

Catalytic Detoxification of Organophosphorus Nerve Agents by Butyrylcholinesterase-Polymer-Oxime Bioscavengers

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Catalytic Detoxification of Organophosphorus Nerve Agents by Butyrylcholinesterase-Polymer-Oxime Bioscavengers

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18 KEYWORDS: Fluorogenic Organophosphorus nerve agents (Flu-OPNA) analogues,
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21 Butyrylcholinesterase (BChE), Atom Transfer Radical Polymerization (ATRP), Oxime,
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25 Protein-Polymer Conjugate
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34 ABSTRACT

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39 Organophosphorus nerve agents (OPNA), used in chemical warfare, irreversibly inhibit
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42 essential cholinesterases (ChEs) in the cholinergic neurotransmission system. Several
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45 potent nucleophilic oximes have been approved for the treatment of acute poisoning by
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49 OPNAs, but they are rapidly cleared from blood circulation. Butyrylcholinesterase (BChE)
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53 stoichiometrically binds nerve agents, but since the molecular weight of a nerve agent is
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4 about 500-fold less than the enzyme, the bioscavenger has had limited utility. We have
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6
7 synthesized BChE-polymer-oxime conjugates using atom transfer radical polymerization
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10 (ATRP) and azide-alkyne “click” chemistry. The activity of the BChE-polymer-oxime
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13 conjugates was dependent on the degree of oxime loading within the copolymer side
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16 chains. The covalent modification of oxime-containing copolymers prolonged the activity
17
18
19 of BChE in the presence of the VX- and cyclosarin-fluorogenic analogues EMP-MeCyC
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22 and CMP-MeCyC, respectively. After complete inactivation by VX and cyclosarin
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25 fluorogenic analogues, the conjugates demonstrated efficient self-reactivation of up to 80%
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28 within 3-6 hours. Repeated inhibition and high-level self-reactivation assays revealed that
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31 the BChE-polymer-oxime conjugates were excellent reactivators of OPNA-inhibited
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34 BChE. Recurring self-reactivation of BChE-polymer-oxime conjugates following repeated
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37 BChE inhibition by Flu-OPs opens the door to developing the next generation of nerve
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40 agent “catalytic” bioscavengers.
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50 INTRODUCTION

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3 Initially developed in the 1930s, OPNAs have been used as pesticides, tested on criminal
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7 subjects, used in terrorist acts and employed as chemical warfare agents.¹ Tabun and
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10 sarin, for example, were used in the Iran-Iraq war in the 1980s, sarin was used against
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13 civilians in Syria and by a terrorist sect in the Tokyo subway, and VX was used to
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16 assassinate the North Korean, Kim Jong-nam, in Malaysia (2017).²⁻⁵ Accidental and self-
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18
19 poisoning by organophosphorus pesticides results in around 100,000 deaths and affects
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21
22 more than 1 million people each year worldwide.⁶ OPNAs inhibit cholinesterases (ChEs)
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24
25 in the cholinergic neurotransmission system⁷ by phosphorylation of a catalytic serine
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27
28 residue in the active site. OPNA toxicity results from the accumulation of
29
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31 neurotransmitters in cholinergic synapses leading to paralysis, hypotension, breathing
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33
34 failure and death.⁸⁻¹¹ Hydrophobic OPNAs cross the blood-brain barrier (BBB) and
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37 inactivate acetylcholinesterase (AChE) in the central nervous system (CNS).
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41 Accumulated OPNAs, retained in the CNS, can also partition back into blood causing
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48 "cholinergic crisis" and irreversible brain damage.^{12, 13}
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4 Currently approved treatment of acute OPNA poisoning includes administration of a
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7 mixture of a pyridinium aldoxime, an anticonvulsant (benzodiazepine) and a muscarinic
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10 acetylcholine receptor antagonist (atropine).^{14, 15} Since the seminal work of Wilson and
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13 colleagues in the mid-1950s, thousands of aldoximes have been synthesized and
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16 screened.^{16, 17} Few molecules, for example pralidoxime (2-PAM), asoxime (HI-6), and
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19 obidoxime (toxogonin), have been developed as OPNA poisoning therapeutics.^{14, 15}
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24 Aldoximes, in the charged oximate nucleophilic state, attack the phosphoryl bond of
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26
27 covalently-inhibited ChEs at the active site serine, releasing the OP moiety from the active
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30 site and restoring ChE activity.¹⁸⁻²⁰ Over several decades, numerous imidazolium oximes
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34 have been developed as OP-ChE reactivators.^{12, 21-24} Some of these derivatives were
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38 shown to be efficient reactivators in specific *in vitro* and *in vivo* reactivation assays using
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42 VX- and tabun-inhibited ChEs.²³⁻²⁷ Antidotal therapy by oximes, unfortunately, requires
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45 repeated administration because low molecular weight oximes have very short half-lives
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49 in circulation.^{28, 29} This short half-life also limits the use of oximes as effective long-term
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52 prophylactic drugs.
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4 A new concept of acute OPNA poisoning treatment, by the administration of
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7 bioscavengers, emerged at the end of the 1980s.³⁰⁻³⁵ Bioscavengers are proteins that
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10 react with and neutralize OPNAs. Human butyrylcholinesterase (BChE) is effective as a
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13 bioscavenger at both pre- and post-OPNA exposure.³⁶⁻⁴⁰ The enzyme is stable in human
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16 blood circulation, with a half-life of 12 days, in concentration ranges between 3.5 and 9.3
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19 mg/L.⁴¹⁻⁴⁵ BChE is found in plasma, organs, and tissues including liver, skin, striated
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22 muscle, smooth muscle, lung, and brain.⁴⁶ Administration of BChE has proven to be safe
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25 with no toxic, immunogenic or behavioral effects.⁴⁷ As a stoichiometric bioscavenger, with
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28 a molecular weight of about 85 kDa, 500-600 times higher than that of OPNAs, a relatively
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31 high dose of BChE (100-500 mg) is required to effectively protect a 70 kg patient.⁴⁸
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35 Unfortunately, this dose costs more than \$2,000, and therefore BChE therapy has not
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42 been deployed as a prophylactic drug.³⁰
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46 Grafting poly(ethylene glycol) (PEG)⁴⁹⁻⁵² and poly(sialic acid) to the surface of BChE has
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49 been used to increase the half-life of the enzyme in the blood.⁵³⁻⁵⁶ Nevertheless, even
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53 extended lifetime BChE does not overcome the inherent limitations of a stoichiometric
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3 bioscavenger. We have become interested in whether polymers could serve to increase
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7 BChE protein lifetime while also enabling reactivation and protection of the enzyme,
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10 thereby defeating the stoichiometric limitations that drive up the necessary dose. Such a
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13 self-reactivating BChE protein-polymer conjugate could also be used as a critical
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17 component in decontamination of broad areas.
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22 A “grafting from” high yield approach to protein-polymer conjugate synthesis, involving
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25 the *in situ* growth of polymers from biomacromolecular initiators, emerged in the early
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28 2000s.⁵⁷⁻⁵⁹ Developed in the early 1990s, atom transfer radical polymerization (ATRP)
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32 has proved to be a controlled and powerful technique to prepare well-defined polymers
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35 and protein-polymer conjugates.^{51, 60-65} With a long term interest in polymer-based protein
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39 engineering, we have generated a large library of protein-polymer conjugates.⁶⁶⁻⁶⁹
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44 In the past decade, first generation “pseudo-catalytic” bioscavenger systems, composed
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47 of BChE delivered with an efficient oxime reactivator, were developed to reduce the
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50 amount of BChE needed for efficient protection.^{23, 24, 36, 70} Unfortunately, the fast
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54 elimination of oxime reactivators from blood circulation, requiring continuous infusion of
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3 the reactivator to sustain reactivation kinetics, limited the use of pseudo-catalytic
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7 bioscavengers *in vivo*.³⁰ Most recently, we have used ATRP to synthesize BChE-polymer
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10 conjugates with multiple pendant azido groups. Azide-alkyne “click” chemistry was then
11
12
13 used to covalently couple oximes to the surface of the enzyme.⁷¹ The BChE-polymer-
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16
17 oximes exhibited a slowed rate of inactivation by organophosphates and some evidence
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19
20 of inter- and intramolecular reactivation. Herein, fluorogenic analogues of VX and
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23 cyclosarin (EMP-MeCyC and CMP-MeCyC, respectively)⁷² were used to study the
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27 kinetics of inactivation and reactivation of next-generation BChE-polymer-oxime
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31 conjugates. The BChE-polymer-oxime conjugate recovered about 85% activity after
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34 complete inactivation induced by fluorogenic analogues of VX in three successive
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38 inactivation-reactivation “catalytic” cycles. The conjugates should also be useful in
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42 surface decontamination of OPNAs.
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EXPERIMENTAL SECTION

