Lockridge, University of Nebraska), which had been equilibrated overnight with 20 mM potassium phosphate, 1 mM EDTA pH 7.0. The beads were then loaded onto a glass column and washed with 20 mM potassium phosphate, 1 mM EDTA, pH7.0, followed by 0.2 M NaCl in 20 mM potassium phosphate, 1 mM EDTA, pH7.0. BChE protein was eluted with 1 M NaCl in the same buffer. The two eluents with the highest BChE activity were combined, then desalted and concentrated over Amicon Ultra Centrifugal Filters (Ultracel-50K). Purity assessed by SDS PAGE gel electrophoresis (reducing conditions) was greater than 90%, with a prominent band at approximately 85 kDa. The purified enzyme was stable during storage at 4°C in 20 mM potassium phosphate, 1 mM EDTA, pH7.0 containing 0.02% NaN₃.

2.3. Copolymer Synthesis

A library of nine copolymers was prepared using three different sizes of poly-L-lysine (PLL) and three different grafting ratios of poly(ethylene glycol) (PEG). Methoxypoly(ethylene glycol, mPEG) with an amine-reactive, n-hydroxysuccinimide ester (NHS) on the distal end was reacted with PLL to produce a PLL-grafted-PEG (PLL-g-PEG) copolymer. A 50/50 mixture of 2 and 5 kDa PEG was used along with each of the three PLLs (high molecular weight (MW), 30-70 kDa; medium MW, 15-30 kDa; low MW, 4-15 kDa). The ratio of PEG to PLL was varied to produce three grafting ratios (2:1, 10:1 and 20:1). The mPEG-NHS and PLL were dissolved in PBS and allowed to react at room temperature for 2 hours. The resulting copolymer was purified using a centrifugal concentrator with a 10 kDa cutoff filter, rinsed with ultrapure water,

then lyophilized and stored at -20°C. Grafting ratios were determined by ¹H NMR spectroscopy using a Bruker Avance INOVA 400 MHz spectrometer.

2.4. Copolymer-BChE Complex Preparation

The method for synthesis of copolymer-BChE complexes (C-BCs) was similar to that reported by Gaydoss and coworkers [26]. As BChE and PLL carry opposing net charges at physiological pH, mixing BChE and the copolymer at neutral pH leads to spontaneous complex formation. Each copolymer was dissolved in PBS immediately prior to adding to the enzyme. A constant amount of rhBChE (25 µl, 0.266 mg/ml) was first aliquoted into a 1.5 ml Eppendorf tube, and then 7.5 µl of either PBS (to produce the free enzyme) or copolymer in PBS (with either 1:1, 2:1, 3:1, 7:1, 10:1 or 14:1 proportionate amounts of copolymer relative to protein) was added drop-wise with continuous mixing. The tubes were allowed to incubate at room temperature for one hour. Either PBS (5 µl; for free enzyme) or glutaraldehyde (5 µl of 0.025% in PBS; for C-BCs) was then slowly added with continuous mixing and the reactions were incubated at room temperature for another three hours. All resulting free enzyme and C-BC preparations thus contained a final concentration of 0.177 mg/ml protein. These were either used immediately or maintained at 4°C until use.

2.5. BChE Activity Measurements

Free enzyme and C-BCs were diluted with PBS containing 0.05% BSA (PBS/BSA) to maintain enzyme activity. BChE activity was measured either with the radiometric method of Johnson and Russell [27] or the photometric method using a modification of Ellman and colleagues [28]. For the radiometric method, 20 µl of a diluted sample was

added to 60 μ l of 10 mM Tris HCl containing 1 mM EDTA, pH 7.2 (25°C), followed by addition of [^{3} H]acetylcholine iodide (1 mM final concentration) and incubation at 37°C. Activity in unknown samples was normalized relative to activity in reactions allowed to undergo complete substrate hydrolysis. For the photometric method, 25- μ l aliquots of sample were transferred to individual wells in a 96-well plate. A cocktail (175 μ l) containing 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB, 100 μ M final concentration) and 1 mM butyrylthiocholine iodide (1 mM final concentration) in 10 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.2 was then added to each well and absorbance change at 412 nm was measured in kinetic mode at 37°C in a Spectramax 340PC micro-plate reader (Molecular Devices; Sunnyvale, CA, USA). Activity was calculated based on the molar extinction coefficient of the colored product (13,600 M $^{-1}$ cm $^{-1}$ [28]). One unit (U) of activity was defined as one μ mole of substrate hydrolyzed per minute under the conditions of each assay.

2.6. Non-denaturing Polyacrylamide Gel Electrophoresis and BChE Activity

Staining

Proteins in the free enzyme and copolymer-BChE complexes were separated by electrophoresis using non-denaturing polyacrylamide gradient gels (4-20% acrylamide; 2 hours at 180V, room temperature) and then stained for esterase activity essentially by the method of Karnovsky and Roots [29]. Briefly, gels were immersed in a solution containing 120 mM sodium maleate, pH 6 with 2 mM butyrylthiocholine iodide, 5 mM sodium citrate, 3 mM copper sulfate and 0.5 mM potassium ferricyanide for three hours at room temperature with gentle agitation. Gels were then rinsed in deionized water and photographed. A reddish-brown precipitate formed where butyrylthiocholine was

hydrolyzed, allowing visualization of differential BChE migration in the gel with free vs copolymer-complexed enzyme.

