Published in final edited form as:
Chem Biol Interact. 2010 September 6; 187(1-3): 295–298. doi:10.1016/j.cbi.2010.01.005.

Visualization of exogenous delivery of nanoformulated butyrylcholinesterase to the central nervous system

Andrea Gaydess[1], **Ellen Duysen**[2], **Yuan Li**[1,#], **Vladimir Gilman**[3], **Alexander Kabanov**[1], **Oksana Lockridge**[2], and **Tatiana Bronich**[1]

[1] College of Pharmacy and Center for Drug Delivery and Nanomedicine, University of Nebraska Medical Center, Omaha, NE 68198-5830

[2] Eppley Institute, University of Nebraska Medical Center 985950, Nebraska Medical Center, NE 68198-5950

[3] InfoSciTex Corporation, Waltham, MA 02451

Abstract

Butyrylcholinesterase (BChE) is an efficient bioscavenger of highly toxic organophosphorus poisons and nerve agents. However, BChE administered into the periphery does not provide significant protection of the central nervous system (CNS) due to rejection by the blood brain barrier. In this study, we evaluated the feasibility of delivering BChE to the CNS by packing it into a block ionomer complex of nanoscale size with a cationic poly(L-lysine)-*graft*-poly(ethylene oxide) (PLL-*g*-PEO) copolymer. The multimolecular structure of BChE/PLL-*g*-PEO complexes was further reinforced by formation of cross-links between the polymer chains. The resulting cross-linked complexes were stable against dilution without significant loss of BChE enzymatic activity. In some cases the BChE was labeled with fluorescent IRDye 800CW before it was incorporated into nanoparticles. BChE/PLL-*g*-PEO complexes were injected into mice intramuscularly and intravenously. In vivo imaging showed incorporation of the fluorescently labeled BChE in brain. Activity assays showed that BChE remained active in the brain at 72 hour post injection. It was concluded that nanocomplexes can deliver the 340 kDa BChE tetramer to the brain.

Keywords

Butyrylcholinesterase; nanotechnology; CNS drug delivery; block ionomer complexes

1. Introduction

Butyrylcholinesterase (BChE, EC 3.1.1.8) is of growing interest in recent years [1–6]. The ability to act as a bioscavenger for various nerve agents and organophosphates (OP) before they reach their synaptic targets makes BChE a prospective detoxifying antidote. However, since BChE does not cross the blood-brain barrier (BBB) when delivered *via* intramuscular (IM), intravenous (IV), subcutaneous, or intraperitoneal routes [7], effective protection of the CNS from OP toxicity by this enzyme is limited. Recently, *in vivo* imaging of mice demonstrated that BChE can be effectively delivered to brain and spinal cord only by intrathecal injection [8].

[#]Present address: Department of Chemistry and Biochemistry, University of Maryland, Baltimore, MD 21250

In the present studies, polyion complexation was used as a platform for the development of a polymer carrier for the enhanced delivery of BChE to the brain. The nanofabrication of such carriers is achieved by reacting a protein with oppositely charged block ionomer containing ionic and nonionic blocks, and spontaneous formation of stable aqueous dispersion of block ionomer complexes of nanoscale size [9]. The resulting nanoparticles contain a core of protein-polyion complex surrounded by a shell of water soluble nonionic polymer such as poly(ethylene oxide) (PEO, same as poly(ethylene glycol) or PEG). A similar strategy successfully delivered oligonucleotides across the BBB [10]. This is the first report to use a nanocarrier to deliver BChE across the BBB.

2. Experimental

2.1. Materials

Lyophilized horse BChE (C-1057) (hrBChE) and all reagents used in BChE activity assays were purchased from Sigma (St. Louis, MO). Native human BChE (huBChE) was provided by InfoSciTex Corp. IRDye 800CW protein labeling kit (928-3840) was purchased from LI-COR (Lincoln, NE). PLL-*g*-PEO was synthesized from poly(L-lysine) hydrobromide (PLL, molecular weight 15–30 kDa, Sigma) and mPEG-succinimidyl propionate (molecular weight 5 kDa, Nektar Corp.). From ^{1}H NMR spectra of the synthesized PLL-*g*-PEO, the number of PEO chains grafted onto a PLL backbone was calculated to be 1.6. The huBChE was labeled using IRDye 800CW Protein labeling kit as described previously [7]. The labeled protein (huBChE*) bound 2.3 dye molecules per HuBChE* tetramer and lost 80% of its initial activity.