Materials

Common reagents and solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used as received unless otherwise specified. Dr. Oksana Lockridge (Eppley Institute, University of Nebraska Medical Center) kindly provided the human BChE. EMP-MeCyC and CMP-MeCyC were prepared we have described previously.⁷² MA-PEG₆-N₃ monomer and NHS-Br (ATRP initiator) were prepared in the same way as our previous reports.⁷¹,⁷³ Copper(II) bromide (CuBr₂), copper(II) sulfate (CuSO₄), hydroxylamine hydrochloride, triethylamine, sodium ascorbate (NaAsc), 2-imidazolecarboxaldehyde, *S*-butyrylthiocholine iodide (BTC), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), *N,N*-dimethylacrylamide (DMAA), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA), tris[2-(dimethylamino)ethyl] amine (Me₆TREN), isonicotinaldehyde, 1,4-diiodobutane, 6-iodohex-1-yne, and 4-chlorobenzyl bromide were purchased from Sigma-Aldrich. DMAA, Me₆TREN and HMTETA were purified with a basic alumina column before using. 2-(4-

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4 ((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)

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7 acetic acid (BTAA) and Azide-PEG-Amine were purchased from Click Chemistry Tools

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10 LLC. *N,N*-dimethylprop-2-yn-1-amine was purchased from Fisher Scientific.

11 12 13 14 15 16 17 **Characterization**

18 19 20 21 **Nuclear magnetic resonance (NMR) analysis**

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26 A 400 MHz spectrometer (Bruker Avance) in the Center for Molecular Analysis, Carnegie

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29 Mellon University, Pittsburgh, PA was used to collect ¹H NMR spectra.

30 31 32 33 34 35 36 37 **Ultraviolet-visible (UV-VIS) and fluorescence spectrophotometry**

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42 UV-VIS spectra were analyzed by a UV-VIS spectrophotometer (Lambda 45,

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45 PerkinElmer). Fluorescence spectra were analyzed by a plate reader (Bio-Tek Synergy

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49 H1).

50 51 52 53 **GPC analysis**

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3 The molecular weight (M_n) and the polydispersity (\mathcal{D}) of azide containing copolymers were
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7 determined by GPC with 3-columns on a Waters 2695 Series system (Waters
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9
10 Ultrahydrogel Linear, 500 and 250) as our previous report.⁷¹ The BChE, BChE-Br, BChE-
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12 PDMAA-N₃ and BChE-PDMAA-OX conjugates were analyzed by the same GPC system
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17 with 4-columns.
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22 Protein analysis by the Bicinchoninic acid (BCA) assay

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26 The BChE-PDMAA-OX conjugate (1-2 mg/mL, protein, 20 or 10 μ L) in BP buffer (50 mM,
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29 pH 7.4) was mixed with 200 μ L BCA working reagent solution (Reagent A/Reagent B =
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32 50/1). The mixture was incubated at 60°C for 15 min. 562 nm absorbance of the samples
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34
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36 was recorded by a plate reader (Bio-Tek Synergy H1). Standard curves of PEG-IO, PEG-
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39 PO and native BChE were used to determine the protein concentration of the conjugates
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43 (Figure S1).
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48 SDS-PAGE analysis

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52 BChE, BChE-Br, or BChE-polymer-oxime conjugate solution (1.0 mg/mL protein, 10 μ L)
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55 was mixed with SDS-PAGE loading buffer (2X, 10 μ L) and heated at 95 °C for 10 min. 10
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4 μL of the sample was loaded onto 4-15% precast gel and run at 200 V for 30 min. The
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7 gel was washed with distilled water three times and stained with 50 mL PageBlue staining
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10 solution. 1 hour later, the gel was de-stained overnight by distilled water.
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14 15 **Methods**

16 17 18 19 **Synthesis of alkyne-imidazolium-oxime**

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23 Alkyne-imidazolium-oxime was synthesized as described previously.⁷¹ Briefly, 2-
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26 imidazolecarboxaldehyde (12 mmol) and K_2CO_3 (24 mmol) were mixed in 30 mL DMF. 4-
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29 Chlorobenzyl bromide (24 mmol) was added and stirred at room temperature overnight.
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34 The reaction mixture was filtered, and 250 mL distilled water was added to the filtrate.
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38 The resulting solution was extracted with ethyl acetate (200 mL \times 3), then the organic
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41 phase was dried with MgSO_4 . After removing the solvent by rotary evaporator, the
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43
44 alkyylimidazole-2-carbaldehyde yield was 74%.
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49 Hydroxylamine hydrochloride (13.5 mmol) was dissolved in 30 mL water, and added to
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52 Na_2CO_3 (13.5 mmol) and alkyylimidazole-2-carbaldehyde (9 mmol). The reaction mixture
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56 was stirred for 3.5 h at room temperature, and the precipitate was filtered and washed by
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4 distilled water three times and Et₂O four times. The product was dried over a vacuum,
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7 generating an 87% yield.
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11 6-Iodo-hex-1-yne (1.5 mmol) in 10 mL ACN and 1 mL DMSO were added to the
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14 alkyimidazole-2-carbaldehyde oxime (0.4 mmol). The reaction mixture was stirred at 67
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17 °C for 5 days. Removed the solvent by rotary evaporator. The crude product was purified
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19
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21 by silica gel column (from hexane/acetone = 1/1 to acetone/MeOH = 3/1). White powder,
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24
25 48% yield. ¹H NMR (400 MHz, MeOD-d₄) δ 8.52 (s, 1H, CH), 7.82 (d, J = 3, 1H, Ar H),
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28 7.74 (d, J = 3, 1H, Ar H), 7.44 (d, J = 3, 2H, Ar H), 7.35 (d, J = 3, 2H, Ar H), 5.63 (s, 2H;
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31 CH₂), 4.43 (t, J = 3, 2H; CH₂), 3.36 (s, 1H; CH), 2.27 (d, J = 3, 2H, CH₂), 2.02-2.00 (m,
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35 2H; CH₂), 1.62-1.59 (m, 2H; CH₂) (Figure S2).
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40 **Synthesis of PEG-IO**