2.7. In Vitro Sensitivity of BChE Activity in Free BChE and Copolymer-BChE Complexes to Inhibition by Paraoxon

The different enzyme preparations were incubated with vehicle (PBS/BSA containing 0.1% ethanol) or one of a range of paraoxon concentrations (0.1 – 300 nM) at 37°C for 20 minutes. Residual BChE activity was then measured by the photometric method as described in Section 2.5. Enzyme activity in the reactions containing paraoxon was normalized relative to activity in the sample incubated with vehicle alone (control) and IC₅₀s along with the respective 95% confidence intervals were calculated using nonlinear regression.

2.8. Stability of Free BChE and Copolymer-BChE Complexes

Preparations were incubated in a shaking water bath at 37°C for up to 28 days and BChE activity measured at selected times to estimate stability. All samples were kept in capped 1.5 ml Eppendorf tubes and aliquots removed to assay BChE activity using the radiometric method, as described in Section 2.5.

Sensitivity of BChE activity in the free enzyme was compared to that in the C-BCs after exposure to chymotrypsin, using a modification of the method of Chilikuri and coworkers [18]. Free enzyme and C-BCs were first diluted to a protein concentration of 0.044 mg/ml using PBS/BSA. Six µl of PBS was added to individual 1.5 ml Eppendorf tubes, followed by 3 µl of either free enzyme or C-BC, and then 3 µl of chymotrypsin (0.15 mg/ml). These reactions were mixed and incubated at 37°C, with aliquots (1.5 µl)

removed at 0, 1, 2, 4, 6 and 8 hours. These aliquots were further diluted another 100-fold using PBS/BSA and assayed for residual BChE activity by the photometric method as described in Section 2.5.

2.9. In vitro Bioscavenger Assay

An indirect enzyme inhibition assay was used to evaluate *in vitro* bioscavenger potential of free enzyme and selected C-BCs using paraoxon as the inhibitor. Pig liver carboxylesterase (CarbE) was used as a marker enzyme and was assayed essentially as described by Wei and coworkers [30]. First, *in vitro* sensitivity of CarbE to paraoxon was evaluated. CarbE (10 μ l, 0.4 U/ml) was added to wells in a 96-well plate on ice followed by addition of 10 μ l of either vehicle (10 mM Tris-HCl containing 1 mM disodium EDTA and 4% ethanol, pH 7.2) or paraoxon (0.3-1,000 nM). Reactions were allowed to incubate for 20 minutes at 37°C before adding substrate (*p*-nitrophenyl acetate, 3 mg/ml in acetonitrile). Absorbance change at 405 nm was monitored in kinetic mode at 37°C. Under these conditions, the IC₅₀ for paraoxon against CarbE activity was 7 nM (data not shown). A concentration of paraoxon that elicited near complete inhibition (40 nM) was chosen for use in the *in vitro* bioscavenger assay.

Paraoxon inactivation *in vitro* (as a measure of bioscavenger potential) was evaluated essentially as described above, but with an additional pre-incubation step. In 1.5 ml Eppendorf tubes, 6 μ l of either vehicle (PBS/BSA) or one of a series of dilutions of either the free or the copolymer-complexed enzyme was added to 6 μ l of either vehicle or paraoxon (40 nM), followed by incubation at 37°C for 20 minutes. Twelve μ l of CarbE (0.4 U/ml in 10 mM Tris HCl containing 1 mM EDTA, pH 7.2) was then added to each

tube and incubated for another 20 minutes at 37°C. The tubes were then transferred to ice and aliquots (20 µl) were subsequently added to wells in a 96-well plate on ice. Buffer (10 mM Tris-HCl, pH 7.2, containing 1 mM disodium EDTA; 177 µl) was added to each well followed by addition of 3 µl of a mixture of *p*-nitrophenyl acetate (3 mg/ml) and ethopropazine (a specific inhibitor of BChE, 665 µM) in acetonitrile. Ethopropazine was added to block any residual BChE from hydrolyzing the CarbE substrate. Absorbance change at 405 nm was recorded in kinetic mode as above. CarbE activity in the reactions that were pre-incubated with only vehicles was defined as vehicle control. Activity in 1) reactions that were pre-incubated without either the free enzyme or copolymer-BChE complex but with paraoxon, as well in 2) reactions that included both the enzyme (free enzyme or C-BC) and paraoxon were plotted as percent of vehicle control. The data were transformed to calculate EC₅₀ values (i.e., protein content for each preparation that inactivated 50% of the paraoxon) by nonlinear regression.