2.2 Preparation of BChE/PLL-g-PEO block ionomer complexes

The complexes were prepared by a simple mixing of buffered solutions (phosphate buffer, 10 mM, pH 7.4) of the protein and copolymer at various compositions of mixtures. The composition of the mixtures ($Z_{+/-}$) was calculated as a ratio of concentration of amino groups in PLL-*g*-PEO copolymer to the total concentration of carboxylic groups in BChE (Glu, Asp, and sialic acid residues). As example, the BChE/PLL-g-PEO mixture at $Z_{+/-} = 2$ was prepared by mixing 50 uL BChE (0.45 mg/ml) and 100 uL of PLL-*g*-PEO (0.35 mg/ml). Cross-linking of BChE/PLL-g-PEO complexes was carried out in phosphate buffer using glutaraldehyde (GA). Targeted cross-linking ratios were calculated as the molar ratio of aldehyde groups to Lys residues in solution and expressed in %. The mixtures were kept for 5 hours at room temperature to insure completion of the cross-linking reaction. The formation of BChE/PLL-*g*-PEO complexes was evaluated by nondenaturating polyacrylamide gel electrophoresis followed by Coomassie blue staining or by staining for BChE activity [11]. Size and size distribution of BChE/PLL-*g*-PEO complexes was determined by dynamic light scattering using Nano ZS Zetasizer (Malvern Instruments, UK) at a fixed 173° scattering angle.

2.3 In vivo imaging of live mice and fluorescence in frozen brain sections

Animal work was conducted in accordance with national animal welfare guidelines and approved by the Institutional Animal Care and Use Committee at UNMC. Wild type and butyrylcholinesterase nullizygous (BChE −/−) mice used in this study were bred at UNMC [12]. Mice were fed a special chlorophyll-free diet (Harlan Teklad, TD94048) for 7 days before the start of imaging and for the duration of the imaging experiments because the diet reduced background fluorescence. Imaging of the animals injected with the complexes containing huBChE* was performed using a Pearl ™ Imager (LI-COR Biosciences) according to our previously reported protocol [8]. Images were analyzed using Pearl Cam Software provided by LI-COR. Mice euthanized by CO_2 asphyxiation, were thoroughly perfused intracardially with 75 ml of phosphate buffered saline before major organs were

collected. Frozen brain sections were prepared as described in [8] and examined using a Zeiss 510 Meta confocal laser scanning microscope at 680 nm. Regions from each section were photographed under constant acquisition conditions.

2.4 BChE activity in brain homogenate

Brain tissues were homogenized in 10 volumes of buffer (50mM potassium phosphate, pH 7.4, 0.5% Tween 20) and centrifuged. The supernatant was assessed by electrophoresis on non-denaturing 4–30% polyacrylamide gels stained for BChE activity with butyrylthiocholine (BTC) and by activity assay using BTC as the substrate. [11].

3. Results and Discussion

3.1 BChE/PLL-g-PEO block ionomer complexes

BChE has an isoelectric point of 4.2–4.9 (the exact value depends on the source of enzyme) and thus, at physiological conditions, has a net negative charge. Simple mixing of BChE with cationic copolymer PLL-*g*-PEO (pKa of amino groups in the PLL segment is 10.8) resulted in spontaneous formation of block ionomer complexes. The formation of huBChE/PLL-*g*-PEO complexes was confirmed by gel electrophoresis (Fig. 1A). The band intensity of free huBChE significantly decreased as the amount of the copolymer in the mixture was increased. Retardation of protein migration was observed at the composition of huBChE/PLL-*g*-PEO mixtures close to $Z_{+/-} = 2$. This suggested that huBChE/PLL-*g*-PEO complexes formed and were unable to enter the gel. Dynamic light scattering measurements for the huBChE/PLL-*g*-PEO mixtures ($Z_{+/-} = 2$) revealed the formation of particles with diameters of ca. 15 nm that had spherical morphology as was confirmed by atomic force microscopy imaging (data not shown). The multimolecular structure of huBChE/PLL-*g*-PEO complexes was further stabilized by formation of cross-links between the copolymer chains using GA as cross-linker. As seen in Fig. 1B, at 40% of targeted cross-linking ratio huBChE/PLL-*g*-PEO complexes were stable against dilution while retaining up to 80% of initial enzymatic activity of free huBChE. Similar procedures were employed to prepare hrBChE/PLL-*g*-PEO complexes and the complete incorporation of hrBChE into the complex was achieved at higher excess of the cationic copolymer, at $Z_{+/-} = 7$ (data not shown). There is 89% homology between human and horse BChE that may account for difference in charge density and thus in protein binding to cationic copolymer.