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44 PEG-IO was synthesized as described previously.⁷¹ Briefly, BTTAA (2.4 mg in 120 μL
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47 H₂O) and sodium ascorbate (NaAsc, 2 mg in 100 μL H₂O) was added to a CuSO₄ solution
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51 (100 mM, 60 μL). Alkyne-imidazolium-oxime (22 mg) and NH₂-PEG₆-N₃ (20 mg in 300 μL
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55 DMF) were added to the solution. The reaction mixture was incubated at room
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3 temperature for 1 hour. The product was purified by precipitation in ether/acetone = 10/1
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7 four times and dried by a vacuum. Dark oil, 75% yield. ^1H NMR (400 MHz, MeOD- d_4) δ
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10 8.60 (s, 1H; CH), 7.94 (s, 1H, Ar H), 7.92 (s, 1H, Ar H), 7.81 (d, J = 3, 1H, Ar H), 7.49 (d,
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12
13 J = 3, 2H, Ar H), 7.33 (d, J = 6, 2H, Ar H), 5.59 (s, 2H; CH₂), 4.45 (t, J = 3, 2H; CH₂), 4.35
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16
17 (t, J = 3, 2H; CH₂), 3.78 (s, 2H; CH₂), 3.50-3.44 (m, 24H; 12CH₂), 2.62 (d, J = 3, 2H, CH₂),
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21 1.81-1.79 (m, 2H; CH₂), 1.59 (d, J = 3, 2H, CH₂) (**Figure S2**). PEG-IO was used as a
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24 standard for the calculation of oxime to BChE ratios of BChE-polymer-oxime conjugates.
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29 **Synthesis of alkyne-pyridinium-oxime**

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33 Isonicotinaldehyde (50 mmol), hydroxylamine hydrochloride (55 mmol) and K₂CO₃ (7.6 g,
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36 55 mmol) were mixed in 250 mL MeOH at room temperature overnight. After removing
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38
39 the solvent by rotary evaporator, the residue was diluted with water (250 mL) and
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41
42 extracted with ethyl acetate (5 × 150 mL). The combined extract was washed with brine
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45 (2 × 150 mL) and dried over MgSO₄. Remove the solvent by rotary evaporator and got
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47
48 the product isonicotinaldehyde oxime, 84% yield.
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55 Isonicotinaldehyde oxime (10 mmol) and 1,4-diiodobutane (30 mmol) were dissolved in
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4 50 mL ACN and stirred at room temperature for 5 days. The resulting precipitate was
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7 collected by filtration, then concentrated and added to 50 mL ethyl acetate. We next
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10 collected and combined the precipitate, then purified by silica gel (EA/MeOH/HCOOH =
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13 4/1/0.25) to generate the product 4-((hydroxyimino)methyl)-1-(4-iodobutyl)pyridin-1-ium
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17 iodide in 77% yield.

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22 4-((hydroxyimino)methyl)-1-(4-iodobutyl)pyridin-1-ium iodide (0.5 mmol) was dissolved in
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25 5 mL ACN and 400 μ L DMSO. *N,N*-dimethylprop-2-yn-1-amine (1 mmol) was added to
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28 the ACN/DMSO solution and stirred at room temperature for 2 days. The solvent was
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31 removed by rotary evaporation. The product alkyne-pyridinium-oxime was purified by
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33
34 precipitation in ethyl acetate/acetone (2/1) three time and dried by vacuum. Yellow
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36
37 powder, 65% yield. ^1H NMR (400 MHz, MeOD- d_4) δ 8.82 (d, J = 6, 2H, Ar H), 8.35 (s,
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39
40 1H, Ar H), 8.19 (d, J = 6, 2H, Ar H), 4.68-4.63 (m, 2H; CH_2), 4.23 (d, J = 3, 2H, CH_2), 3.49-
41
42
43 3.46 (m, 2H; CH_2), 3.23 (t, J = 3, 1H; CH), 3.15 (s, 6H; 2CH_3), 2.18-2.07 (m, 2H; CH_2),
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46 1.91-1.87 (m, 2H; CH_2) (**Figure S3**).

53 54 **Synthesis of PEG-PO**

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3 PEG-PO was synthesized as described previously.⁷¹ Briefly, BTAA (13 mg in 300 μ L
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7 H₂O) and sodium ascorbate (NaAsc, 20 mg in 200 μ L H₂O) were added to a CuSO₄
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9
10 solution (100 mM, 300 μ L). Alkyne-pyridinium-oxime (52 mg) and NH₂-PEG₃-N₃ (55 mg
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14 in 600 μ L DMF) were then added to the solution and the reaction mixture was incubated
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18 at room temperature overnight. The product was purified by precipitation in ether/acetone
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21 = 10/1 four times and dried by a vacuum. Dark oil, 53% yield. ¹H NMR (400 MHz, MeOD-
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24 d₄) δ 8.82 (s, 1H; Ar H), 8.80 (s, 1H; Ar H), 8.34 (d, J = 3, 2H, Ar H), 8.17 (d, J = 6, 2H,
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26
27 Ar H), 4.69-4.62 (m, 6H; 3CH₂), 3.95 (t, J = 3, 2H, CH₂), 3.70 (t, J = 3, 2H, CH₂), 3.65-
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31 3.59 (m, 8H; 4CH₂), 3.35-3.32 (m, 2H; CH₂), 3.16 (t, J = 3, 2H, CH₂), 3.04 (s, 6H; 2CH₃),
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35 2.08-2.03 (m, 2H; CH₂), 2.01-1.95 (m, 2H; CH₂) (**Figure S3**). PEG-PO was used as a
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39 standard for the calculation of oxime to BChE ratios of BChE-polymer-oxime conjugates.
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47 **Synthesis of BChE-polymer-oxime conjugates**

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51 BChE-PDMAA-N₃ conjugate was synthesized as described previously.⁷¹ Alkyne-
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55 imidazolium-oxime or alkyne-pyridinium-oxime was attached to the side chains of BChE-
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3 PDMAA-N₃ conjugate by “click” chemistry.⁷⁴ NaAsc (10 mM × 35 μL), CuSO₄ (10 mM ×
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7 17.6 μL), and BTAA (10 mM × 35 μL) in distilled water were added into a BChE-PDMAA-
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9
10 N₃ (27 μM BChE, 650 μL) solution. Alkyne-imidazolium-oxime or alkyne-pyridinium-oxime
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12
13 (50 mM) in distilled water was added in different oxime to BChE ratios (40-, 80- or 160-
14
15
16
17 fold). The reaction mixture was incubated at room temperature for 3 h. The BChE-
18
19
20 PDMAA-OX conjugates were purified by ultrafiltration (50 kDa cut-off membrane) four
21
22
23
24 times. The number of oximes per BChE monomer of the conjugates was determined by
25
26
27
28 BCA assay and UV absorbance at 280 nm (**Figure S1**). BChE-PDMAA-OX conjugates
29
30
31 were characterized by GPC and SDS-PAGE analysis.
32
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35