2.10. Statistical Analyses

IC₅₀ and EC₅₀ values were calculated using a non-linear curve fit program [i.e., log (inhibitor) vs. normalized response (variable parameter)]. Characterization of BChE activity in the free enzyme and C-BCs and paraoxon inactivation in the bioscavenger assay both used one way ANOVA followed by Dunnett's test. The influence of PLL size and PEG grafting ratio on paraoxon sensitivity *in vitro* was evaluated using two way ANOVA. Enzyme stability with prolonged 37°C and chymotrypsin exposure was evaluated by two way repeated ANOVA with enzyme and time as main effects, followed by Dunnett's *post hoc* test. All procedures were conducted using GraphPad Prism® software, version 6.0.

3. Results

Figure 1 shows separation of proteins in the free enzyme and C-BCs formed with the nine different copolymers using nondenaturing gels stained for BChE activity. Six copolymer-to-protein mass ratios (1:1, 2:1, 3:1, 7:1, 10:1 and 14:1) were used with each of the copolymers (high MW-based, Figure 1A-C; medium MW-based, Figure 1D-F; low MW-based, Figure 1G-I). Grafting ratios (2:1, 10:1 and 20:1) are shown from left to right in the different gels, across each PLL size. The free enzyme is shown in the left-most lane in each gel, while the remaining lanes show the increasing copolymer-to-protein ratios (from left to right).

The free enzyme showed two primary bands of activity in all gels (although a lower band was sometimes apparent). These two primary bands of BChE activity appeared to migrate less into the gel matrix with increasing copolymer-to-protein ratios with all copolymers, suggesting formation of copolymer-BChE complexes (C-BCs). In general, there were PLL size-based differences in migration of BChE into the gel matrix (extent of migration: low MW>medium MW≥high MW). The 10:1 grafting ratio with the high MW-based copolymers showed lesser protein migration compared to 2:1 and 20:1 ratios, especially with the lower (1:1, 2:1 and 3:1) copolymer-to-protein ratios. On the other hand, protein migration with the 2:1 grafting ratio and medium MW-based copolymer was less than when using the other two grafting ratios. Finally, grafting ratios with the low MW-based copolymers appeared to have relatively little effect on copolymer concentration-dependent effects on migration. The results suggested however that incubation of rhBChE with any of the copolymers led to copolymer concentration-dependent C-BC formation.

Figure 2 shows enzyme activity in these same preparations. In general, activity was reduced to in the different C-BCs relative to the free enzyme. The highest retention of BChE activity among the nine copolymers was with the medium MW- (10:1 grafting, Figure 2E), low MW- (10:1 grafting, Figure 2H), and low MW- (20:1 grafting, Figure 2I) based copolymers.

For a copolymer-modified BChE to be an effective bioscavenger, it needs to interact freely with OP molecules. We therefore compared the inhibition of BChE activity in the free and copolymer-complexed forms by paraoxon, a prototype OP inhibitor. Table 1 shows that paraoxon elicited similar concentration-dependent inhibition of BChE activity with either the free enzyme or any of the C-BCs tested (IC_{50} values = 4.0 – 10.1 nM). When these data were analyzed by PLL size and grafting ratio, there was no effect of PLL size but a significant main effect of grafting ratio was noted ($F_{2,18}$) = 16.17, p < 0.0001), with higher IC_{50} values in complexes made with the 20:1 grafting ratio (Table 1). Based on relative differences in migration in the activity gels (Figure 1), differences in retention of BChE activity (Figure 2), and slight differences in sensitivity to *in vitro* inhibition by paraoxon (Table 1), we selected three copolymers (high, medium and low MW, all 10:1 grafting) for further study.

Figure 3 shows BChE activity from 3-4 independent preparations of the selected C-BCs. As noted in the initial screening assays (Figure 2), copolymer-BChE complex (C-BC) formation led to a loss of enzyme activity with A) the high MW- ($F_{(3,8)}$ = 12.77, p = 0.002), B) medium MW- ($F_{(3,12)}$ = 17.46, p < 0.0001) and C) low MW-based ($F_{(3,8)}$ = 6.731, p = 0.014) copolymers. Enzyme activity was significantly lower relative to the free enzyme with all three copolymers using the 7:1 and 14:1 copolymer-to-protein ratios. With the

1 medium MW-based complexes (Figure 3B), BChE activity was significantly lower with
2 the 7:1 and 14:1 compared to 1:1 copolymer-to-protein ratio. Compared to the free
3 enzyme, C-BCs had between 29-52% less BChE activity, with the most extensive
4 reduction noted in the high MW-based samples (Figure 3A).

5 A major goal for copolymer modification of rhBChE is to increase circulation time. The
6 modified enzyme needs to be more stable with prolonged time at body temperature and
7 less sensitive to proteases, relative to the free enzyme. We first compared the *in vitro*
8 stability of the free and copolymer-complexed enzymes maintained at 37°C in PBS/BSA
9 for up to 28 days. As shown in Figure 4, although BChE in the free enzyme preparations
10 had the highest activity, they showed 26-35% loss of activity during the 28-day
11 incubation (dashed lines, Figure 4A-C). The C-BCs using the high MW-based
12 copolymer with 7:1 and 14:1 copolymer-to-protein ratios also showed substantial loss of
13 activity (35-38%, Figure 4A), but there were no significant differences compared to the
14 free enzyme ($F_{3,8} = 2.174$, $p=0.17$). In contrast, significantly less reduction (11-16%)
15 was noted in the C-BCs made with either medium MW- ($F_{3,8} = 7.60$, $p=0.01$) or low MW-
16 based copolymer ($F_{3,8} = 4.1$, $p=0.049$) (Figure 4B,C) compared to the free enzyme,
17 suggesting these C-BCs are relatively more stable at 37°C than the free enzyme.