3.2 In vivo imaging

The *in vivo* migration and localization of fluorescently labeled huBChE* delivered by means of polymer complexes was evaluated in BChE−/− mice using optical imaging (Fig. 2). Fluorescence signal corresponding to huBChE* was detected in the brains of the animals 2h post IM (Fig. 2B) or IV (Fig. 2D) injections of cross-linked huBChE*/PLL-*g*-PEO complexes and remained elevated up to 72 h post-dosing. An intense fluorescent signal was observed in brains excised from the animals treated with huBChE*/PLL-*g*-PEO complexes (Fig. 2F and G). This is in contrast to the remarkably lower signal that was observed in the brain of the animal at 24 h after injection of an equivalent dose of free huBChE* (Fig. 2E). These results were confirmed by confocal scanning microscopy of brain sections prepared from treated and untreated mice. As seen in Fig. 3C considerably higher fluorescence signal corresponding to huBChE* was clearly detected in the brain striatum of the animals treated with cross-linked huBChE*/PLL-*g*-PEO complexes. Very low signal comparable to the background was observed in the sections from free huBChE* treated mice (Fig. 3A, B).

3.3 BChE activity in the brain

BChE−/− mice were treated in one experiment with non cross-linked hrBChE/PLL-g-PEO complexes or free BChE via IM injection. In a second experiment wild type mice were treated with similar complexes via IV injection. Mice were sacrificed 23 h post treatment; thoroughly perfused brains were collected and homogenized. Brain supernatants were electrophoresed on native gels and stained for BChE activity. An increased BChE activity in the brains of BChE−/− mice 23 h post IM injection with hrBChE/PLL-*g*-PEO complex (lanes 3, 4, and 5) was detected compared to activity in the brains of mice treated IM with an equivalent dose of free hrBChE (lanes 1 and 2) (Fig. 4A). BChE levels in the brain homogenates of mice treated IM with free hrBChE were an average of 0.04±0.01 units/mg while mice treated with the complexes had average 0.1±0.07 units/mg. Similarly, higher level of BChE activity was found in the brains of mice treated IV with hrBChE/PLL-*g*-PEO complexes (lanes 1, 2 and 3) compared to endogenous BChE levels in the brain of a wild type mouse (lane 4) (Fig. 4B). Activity assays show that the endogenous levels of BChE in the wild type brain was 0.16 units/mg. Mice treated via IV injection with hrBChE/PLL-g-PEO complexes showed average levels of 0.27±0.1 units/mg in brain homogenates.

Overall, these studies provide initial evidence that BChE administered in block ionomer complexes via IM or IV injections exhibited increased brain accumulation compared to BChE alone. The prolonged activity and retention of enzyme delivered to the CNS might facilitate an extended protection from OP exposure. However, it is unclear whether BChE was able to cross the BBB. Although saline perfusion of the mice was performed to ensure that none of the BChE brain activity could be attributed to enzyme present in the vasculature, we cannot exclude the possibility that BChE/PLL-*g*-PEO complex was trapped by the capillaries and was not able to enter the brain parenchymal space. BChE/PLL-g-PEO complex within the capillaries may provide protection to the brain against OP exposure yet our intent is to deliver the enzyme to the parenchyma, Therefore, a capillary depletion method [13] will be implemented in our future studies to assess this possibility and determine the portion of the injected dose that actually crosses the BBB and enters the brain following peripheral administration.