36 **Synthesis of PDMAA-IO and PDMAA-PO**

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40 To synthesize the azide containing copolymer, PDMAA-N₃, DMAA (1.20 mmol), MA-
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42
43 PEG₆-N₃ (60 μmol) and ATRP initiator (1 μmol) were mixed in 2 mL of PB buffer (50 mM,
44
45
46
47 pH 7.4), sealed, and bubbled with argon for 30 min at room temperature. Deoxygenated
48
49
50 Me₆TREN (8 μmol), CuBr₂ (3 μmol) and NaAsc (4 μmol) in distilled water (1 mL) were
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54 added to the initiator-monomer solution, sealed, and stirred for 2 h at room temperature.
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3 The copolymer PDMAA-N₃ was purified by dialysis (8 kDa cut-off membrane) in distilled
4 water and lyophilization. The molecular weight (M_n) and dispersity (\mathcal{D}) of PDMAA-N₃ were
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6
7 64.4 kDa and 1.87, respectively (Figure S4 and S5). GPC calibration was based on
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9
10
11
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14 poly(ethylene glycol) standards.
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16
17

18 Alkyne-imidazolium-oxime or alkyne-pyridinium-oxime was attached to the side chains of
19
20
21 PDMAA-N₃ conjugate by “click” chemistry to prepare PDMAA-IO or PDMAA-PO.⁷⁴ NaAsc
22
23
24 (10 mM × 80 μL), CuSO₄ (10 mM × 40 μL), and BTAA (10 mM × 80 μL) in distilled water
25
26
27
28 were added into a PDMAA-N₃ (14 mg in 650 μL H₂O) solution. Alkyne-imidazolium-oxime
29
30
31 or alkyne-pyridinium-oxime (2 mg in 80 μL DMSO) was added and incubated at room
32
33
34
35 temperature overnight. PDMAA-IO or PDMAA-PO was purified by ultrafiltration (30 kDa
36
37
38 cut-off membrane) four times.
39
40
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43 The oxime content of PDMAA-IO and PDMAA-PO, determined from the UV-VIS spectra,
44
45
46 were 12.5 wt% and 4.5 wt%, respectively (Figure S1 and S4).
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51 Activity assay of BChE and BChE-PDMAA-OX conjugates

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55 BTC was used as a substrate to determine the activity of BChE and BChE-PDMAA-OX
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3 conjugates at room temperature. 1 mL of BTC (1 mM) and DTNB (Ellman assay reagent,
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6
7 0.1 mM) in PB buffer (50 mM, pH 7.4) was added to a 1.5 mL cuvette. Native BChE or
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9
10 BChE-PDMAA-OX conjugates (20 nM protein, 100 μ L) was then added to the cuvette.⁷⁵
11
12
13
14 The activity of BChE or BChE-PDMAA-OX conjugates was evaluated by monitoring BTC
15
16
17 hydrolysis, resulting in an increase in absorbance at 412 nm from TNB (extinction
18
19
20 coefficient of 14,000 $M^{-1} cm^{-1}$), using a Lambda 2 PerkinElmer UV-VIS spectrophotometer.
21
22
23
24

25 **Inhibition assay of BChE and BChE-polymer-oxime conjugates**

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29 BChE or BChE-PDMAA-OX conjugate (20 or 10 nM) was incubated with a 5-fold
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31
32 stoichiometric excess of EMP-MeCyC or CMP-MeCyC at room temperature. An aliquot
33
34
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36 was diluted 10 or 20 times in PB buffer (50 mM, pH 7.4) at specified time intervals. After
37
38
39
40 addition of BTC (1 mM) and DTNB (0.1 mM), we measured the enzymatic activity of each
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44 group. The uninhibited BChE or BChE-PDMAA-OX conjugate was treated as 100%
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46
47 activity at the time zero. Activity evaluation was performed in PB buffer (50 mM, pH 7.4)
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49
50 by the Ellman assay using a Lambda 2 PerkinElmer UV-VIS spectrophotometer.⁷⁵
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55 **Flu-OPNAs degradation assay**

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3 A mixture of BChE, BChE-PDMAA-IO₁₇₂ conjugate (or BChE-PDMAA-PO₂₁₆ conjugate)
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7 and PDMAA-IO (or PDMAA-PO) at various ratios (protein 50 nM; Oxime/BChE = 43 or
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10 54) were incubated with a 5-fold excess of EMP-MeCyC (or CMP-MeCyC) in PB buffer
11
12
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14 at pH 7.4. The total volume of the mixture was 100 μ L. The fluorescence intensity (ex 400
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17 nm; em 446 nm) was measured using a Bio-Tek Synergy H1 plate reader at specified
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21 time intervals.
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25 **Reactivation assay of BChE and BChE-PDMAA-OX conjugates**

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30 A reactivation assay was carried out with BChE or BChE-PDMAA-OX conjugates (1 μ M)
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33 in 1 mg/mL bovine serum albumin (BSA) solution in PB buffer (50 mM, pH 6.0) that we
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35
36 mixed with a 10-fold molar excess of EMP-MeCyC or CMP-MeCyC in PB buffer (50 mM,
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39 pH 6.0) for 10 min. The completely inactive BChE or conjugates were transferred into an
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42
43 ultrafiltration tube (50 kDa cut-off membrane) to remove the excess of EMP-MeCyC or
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47 CMP-MeCyC (10,000 \times g for 5 min, three times), then diluted 50-fold in PB buffer (50 mM,
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50 pH 8.0) at room temperature. At specified time intervals, the activities of BChE or
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3 conjugates were measured by Ellman assay using BTC (1 mM) and DTNB (0.1 mM).
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7 The activity of uninhibited BChE or conjugates were used to determine the 100% activity.
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9

10 11 **Inactivation and reactivation recycle of BChE-PDMAA-OX conjugates** 12 13

14
15 BChE-PDMAA-OX conjugates 1, 2 or 3 (1 μ M) in 1 mg/mL bovine serum albumin (BSA)
16
17 in PB buffer (50 mM, pH 6.0) solution was mixed with a 10-fold molar excess of EMP-
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19 MeCyC in PB buffer (50 mM, pH 6.0) for 10 min. The completely inactive conjugates were
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23 purified by ultrafiltration tube (50 kDa cut-off membrane, 10,000 \times g for 5 min, three times),
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26
27 then diluted 50-fold in PB buffer (50 mM, pH 8.0) and incubated at room temperature for
28
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33 200 min. The conjugates were buffer-exchanged with PB buffer (50 mM, pH 6.0) and
34
35
36 concentrated to 1 μ M, then mixed with a 10-fold molar excess of EMP-MeCyC in PB buffer
37
38
39 (50 mM, pH 6.0) and incubated at room temperature for 10 min. The completely inactive
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43 conjugates were purified by ultrafiltration tube (50 kDa cut-off membrane, 10,000 \times g for
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47 5 min, three times), then diluted 50-fold in PB buffer (50 mM, pH 8.0) and incubated at
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49
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51 room temperature for 200 min. The inactivation and reactivation cycle were repeated
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55 three times. At specified time intervals, the conjugate activity was measured by the Ellman
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3 assay using BTC (1 mM) and DTNB (0.1 mM) as substrates. The activity of uninhibited,
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7 or activity recovered conjugates at pH 8.0 after 200 min, were used to determine the
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9
10 100% activity value.
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14 **Tertiary structure-based prediction of NHS-ATRP initiator modification**

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19 The tertiary structure of BChE was downloaded from Protein Data Bank (PDB code:
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21
22 6I2T). In order of influence on initiator-amino group specificity, the following properties
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26 for each lysine residue and N-termini were determined: exposed surface area (ESA),
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29 steric hindrance, pK_a , secondary structure, local charge, and H-bonding (Table S1).⁷⁶
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38 **RESULTS AND DISCUSSION**