18 We also compared the *in vitro* inactivation of BChE activity in the free enzyme and C-
19 BCs by chymotrypsin. Figure 5 shows that BChE activity in C-BCs was significantly less
20 sensitive than the free enzyme to inactivation by this protease (main effect, $F_{3,10} = 10.33$,
21 $p = 0.002$). There was also a main effect of time in this analysis, as well as a significant
22 interaction between enzyme form and time. Thus pair-wise comparisons were not
23 reported.

Modeling these inactivation data (Figure 5) with one-phase decay led to relatively high R^2 values for the free enzyme and C-BCs made with the low MW- and high MW-based copolymers ($R^2 = 0.75-0.99$). Moreover, maximal loss of activity with the free enzyme was estimated at 97% (95% confidence interval [CI] = 91-102%), while maximal loss with the low MW- and high MW-based C-BCs was 46% (CI = 48-61%) and 46% (CI = 38-69%), respectively. This suggested that a proportion of the BChE molecules in these C-BCs may be effectively shielded from the protease. Interestingly, the data with the medium MW-based C-BCs did not fit well with a one-phase decay model. Further studies will be needed, using extended incubation times and other proteases, to better understand the relative sensitivity to proteases between free and copolymer-complexed enzymes. Overall however the data suggest that BChE in polyionic copolymer-enzyme complexes may be relatively resistant to degradation by chymotrypsin and possibly other proteases and therefore more stable *in vivo*.

While the *in vitro* inhibition data (Table 1) suggested that the free and copolymer-complexed BChE are relatively similar in sensitivity to paraoxon, we also evaluated their respective abilities to inactivate paraoxon, i.e., to act as a bioscavenger *in vitro*. Figure 6 shows there was essentially complete CarbE inhibition when paraoxon was initially pre-incubated with only PBS (PO, Figures 6A-D) before adding the marker enzyme. In contrast, pre-incubation of paraoxon with the free enzyme (Figure 6A; $F_{(7,32)} = 101.3$, $p < 0.0001$) or C-BCs made with high MW- (Figure 6B; $F_{(7,24)} = 47.52$, $p < 0.0001$), medium MW- (Figure 6C; $F_{(7,16)} = 73.44$, $p < 0.0001$) and low MW-based (Figure 6D; $F_{(7,14)} = 7.82$, $p = 0.0006$) copolymers protected against CarbE inhibition in a relatively similar, concentration-dependent manner. Plotting percent protection of CarbE inhibition

vs log of the protein concentration allowed estimation of bioscavenger EC₅₀s. All four preparations (free enzyme, high MW-, medium MW- and low MW-based copolymer-BChE complexes) had EC₅₀ values between 0.04-0.05 µg protein/reaction. Thus even though the free enzyme contained significantly higher enzyme activity than the C-BCs (particularly with the high MW-based C-BC), it did not have higher potency as an *in vitro* bioscavenger.

4. DISCUSSION

A library of a copolymers made with different sizes of poly-L-lysine (PLL) and different grafting ratios of poly(ethylene glycol) (PEG, i.e., PLL-g-PEG) was used for electrostatic self-assembly of polyionic complexes containing recombinant human BChE (C-BCs). Complex formation was visualized as a relative reduction in protein migration into nondenaturing gels subsequently stained for BChE activity. The resulting C-BCs exhibited varying degrees of BChE activity loss, with greater loss in C-BCs assembled with the high MW-based copolymers. While there was lower enzyme activity in the C-BCs compared to the free enzyme, *in vitro* inhibition of BChE activity by paraoxon was relatively similar among all preparations. Enzyme activity in the medium MW- and low MW-based complexes appeared more stable than the free enzyme following prolonged incubation at 37°C, and all C-BCs appeared more resistant to inactivation by chymotrypsin. Moreover, the free enzyme and C-BCs showed similar concentration-dependent inactivation of paraoxon in an *in vitro* bioscavenger assay. Overall, these *in vitro* findings suggest that copolymer-based complexes containing rhBChE have promise in the development of a recombinant enzyme-based bioscavenger.

1 As noted above, our C-BC synthesis protocol follows the work of Gaydess and
2 coworkers [26]. These investigators synthesized PLL-g-PEG copolymers using the
3 medium MW poly-L-lysine (PLL, 15-30 kDa), while our studies compared complex
4 formation using copolymers made with three different molecular sizes of PLL (4-15 kDa,
5 15-30 kDa and 30-70 kDa). Moreover, these investigators [26] used one PEG:PLL
6 grafting ratio (1.6:1) while we studied three different grafting ratios (2:1, 10:1 and 20:1).
7 Human serum and horse serum BChE were used previously whereas we used
8 recombinant human BChE in our studies. A primary goal of their study was to determine
9 if the copolymer-BChE complexes could gain access to the brain after systemic
10 administration, while our studies focused on *in vitro* comparisons of enzyme activity,
11 sensitivity to inhibition by an OP inhibitor, stability and potential to act as a bioscavenger.
12 Thus while there are obvious comparisons in the approaches, our findings extend the
13 work of Gaydess and colleagues to show activity- and inhibitor-based characteristics of
14 copolymer-modified recombinant BChE that could be of use in design of bioscavengers.