Acknowledgments

The support of the US Army (contract no. W81XWH-05-C-0053) and helpful discussions with Dr. Marti Jett (Walter Reed Army Institute of Research, Silver Spring, USA) are greatly acknowledged. We thank Janice A. Taylor and James R. Talaska for assistance with confocal microscopy studies (UNMC Core Facility is supported by NIH Cancer Center Grant P30CA036727). We thank LI-COR for use of the Pearl Imager.

References

1. Geula C, Nagykery N. Butyrylcholinesterase activity in the rat forebrain and upper brainstem: postnatal development and adult distribution. Exp Neurol 2007;204(2):640–657. [PubMed: 17274983]
2. Mehrani H. Protective effect of polyurethane immobilized human butyrylcholinesterase against parathion inhalation in rat. Environmental Toxicology and Pharmacology 2004;16(3):179–185.
3. Schumacher I, Arad A, Margalit R. Butyrylcholinesterase formulated in liposomes. Biotechnol Appl Biochem 1999;30(Pt 3):225–230. [PubMed: 10574691]
4. Lockridge O, Bartels CF, Vaughan TA, Wong CK, Norton SE, Johnson LL. Complete amino acid sequence of human serum cholinesterase. J Biol Chem 1987;262(2):549–557. [PubMed: 3542989]
5. Nicolet Y, Lockridge O, Masson P, Fontecilla-Camps JC, Nachon F. Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. J Biol Chem 2003;278(42): 41141–41147. [PubMed: 12869558]

6. Altamirano CV, Lockridge O. Association of tetramers of human butyrylcholinesterase is mediated by conserved aromatic residues of the carboxy terminus. Chem Biol Interact 1999:119–120. 53–60. [PubMed: 10421445]

7. Duysen EG, Lockridge O. Whole body and tissue imaging of the butyrylcholinesterase knockout mouse injected with near infrared dye labeled butyrylcholinesterase. Chem Biol Interact 2008;175(1–3):119–124. [PubMed: 18486120]

8. Johnson ND, Duysen EG, Lockridge O. Intrathecal delivery of fluorescent labeled butyrylcholinesterase to the brains of butyrylcholinesterase knock-out mice: visualization and quantification of enzyme distribution in the brain. Neurotoxicology 2009;30(3):386–392. [PubMed: 19442823]

9. Vinogradov SV, Batrakova EV, Kabanov AV. Nanogels for oligonucleotide delivery to the brain. Bioconjug Chem 2004;15(1):50–60. [PubMed: 14733583]

10. Batrakova, EV.; Bronich, TK.; Vetro, JA.; Kabanov, AV. Polymer micelles as drug carriers. In: Torchilin, VP., editor. Nanoparticulates as Drug Carriers. Imperial College Press; London: 2006. p. 57-93.

11. Karnovsky MJ, Roots L. A “Direct-Coloring” Thiocholine Method for Cholinesterases. J Histochem Cytochem 12(1964):219–221. [PubMed: 14187330]

12. Li B, Duysen EG, Saunders TL, Lockridge O. Production of the butyrylcholinesterase knockout mouse. J Mol Neurosci 2006;30(1–2):193–195. [PubMed: 17192674]

13. Gutierrez EG, Banks WA, Kastin AJ. Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. J Neuroimmunol 1993;47(2):169–176. [PubMed: 8370768]

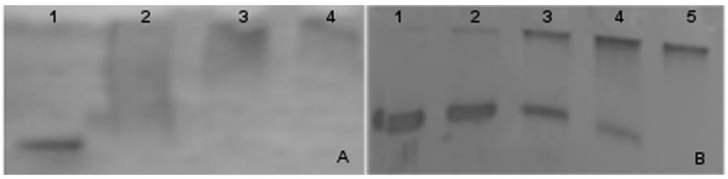

Figure 1.