39 40 41 42 **Synthesis and Characterization of BChE-polymer-oxime conjugates**

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47 To synthesize BChE-polymer-oxime conjugates, an *N*-hydroxysuccinimide (NHS) ester
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51 modified ATRP initiator (NHS-Br) was reacted with accessible amine groups on the
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54 surface of BChE generating the macroinitiator BChE-Br. On average, 6.5 initiators were
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3 attached to each BChE monomer.⁷¹ To pinpoint where these modification sites were
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6 located, we employed a tertiary structure-based predictive model that we had previously
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8
9 developed to identify highly reactive amine groups on the surface BChE.⁷⁶ This approach
10
11 enabled us to isolate the N-termini and a small group of lysine residues (K9, K60, K105,
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13
14 K190, and K558) as the likely sites of reaction with the ATRP initiators (Table 1). Azide-
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17 containing copolymers were grown from BChE-Br at fast-modified lysine residues and the
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19
20 N-termini by *in situ* ATRP with a neutral monomer, *N,N*-dimethylacrylamide (DMAA), in
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23 the presence of an azido monomer (MA-PEG₆-N₃), to generate BChE-PDMAA-N₃
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25
26 conjugates, as described previously.⁷¹ Copper catalyzed azide-alkyne cycloaddition “click”
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28
29 chemistry was then used to couple an alkyne-imidazolium-oxime or an alkyne-pyridinium-
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31
32 oxime to the azido-containing copolymers of BChE-PDMAA-N₃ conjugates (**Figure 1**).
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35 Stoichiometric alkyne-imidazolium-oxime (IO) or alkyne-pyridinium-oxime (PO) excesses
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38 of 40-fold, 80-fold or 160-fold were used to modify BChE-PDMAA-N₃ in the preparation
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41 of BChE-PDMAA-OX conjugates (BChE-PDMAA-IO and BChE-PDMAA-PO). The BChE-
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44 PDMAA-OX conjugates were purified by ultrafiltration (100 kDa cut-off membrane) and
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46
47 the oxime to BChE ratios were determined using the bicinchoninic acid (BCA) assay and
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3 absorbance at 280 nm (**Figure S1**). The number of imidazolium-oximes per BChE
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6 tetramer was 28, 84 and 172 for BChE-PDMAA-IO conjugates (we refer to these
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8
9
10 conjugates as BChE-PDMAA-IO₂₈, BChE-PDMAA-IO₈₄, and BChE-PDMAA-IO₁₇₂,
11
12
13 respectively). The number of pyridinium-oximes per BChE tetramer was 44, 104 and 216
14
15
16 for BChE-PDMAA-PO conjugates BChE-PDMAA-PO₄₄, BChE-PDMAA-PO₁₀₄, and
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20
21 BChE-PDMAA-PO₂₁₆, respectively. The molecular weights and purities of the BChE-
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24 PDMAA-OX conjugates were compared by gel permeation chromatography (GPC) and
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27 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Table**
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31 **1, Figure 2**). It is important to note that there are no GPC standards for these kinds of
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34 protein-polymer conjugates, so we must be cautious when interpreting observed
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37 conjugate molecular weights. That's said, GPC can be useful when interpreting the
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42 differences in apparent molecular weight between various conjugates.
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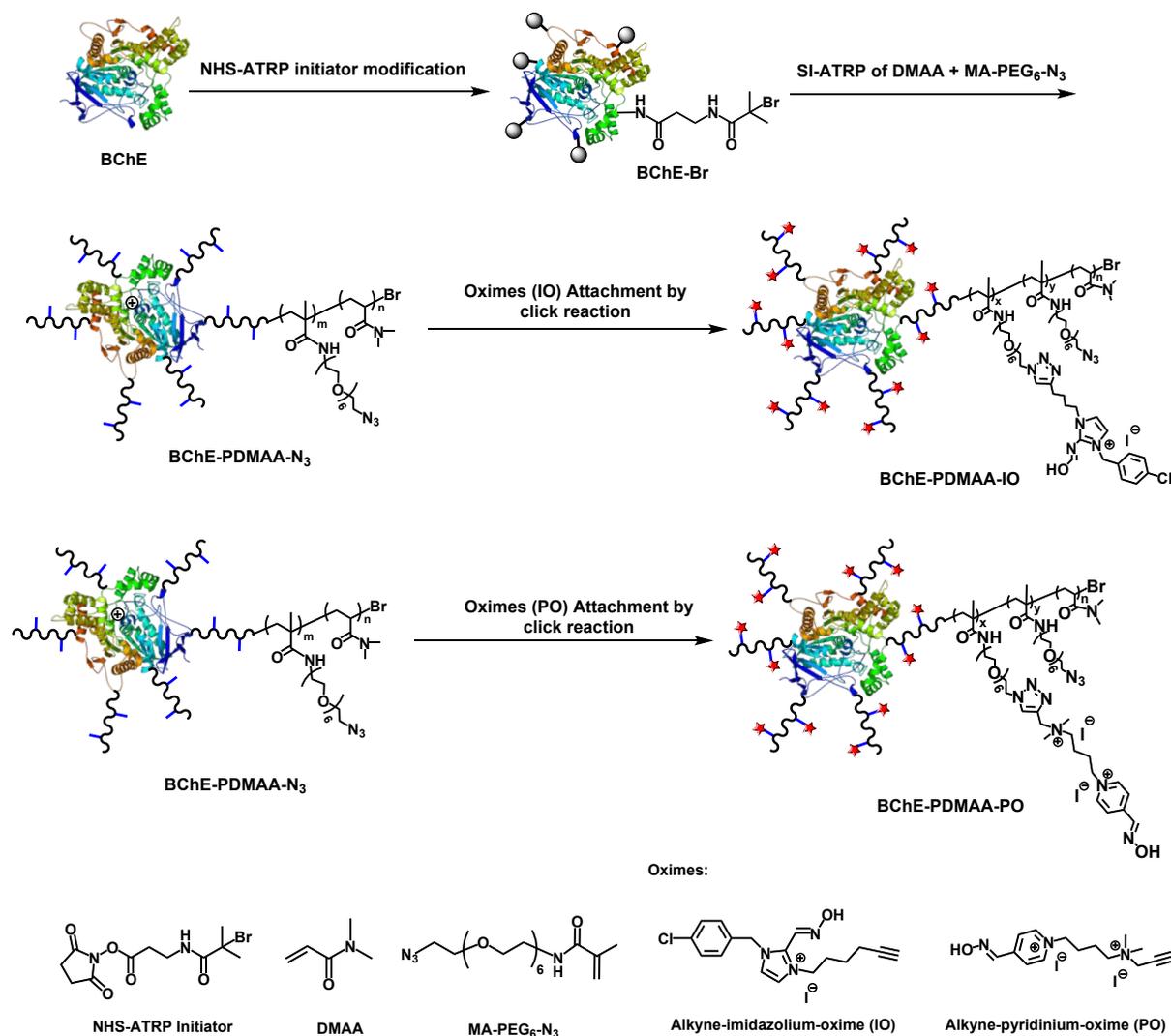


Figure 1. Synthesis of butyrylcholinesterase-polymer-oxime conjugates using atom-transfer radical polymerization (ATRP) and “click” chemistry. Additional acronyms: *N*-hydroxysuccinimide (NHS), *N,N*-dimethylacrylamide (DMAA), *N*-(20-azido-3,6,9,12,15,18-hexaoxaicosyl)methacrylamide (MA-PEG₆-N₃), butyrylcholinesterase (BChE).