15 Copolymer modification of rhBChE led to a reduction in BChE activity in a PLL size- and
16 copolymer-to-protein ratio-dependent manner (Figures 2,3). Lesser reductions were
17 generally noted with the lowest amount of copolymer added (1:1 copolymer-to-protein
18 ratio) compared to the higher amounts (7:1 and 14:1). Thus, only on the basis of
19 retention of BChE enzyme activity, one might conclude that complexes made with equal
20 proportions of copolymer and enzyme would retain more enzyme activity and thus be
21 more effective bioscavengers. Electrophoretic separation and activity staining (Figure 1)
22 suggested however that the 1:1 copolymer-to-protein ratio led to incomplete complex

1 formation and that the higher activity may be merely because there was more free
2 enzyme in those preparations.

3 Relatively similar sensitivity to *in vitro* inhibition of BChE activity was noted with the free
4 enzyme and various C-BCs (Table 1). There was, however a significant difference in *in*
5 *vitro* potencies of paraoxon based on the grafting ratio, with higher IC₅₀s (lower
6 potencies) generally seen with C-BCs containing copolymer using the 20:1 grafting ratio.
7 Along with the generally lower retention of enzyme activity with the 2:1 grafting ratio
8 (Figure 2), we concluded that further investigations should focus on C-BCs made with
9 the 10:1 grafting ratio, using the three different sizes of PLL (Figure 3).

10 The greater stability of medium MW- (Figure 4B) and low MW- (Figure 4C) based C-
11 BCs to prolonged incubation at 37°C and to *in vitro* incubation with chymotrypsin (Figure
12 5) provided support for selection of these C-BCs for future *in vivo* studies. Any potential
13 benefits of increased stability could be countered however if the enzyme activity was
14 concurrently reduced (Figure 3). Surprisingly, while the free enzyme had significantly
15 higher enzyme activity compared to the three C-BCs (in particular with the high MW-
16 based), all exhibited relatively similar concentration-dependent inactivation of paraoxon,
17 i.e., *in vitro* bioscavenging (Figure 6). An interesting possibility posed by these data is
18 that while enzyme activity is reduced with the formation of the copolymer-enzyme
19 complex, the ability of the BChE molecules in these complexes to bind to and inactivate
20 an inhibitor may not be. Charged BChE substrates may have lesser ability than neutral
21 inhibitors to access some active site regions in copolymer-complexed enzymes. This
22 possibility requires additional investigation but could be important in determining the
23 utility of different C-BCs for further development.

1 The copolymer used herein consists of polymers of the amino acid L-lysine and
2 ethylene glycol. With a high positive charge density, the poly-L-lysine (PLL) backbone is
3 essential for electrostatic self-assembly of the protein into the polyionic complex. The
4 PEG grafting to PLL provides surface residues that can impair the clearance of proteins
5 from the blood. In a related manner, the tetramer form of BChE is thought to have
6 longer residence in the circulation than monomers or dimers because of the presence of
7 sugar residues on its surface [10]. PEG residues are thought to form a surface
8 hydrophilic “corona” on various structures that can effectively modify distribution
9 (reviewed in [31]). Chilukuri and coworkers [18] reported an approximate doubling of
10 circulation time for recombinant human BChE in mice when it was PEGylated and that
11 PEGylated BChE was resistant to inactivation by proteases. Song and coworkers [32]
12 reported resistance of the DNA to nuclease-mediated degradation in a PEGylated DNA-
13 gold nanoparticle complex. Tsiourvas and colleagues [33] reported resistance to
14 proteases (trypsin, chymotrypsin) with PEGylation of an oligopeptide-insulin
15 nanocomplex. Thus the relatively greater stability of BChE in the C-BCs compared to
16 the free enzyme may be mediated by the grafted PEG chains oriented at the surface of
17 the copolymer-BChE complex.

18 While numerous studies have reported increased circulation and changes in
19 biodistribution of proteins, drugs and nanomaterials following PEGylation, another
20 important use for surface PEG chains is the attachment of targeting ligands. Various
21 antibodies, peptides, drugs and toxins have been conjugated to PEG-modified proteins
22 to aid in selective targeting [34-36]. Copolymer-BChE complexes such as those

1 described herein may also benefit by molecular targeting strategies enabled *via* surface
2 PEG residues.