Gel analysis of (**A**) free huBChE and huBChE/PLL-*g*-PEO complexes: (1) free huBChE; (2) $Z_{+/-}$= 0.94; (3) $Z_{+/-}$= 1.85: (4) $Z_{+/-}$= 2.79. (30μg HuBChE/well) The gel was stained with Coomassie blue. (**B**) free huBChE, and huBChE/PLL-*g*-PEO complexes, $Z_{+/-}$= 2.0, (0.06 μg/well of huBChE): (1) free huBChE; (2) non cross-linked; and cross-linked at (3) 10%; (4) 20%: (5) 40% degree of cross-linking. The gel was stained for BChE activity.

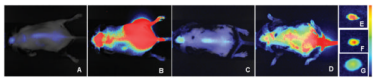

Figure 2.
Images of BChE−/− mice injected IM or IV with cross-linked huBChE*/PLL-*g*-PEO complexes, $Z_{+/-}$ = 2, 40% degree of cross-linking. The fluorescence was from BChE labeled with IRDye 800CW. **A**) Pre-dosing and **B**) 2 hours post IM injection; **C**) Pre-dosing and **D**) 2 hours post IV injection into the tail vein. Images of the brain tissues at 24 h after **E**) IM treatment with huBChE*/PLL-*g*-PEO complexes; **F**) IV treatment with huBChE*/PLL-*g*-PEO complexes; **G**) IM treatment with free huBChE*.

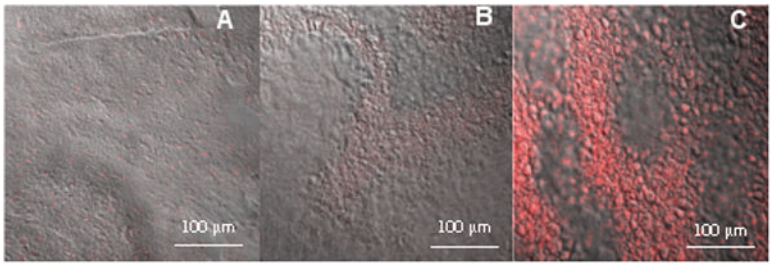

Figure 3.

Confocal Images of brain striatum from BChE −/− mice: (A) untreated; (B) treated with free huBChE* via IM injection; (C) treated with cross-linked huBChE*/PLL-*g*-PEO complex ($Z_{+/-}$=2) via IM injection. Bar is equal to 100 μm.

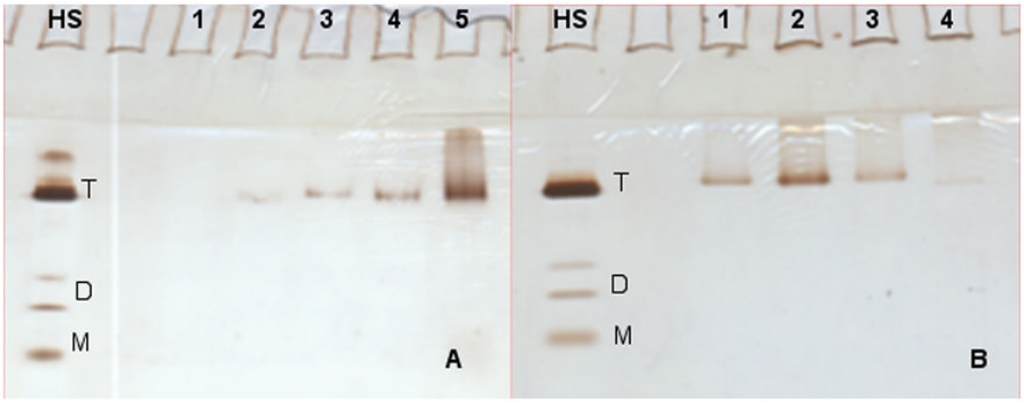

Figure 4.

BChE activity in mouse brain supernatants: (**A**) at 23h post IM injection of free hrBChE into BChE−/− mice (1, 2); hrBChE/PLL-*g*-PEO complex, $Z_{+/-}$=7 (3–5); (**B**) at 23h post IV injection of hrBChE/PLL-*g*-PEO complex, $Z_{+/-}$=7 into wild-type mice (1–3); and control wild type mouse brain (4). Human plasma (lane marked HS) was used as a control with T representing the tetramer, D the dimer, and M the monomer forms of BChE.