We next investigated whether the BChE-PDMAA-OX conjugates retained bioactivity.

Using S-butyrylthiocholine iodide (BTC) as a substrate (10 μM, pH 8.0), we compared

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3 conjugate activity to that of native BChE. For BChE-PDMAA-IO₂₈, BChE-PDMAA-IO₈₄,
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7 and BChE-PDMAA-IO₁₇₂, activity was inversely proportional to oxime content (76%, 67%
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10 and 54%, respectively). The BChE-PDMAA-PO conjugates also exhibited reduced
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13 activity, but with a less pronounced inverse dependence on oxime content (63%, 48%
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15
16 and 48% for BChE-PDMAA-PO₄₄, BChE-PDMAA-PO₁₀₄, and BChE-PDMAA-PO₂₁₆
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19 respectively) (**Figure 3**). Previous work lead us to hypothesize that the activity loss of
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21 BChE-PDMAA-OX conjugates resulted from a combination of polymer attachment and
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24 reversible direct oxime inhibition of the enzyme.^{23, 71} In addition, Cu²⁺ and Cu-ligand
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27 complexes can inhibit cholinesterase activity.^{77, 78} The alkyne-imidazolium-oxime had a
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29
30 BChE IC₅₀ of approximately 1 μM (**Figure S6**) and the alkyne-pyridinium-oxime BChE IC₅₀
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33 was > 50 μM (**Figure S7**). The BChE-PDMAA-OX conjugates reported herein were the
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35
36 highest activity BChE-polymer-oxime conjugates that we have studied to date. We have
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38
39 recently determined the Michaelis-Menten kinetics for the BChE, BChE-Br, BChE-
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42 PDMAA-N₃ starting materials.⁷¹
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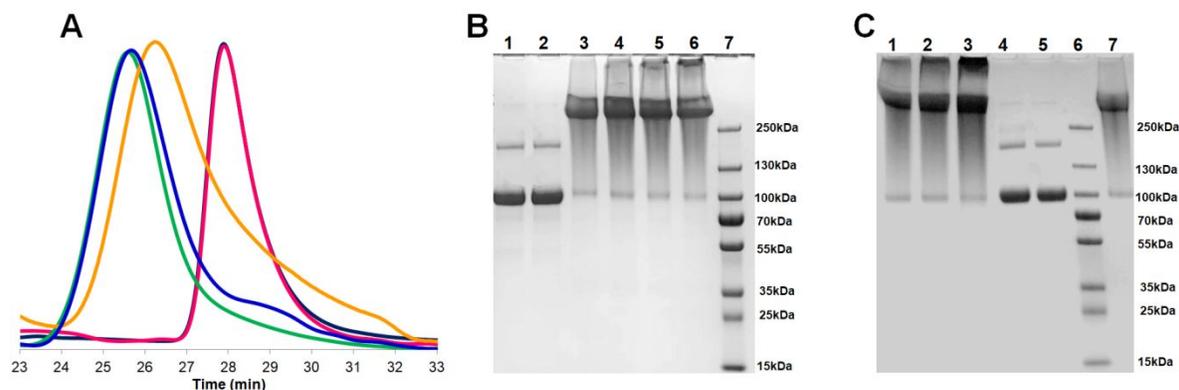
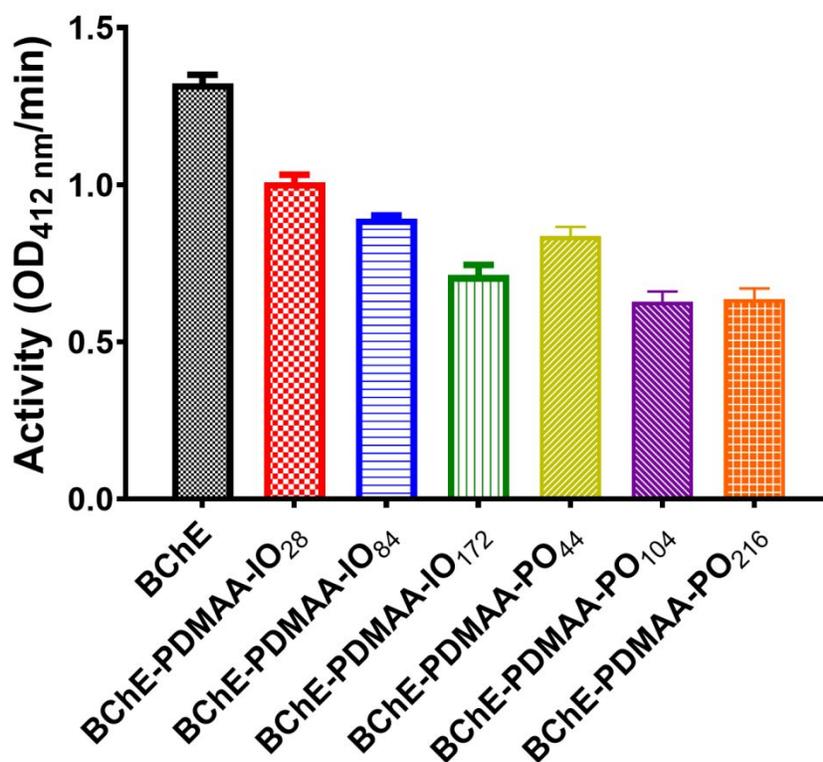


Figure 2. Characterization of BChE, BChE-Br, BChE-PDMAA-N₃ and BChE-PDMAA-OX conjugates. A) GPC analysis of BChE (black curve), BChE-Br (red curve), BChE-PDMAA-N₃ (green curve), BChE-PDMAA-IO₈₄ (yellow curve) and BChE-PDMAA-PO₁₀₄ (blue curve) conjugates. B) SDS-PAGE analysis of BChE, BChE-Br, BChE-PDMAA-N₃ and BChE-PDMAA-IO conjugates. Lane 1: BChE; Lane 2: BChE-Br; Lane 3: BChE-PDMAA-N₃; Lane 4: BChE-PDMAA-IO₂₈; Lane 5: BChE-PDMAA-IO₈₄; Lane 6: BChE-PDMAA-IO₁₇₂; Lane 7: Marker. C) SDS-PAGE analysis of BChE, BChE-Br, BChE-PDMAA-N₃ and BChE-PDMAA-PO conjugates. Lane 1: BChE-PDMAA-PO₄₄; Lane 2: BChE-PDMAA-PO₁₀₄; Lane 3: BChE-PDMAA-PO₂₁₆; Lane 4: BChE-Br; Lane 5: BChE; Lane 6: Marker; Lane 7: BChE-PDMAA-N₃.

Since only negatively charged oximates can reactivate BChE active sites, it was important to determine the pK_a 's for the monomeric alkyne-imidazolium-oxime and alkyne-pyridinium-oxime.⁷⁶ We used a spectroscopic assay and thereby surmised that the oximes groups on BChE-PDMAA-IO and BChE-PDMAA-PO had pK_a 's of 8.35 and 8.38, respectively (**Figure S8**). Thus, at pH 6.0 the conjugates would be in their fully protonated

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3 oxime form, and therefore would be unable to undergo either inter- or intramolecular
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7 reactivation. Therefore, in order to minimize any reactivation, we inhibited the BChE-
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11 polymer-oxime conjugates by fluorogenic OPNAs (Flu-OPNA) analogues at pH 6.0 (vide
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15 infra).