3 In summary, electrostatic self-assembly of recombinant BChE with a series of PLL-g-
4 PEG copolymers (and post-exposure to glutaraldehyde) led to stable polyionic
5 complexes that retained at least 50% of the enzyme activity and relatively similar
6 sensitivity to inhibition by paraoxon, compared to the free enzyme. Complexes made
7 with the medium MW- and low MW-based copolymers showed higher stability with
8 prolonged incubation at 37°C, were less sensitive to inactivation by the protease
9 chymotrypsin, and were similarly potent in an *in vitro* bioscavenger assay as the free
10 enzyme. Based on these characteristics and apparent relative degree of complex
11 formation, copolymer-BChE complexes made with the medium MW-based copolymer,
12 10:1 grafting ratio, and the higher copolymer-to-protein ratios appear best suited for
13 further development and *in vivo* performance studies.

14

5. Figure Legends

Figure 1. Nondenaturing polyacrylamide gel electrophoresis and BChE activity staining.

Vehicle (PBS; left lane in Figures 1A-I) or copolymer in PBS was added to a fixed amount of rhBChE (6.65 μ g in PBS) using increasing proportions of copolymer-to-protein (1:1, 2:1, 3:1, 7:1, 10:1, and 14:1; left to right in Figure 1A-I). Vehicle (for the free enzyme) or glutaraldehyde (0.003% final, for the copolymer-BChE complexes) was then added for stability. Samples were separated on nondenaturing gradient gels and stained as described in Section 2.6. Migration of BChE activity in preparations using the high MW-based copolymer with 2:1 (Figure 1A), 10:1 (Figure 1B) and 20:1 (Figure 1C) grafting ratios, medium MW-based copolymer with 2:1 (Figure 1D), 10:1 (Figure 1E) and 20:1 (Figure 1F) grafting ratios, and low MW-based copolymer with 2:1 (Figure 1G), 10:1 (Figure 1H) and 20:1 (Figure 1I) grafting ratios are each shown as a function of copolymer-to-protein ratio.

Figure 2. BChE enzyme activity following complex formation. Conditions for subfigures (2A-I) are identical to Figure 1. Enzyme activity was assayed by the radiometric method as described in Section 2.5. The data represent percent activity relative to the mean enzyme activity in nine different free enzyme preparations (47 ± 3 U/ml).

Figure 3. BChE activity in selected copolymer-BChE complexes. BChE-containing polyionic complexes were produced using A) high MW- (n=3), B) medium MW- (n=4) and C) low MW- (n=3) based copolymer, each using the 10:1 grafting ratio and three selected copolymer-to-protein ratios (1:1, 7:1 or 14:1). BChE activity was assayed by the radiometric method and reported as percent of activity relative to mean activity in all

free enzyme preparations (47.8 ± 1.6 U/ml, $n = 10$). A significant difference from the free enzyme is represented by “a”, while “b” indicates a significant difference between respective C-BCs made with either the 7:1 or 14:1, relative to the 1:1 copolymer-to-protein ratio.

Figure 4. Stability of BChE activity in free enzyme and copolymer-BChE complexes (C-BCs) at 37°C. C-BCs were prepared with A) high MW-, B) medium MW- and C) low MW-based copolymers using the 10:1 grafting ratio, with 1:1 (filled squares), 7:1 (filled triangles) or 14:1 (open squares) copolymer-to-protein ratios. Free enzyme is represented by the open circles. Samples were diluted in PBS containing 0.05% BSA and incubated for 28 days. Aliquots were withdrawn and BChE activity measured by the radiometric method at selected times. Data from 3-4 independent preparations of each are reported as percent of activity at time 0 (mean $\pm$ SEM). A significant difference in activity between the free enzyme and the C-BCs using the 1:1 copolymer-to-protein ratio is represented by “a”, between free enzyme and C-BCs using the 7:1 ratio by “b”, and between free enzyme and C-BCs using the 14:1 ratio by “c”.

Figure 5. Sensitivity of BChE activity in free enzyme and copolymer-BChE complexes to inactivation by chymotrypsin. Preparations of free enzyme and C-BCs were added to chymotrypsin and incubated at 37°C as described in Section 2.8. Aliquots were removed at selected times up to 8 hours and BChE activity measured. Data are reported as percent of activity at time 0 (mean $\pm$ SEM; $n = 3-4$ independent replicates for each preparation).

Figure 6. *In vitro* inactivation of paraoxon by free enzyme and copolymer-BChE complexes (C-BCs). Free enzyme (A) and C-BCs using either the B) high MW-, C) medium MW- or D) low MW-based copolymer, 10:1 grafting ratio, 7:1 copolymer-to-protein ratio, were prepared as described in Section 2.4. Vehicle (PBS) and dilutions of the free enzyme and C-BCs were first pre-incubated with either vehicle or paraoxon (PO) for 20 min at 37°C. A marker enzyme (pig liver carboxylesterase, CarbE) was then added followed by incubation for an additional 20 min at 37°C. Substrate (p-nitrophenyl acetate) and the butyrylcholinesterase inhibitor ethopropazine were then added and residual CarbE activity was measured. Activity in reactions pre-incubated without paraoxon and BChE were used as vehicle controls (VEH). Data were plotted as percent of control (mean $\pm$ SEM) from 3-4 independent preparations. An asterisk indicates a significant difference compared to reactions pre-incubated with paraoxon only (PO).