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45 **Figure 3.** Enzymatic activity of BChE, BChE-PDMAA-IO and BChE-PDMAA-PO
46 conjugates at a single concentration of BTC in PB (50 mM) buffer at pH 8.0. Results are
47 presented as mean values \pm standard deviation ($n = 3$).
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Oxime containing copolymers protect BChE against OPs

Two racemic Flu-OPNAs analogues, which were designed to be analogues of VX or cyclosarin after reaction with BChE, were used to inhibit BChE-polymer-oxime conjugates.

The Flu-OPNAs contained either an ethyl (E) or cyclohexyl (C) O-alkyl attached to a methyl-phosphoryl (MP) moiety. The fluorescent moiety, 3-cyano-4-methyl-7-hydroxy coumarin (MeCyC-OH), was released upon reaction between the BChE active site serine and Flu-OPNAs (EMP-MeCyC or CMP-MeCyC) resulting in a significant and measurable increase in fluorescence intensity.⁷² BChE-PDMAA-OX conjugates (10 or 20 nM) were exposed to a 5-fold stoichiometric excess of Flu-OPNA in sodium phosphate buffer (pH 7.4, 50 mM) and the activity loss was measured over time by removing aliquots and performing an activity assay as described above. Following the addition of Flu-OPNAs, native BChE lost all activity within 10 min (**Figure S9A**). After exposure to EMP-MeCyC, BChE-PDMAA-IO₂₈, BChE-PDMAA-IO₈₄, and BChE-PDMAA-IO₁₇₂ retained 7%, 10% and

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3 15% activity, respectively (**Figure S9A**). The BChE-PDMAA-PO₄₄, BChE-PDMAA-PO₁₀₄,
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7 and BChE-PDMAA-PO₂₁₆ conjugates retained 27%, 28% and 35% activity, respectively
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10 (**Figure S9B**). Thus, all the BChE-PDMAA-OX conjugates were all moderately resistant
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14 to inactivation by EMP-MeCyC, with the degree of protection being proportional to oxime
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17 content and related to oxime structure. Imidazolium-oxime containing copolymers also
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20 protected BChE against CMP-MeCyC (**Figure S10A**), though the highest oxime content
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24 BChE-PDMAA-PO did not resist inactivation by EMP-MeCyC (**Figure S10B**). The BChE-
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28 PDMAA-OX conjugates were all inactivated eventually by a large excess of paraoxon
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31 (POX) (**Figure S11**).
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36 To elucidate whether the protection of oxime containing BChE-PDMAA-OX conjugates
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39 against Flu-OPNAs was consistent with intramolecular and/or intermolecular interactions,
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42 inhibition was monitored in mixtures of native BChE, BChE-PDMAA-OX conjugates and
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45 oxime containing copolymers (PDMAA-IO and PDMAA-PO) using the release of
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49 fluorescence (the leaving group is MeCyC-OH) upon covalent coupling of Flu-OPNAs to
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53 the enzyme (**Figure 4**). The total oxime content was kept constant at 172 and 216
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4 equivalents for BChE-PDMAA-IO and BChE-PDMAA-PO respectively, delivering the
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7 oxime by polymer only or enzyme-linked oxime. The presence of free PDMAA-IO
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10 enhanced EMP-MeCyC hydrolysis. BChE-PDMAA-IO₁₇₂ with no free PDMAA-IO had the
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13 lowest fluorescence intensity increase. The degree of fluorescence intensity increased
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16 steadily as the fraction of free PDMAA-IO increased over 20 min (**Figure 4A**). These data,
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19 when combined with control experiments, showed that free PDMAA-IO could protect
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22 BChE against EMP-MeCyC, thereby indicating that intermolecular protection accounted
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25 for the majority of the observed protection. A similar phenomenon was observed in the
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28 fluorescence monitoring assay of CMP-MeCyC mixed with BChE, PDMAA-PO and
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31 BChE-PDMAA-PO₂₁₆ (**Figure 4B**). CMP-MeCyC did not significantly hydrolyze in PB
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34 buffer at pH 7.4 at room temperature over 40 min. PDMAA-PO addition reduced the
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37 fluorescence intensity of CMP-MeCyC (the leaving group), and the fluorescence intensity
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40 of CMP-MeCyC did not increase upon BChE addition indicating complete inactivation of
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42
43 the active site. Since we know that native BChE stoichiometrically reacts with OPNAs in
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46 a 1 to 1 ratio, and the fluorescence intensity of CMP-MeCyC with BChE-PDMAA-PO was
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49 higher than with BChE after 40 min, we deduced that one molecule of BChE-PDMAA-PO
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could scavenge approximately 1.4 molecules of CMP-MeCyC based on the difference of fluorescence intensity between 100% BChE group and 100% BChE-PDMAA-PO₂₁₆ group.

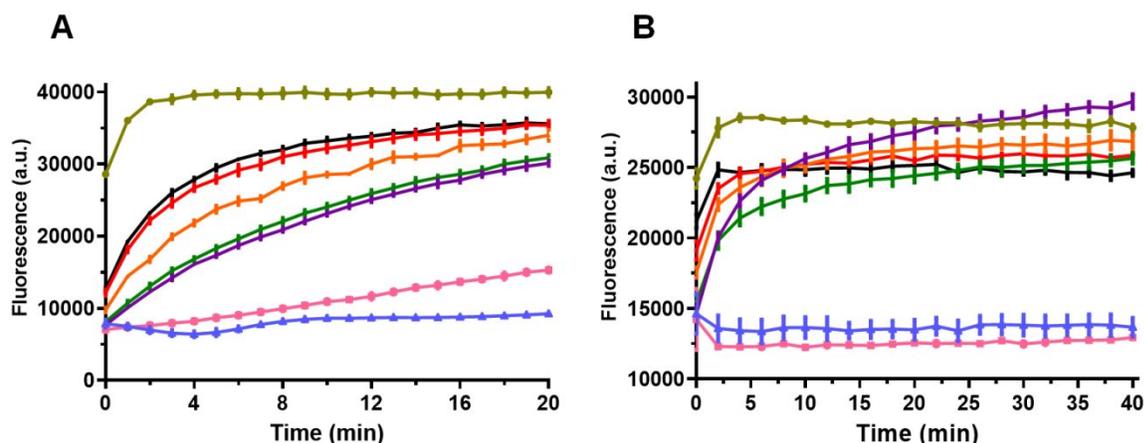


Figure 4. Time-course of Flu-OPNAs (250 nM) degradation by mixtures of BChE, polymer-oxime and BChE-PDMAA-OX conjugates in PB buffer at pH 7.4. (A) Fluorescence intensity of EMP-MeCyC mixed with BChE, PDMAA-IO and BChE-PDMAA-IO₁₇₂ (Imidazolium-oxime/BChE tetramer = 172). (B) Fluorescence intensity of CMP-MeCyC mixed with BChE, PDMAA-PO and BChE-PDMAA-PO₂₁₆ (Pyridinium-oxime/BChE tetramer = 216). Black curve: 100% BChE; Red curve: 80% BChE + 20% BChE-PDMAA-IO₁₇₂ (or BChE-PDMAA-PO₂₁₆); Orange curve: 50% BChE + 50% BChE-PDMAA-IO₁₇₂ (or BChE-PDMAA-PO₂₁₆); Green curve: 20% BChE + 80% BChE-PDMAA-IO₁₇₂ (or BChE-PDMAA-PO₂₁₆); Purple curve: 100% BChE-PDMAA-IO₁₇₂ (or BChE-PDMAA-PO₂₁₆); Yellow curve: only BChE; Pink curve: only PDMAA-IO; Blue curve: PB buffer. Results are presented as mean values \pm standard deviation ($n = 3$).