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References

- [1] Liu J, Uchea C, Wright L, Pope C. Chemical warfare agents and the nervous system. In: Gupta RC, editor. *Handbook of Toxicology and Chemical Warfare Agents*, 2nd edition, London: Academic Press; 2015, p. 463-475.
- [2] Shafferman A, Barak D, Kaplan D, Ordentlich A, Kronman C, Velan B. Functional requirements for the optimal catalytic configuration of the AChE active center. *Chem Biol Interact.* 2005; 157-158:123-131.
- [3] Pope, C, Karanth S, Liu J. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. *Environ. Toxicol. Pharmacol.* 2005; 19:433-446.
- [4] Ashani Y, Shapira S, Levy D, Wolfe AD, Doctor BP, Raveh L. Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem Pharmacol.* 1991; 41:37-41.
- [5] Doctor BP, Raveh L, Wolfe AD, Maxwell DM, Ashani Y. Enzymes as pretreatment drugs for organophosphate toxicity. *Neurosci Biobehav Rev.* 1991; 15:123-128.
- [6] Doctor BP, Saxena A. Bioscavengers for the protection of humans against organophosphate toxicity. *Chem Biol Interact.* 2005; 157-158:167-171.
- [7] Saxena A, Sun W, Dabisch PA, Hulet SW, Hastings NB, Jakubowski EM, Mioduszewski RJ, Doctor BP. Pretreatment with human serum butyrylcholinesterase alone prevents cardiac abnormalities, seizures, and death in Göttingen minipigs exposed to sarin vapor. *Biochem Pharmacol.* 2011; 82:1984-1993.
- [8] Nachon F, Brazzolotto X, Trovaslet M, Masson P. Progress in the development of enzyme-based nerve agent bioscavengers. *Chem Biol Interact.* 2013; 206:536-544.
- [9] Worek F, Seeger T, Goldsmith M, Ashani Y, Leader H, Sussman JS, Tawfik D, Thiermann H, Wille T. Efficacy of the rePON1 mutant IIG1 to prevent cyclosarin toxicity in vivo and to detoxify structurally different nerve agents in vivo. *Arch Toxicol* 2014; 88:1257-1266.
- [10] Lockridge O. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic, and potential therapeutic uses. *Pharmacol Ther.* 2015; 148:34-46.
- [11] Saxena A, Sun W, Luo C, Doctor BP. Human serum butyrylcholinesterase: in vitro and in vivo stability, pharmacokinetics, and safety in mice. *Chem Biol Interact.* 2005; 157-158:199-203.

- [12] Chilukuri N, Duysen EG, Parikh K, diTargiani R, Doctor BP, Lockridge O, Saxena A. Adenovirus-transduced human butyrylcholinesterase in mouse blood functions as a bioscavenger of chemical warfare nerve agents. *Mol Pharmacol*. 2009; 76:612-617.
- [13] Raveh L, Grunwald J, Marcus D, Papier Y, Cohen E, Ashani Y. Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity. In vitro and in vivo quantitative characterization. *Biochem Pharmacol*. 1993; 45:2465-2474.
- [14] Saxena A, Sun W, Luo C, Myers TM, Koplovitz I, Lenz DE, Doctor BP. Bioscavenger for protection from toxicity of organophosphorus compounds. *J Mol Neurosci*. 2006; 30:145-148.
- [15] Myers TM, Sun W, Naik RS, Clark MG, Doctor BP, Saxena A. Characterization of human serum butyrylcholinesterase in rhesus monkeys: behavioral and physiological effects. *Neurotoxicol Teratol*. 2012; 34:323-330.
- [16] Ostergard D, Viby-Mogensen J, Hanel HK, Skovgaard LT. Half-life of plasma cholinesterase. *Acta Anaesthesiol Scand* 1988; 32:266-269.
- [17] Duysen EG, Bartels CF, Lockridge O. Wild-type and A328W mutant human butyrylcholinesterase tetramers expressed in Chinese hamster ovary cells have a 16-hour half-life in the circulation and protect mice from cocaine toxicity. *J Pharmacol Exp Ther*. 2002; 302:751-758.
- [18] Chilukuri N, Parikh K, Sun W, Naik R, Tipparaju P, Doctor BP, Saxena A. Polyethylene glycosylation prolongs the circulatory stability of recombinant human butyrylcholinesterase. *Chem Biol Interact*. 2005; 157-158:115-121.
- [19] Buske J, König C, Bassarab S, Lamprecht A, Mühlau S, Wagner KG. Influence of PEG in PEG-PLGA microspheres on particle properties and protein release. *Eur J Pharm Biopharm*. 2012; 81:57-63.
- [20] Santander-Ortega MJ, Csaba N, Gonzalez L, Bastos-Gonzalez D, Ortega-Vinuesa JL, Alonso MJ. Protein-loaded PLGA-PEO blend nanoparticles: encapsulation, release and degradation characteristics. *Colloid Polymer. Sci*. 2010; 288:141-150.
- [21] Kochendoerfer G. Chemical and biological properties of polymer-modified proteins. *Expert Opin Biol Ther*. 2003; 3:1253-1261.
- [22] Milla P, Dosio F, Cattel L. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. *Curr Drug Metab*. 2012; 13:105-119.
- [23] Aliabadi HM, Brocks DR, Lavasanifar A. Polymeric micelles for the solubilization and delivery of cyclosporine A: pharmacokinetics and biodistribution. *Biomaterials* 2005; 26:7251-7259.

- [24] Kolate A, Baradia D, Patil S, Vhora I, Kore G, Misra A. PEG - a versatile conjugating ligand for drugs and drug delivery systems. *J Control Release*. 2014; 192:67-81.
- [25] Novikov BN, Grimsley JK, Kern RJ, Wild JR, Wales ME. Improved pharmacokinetics and immunogenicity profile of organophosphorus hydrolase by chemical modification with polyethylene glycol. *J Control Release*. 2010; 146:318-325.
- [26] Gaydess A, Duysen E, Li Y, Gilman V, Kabanov A, Lockridge O, Bronich T. Visualization of exogenous delivery of nanoformulated butyrylcholinesterase to the central nervous system. *Chem Biol Interact*. 2010; 187:295-298.
- [27] Johnson CD, Russell RL. A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal Biochem*. 1975; 64:229-238.
- [28] Ellman GL, Courtney KD, Andres Jr. V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 1961; 7:91-95.
- [29] Karnovsky MJ, Roots L. A "direct-coloring" thiocholine method for cholinesterases. *J Histochem. Cytochem*. 1964; 12:219-221.
- [30] Wei Y, Peng AY, Huang J. Inhibition of porcine liver carboxylesterase by phosphorylated flavonoids. *Chem Biol Interact*. 2013; 204:75-79.
- [31] Rabanel JM, Hildgen P, Banquy X. Assessment of PEG on polymeric particles surface, a key step in drug carrier translation. *J Control Release*. 2014; 185:71-87.
- [32] Song L, Guo Y, Roebuck D, Chen C, Yang M, Yang Z, Sreedharan S, Glover C, Thomas JA, Liu D, Guo S, Chen R, Zhou D. Terminal PEGylated DNA-gold nanoparticle conjugates offering high resistance to nuclease degradation and efficient intracellular delivery of DNA binding agents. *ACS Appl Mater Interfaces*. 2015; 7:18707-18716.
- [33] Tsiourvas D, Sideratou Z, Sterioti N, Papadopoulos A, Nounesis G, Paleos CM. Insulin complexes with PEGylated basic oligopeptides. *J Colloid Interface Sci*. 2012; 384:61-72.
- [34] Wang M, Thanou M. Targeting nanoparticles to cancer. *Pharmacol Res*. 2010; 62:90-99.
- [35] Yao XL, Nakagawa S, Gao JQ. Current targeting strategies for adenovirus vectors in cancer gene therapy. *Curr Cancer Drug Targets*. 2011; 11:810-825.
- [36] Duskey JT, Rice KG. Nanoparticle ligand presentation for targeting solid tumors. *AAPS PharmSciTech*. 2014; 15:1345-1354.

Table 1. In vitro inhibition of BChE activity by paraoxon in copolymer-BChE complexes.

		PEG:PLL Grafting Ratio								
		2:1			10:1			20:1		
		Copolymer-to-Protein			Copolymer-to-Protein			Copolymer-to-Protein		
		1:1	7:1	14:1	1:1	7:1	14:1	1:1	7:1	14:1
PLL Molecular Weight	High	8.7 (7.2-10.4)	4.4 (3.7-5.2)	5.0 (4.4-5.6)	6.7 (5.7-7.8)	5.9 (5.2-6.7)	5.8 (4.8-6.9)	9.5 (8.1-11.2)	7.1 (5.9-8.5)	7.8 (6.5-9.4)
	Medium	7.9 (6.4-9.9)	8.4 (6.2-11.3)	7.4 (5.6-9.7)	5.7 (3.4-9.5)	4.0 (2.7-6.0)	4.2 (3.5-5.1)	8.8 (6.1-12.7)	8.7 (6.9-11.0)	10.1 (7.1-14.5)
	Low	6.0 (4.6-7.7)	6.7 (5.6-7.9)	6.7 (5.5-8.0)	6.6 (5.7-7.7)	4.3 (2.9-6.3)	4.2 (3.1-5.9)	7.0 (4.9-9.8)	7.5 (5.8-9.9)	7.6 (5.5-9.9)

Copolymer-BChE complexes were made using one of nine different copolymers made with different sizes of poly-L-lysine and different grafting ratios of PEG. Different relative amounts of each copolymer (1:1, 7:1 or 14:1) were added to a fixed amount of rhBChE, followed by addition of glutaraldehyde for stability. The free enzyme and copolymer-BChE complexes were pre-incubated with paraoxon (0.1-300 nM, 20 minutes, 37°C) prior to adding substrate and measuring residual activity. Data are mean IC₅₀ values with 95% confidence intervals shown in parentheses. The mean IC₅₀ value and standard error from all nine preparations of the free enzyme was 5.8 ± 0.2 nM.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6