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4 To discover whether or not oxime side chains reacted directly with EMP-MeCyC and/or
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7 CMP-MeCyC, we monitored the fluorescence intensity of Flu-OPNAs after the addition of
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10 free imidazolium- or pyridinium-oximes. Alkyne-imidazolium-oxime addition enhanced the
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13 fluorescence of the MeCyC-OH moiety released from EMP-MeCyC and CMP-MeCyC
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16
17 **(Figure S12)**. We observed a reduction in the fluorescence of EMP-MeCyC and CMP-
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19
20 MeCyC after the addition of alkyne-pyridinium-oxime **(Figure S12B)**. EMP-MeCyC
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23 spontaneously hydrolyzed in PB buffer at pH 7.4 at room temperature **(Figure S12A)**. The
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27 decreased inhibition rates of the conjugates by EMP-MeCyC and CMP-MeCyC could
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31 have been caused by a direct reaction between the imidazolium-oxime and Flu-OPNAs.
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35 We were able to rule out, however, that the protection by pyridinium-oxime containing
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38 copolymer on the surface of BChE-polymer-oxime conjugates was related to a direct
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42 reaction between pyridinium-oxime and CMP-MeCyC. The reversible inhibition of the
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45 BChE active site by pyridinium-oxime apparently masked the active site toward inhibition
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49 by CMP-MeCyC.
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53 **Reactivation of Flu-OPNAs-inhibited BChE-PDMAA-OX conjugates**
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4 To determine whether free alkyne-imidazolium-oxime and alkyne-pyridinium-oxime were
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7 effective reactivators of BChE-EMP and BChE-CMP covalent complexes, Flu-OPNA-
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10 inhibited BChE was treated with a 25,000-fold stoichiometric excess of free oxime (0.5
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13 mM) (**Figure S13**). More than 40% of EMP-MeCyC- and CMP-MeCyC- inhibited BChE
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16 activity was recovered after the addition of alkyne-pyridinium-oxime within 24 h (**Figure**
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19 **S13**). Addition of 2-PAM (0.5 mM), a positive control reagent, restored more than 65%
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22 activity over the same time period. There was no significant reactivation after the addition
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25 of the same amount of alkyne-imidazolium-oxime. Alkyne-imidazolium-oxime had an IC_{50}
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28 of 1 μ M (**Figure S6**). The active site of BChE, inhibited by Flu-OPNA, may be reactivated
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31 by the nucleophilic attack of alkyne-imidazolium-oxime, but reversibly inhibited by excess
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34 oxime. To determine whether alkyne-imidazolium-oxime could restore the activity of Flu-
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37 OPNAs inhibited BChE, EMP-MeCyC-inhibited BChE was treated with different
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40 concentrations of alkyne-imidazolium-oxime. Lower concentrations of alkyne-
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43 imidazolium-oxime induced the reactivation of EMP-MeCyC inhibited BChE. More than
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46 25% of enzyme activity was recovered 5 hours after the addition of 2 μ M alkyne-
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3 imidazolium-oxime, whereas a higher concentration of alkyne-imidazolium-oxime
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7 reactivated less than 25% of the activity due to reversible inhibition of BChE (**Figure S14**).
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11 The degree to which the polymer-bound oxime could reactivate Flu-OPNA-inhibited
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13 BChE-PDMAA-OX conjugates was determined by first inhibiting the enzyme at low pH
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15 (6.0) and then tracking recovery of activity at high pH (8.0).⁷¹ At pH 6.0, more than 2 units
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18 below the pK_a of alkyne-imidazolium-oxime and alkyne-pyridinium-oxime, we were able
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21 to inhibit the BChE active site of the BChE-PDMAA-OX conjugates without reactivation
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24 or interference by the polymer oxime side chains. BChE-PDMAA-OX conjugates and
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27 native BChE lost all activity after being mixed with a 10-fold molar excess of Flu-OPNAs
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30 at pH 6.0 within 10 min. The EMP-BChE-PDMAA-OX or CMP-BChE-PDMAA-OX
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33 conjugates were purified by removing excess Flu-OPNAs by ultrafiltration. Fluorescence
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36 intensity analysis showed that more than 99% of free Flu-OPNAs were removed after
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39 three cycles of ultrafiltration (**Figure S15**). The EMP-BChE-PDMAA-OX or CMP-BChE-
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42 PDMAA-OX conjugates were then diluted 50-fold in PB buffer at pH 8.0 and reactivation
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45 was measured using the Ellman assay with BTC as the substrate at pH 8.0 (**Figure 5**).
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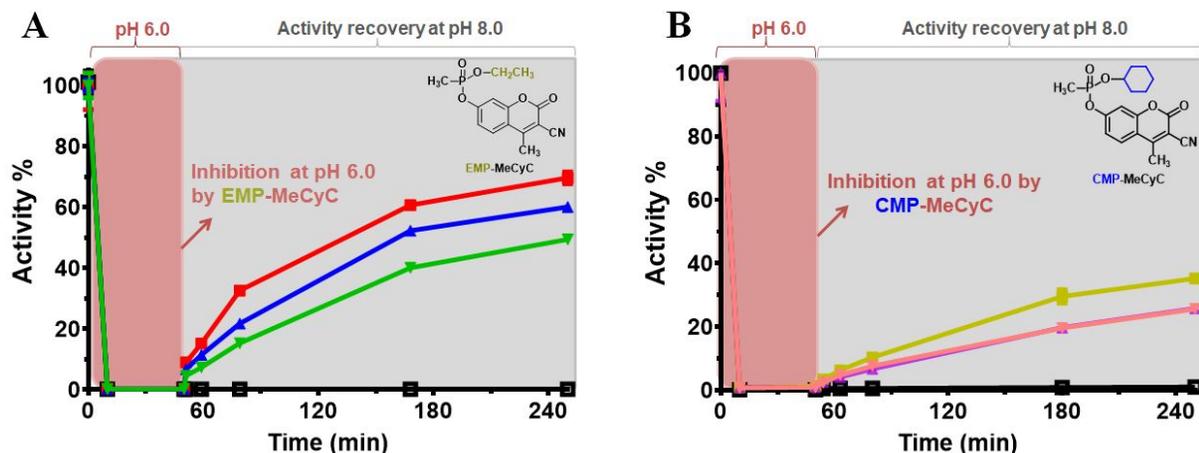


Figure 5. Reactivation assay of BChE-PDMAA-IO conjugates inhibited by EMP-MeCyC (A) and BChE-PDMAA-PO conjugates inhibited by CMP-MeCyC (B). 1 μ M BChE-PDMAA-IO, BChE-PDMAA-PO or free BChE were inhibited by 10-fold excess of Flu-OPNAs in PB buffer at pH 6.0 for 10 min, remove the excess of Flu-OPNAs, then diluted 50-fold in PB buffer at pH 8.0. Black curve: BChE; Green curve: BChE-PDMAA-IO₂₈; Blue curve: BChE-PDMAA-IO₈₄; Red curve: BChE-PDMAA-IO₁₇₂; Pink curve: BChE-PDMAA-PO₄₄; Purple curve: BChE-PDMAA-PO₁₀₄; Yellow curve: BChE-PDMAA-PO₂₁₆. Results are presented as mean values \pm standard deviation ($n = 3$).

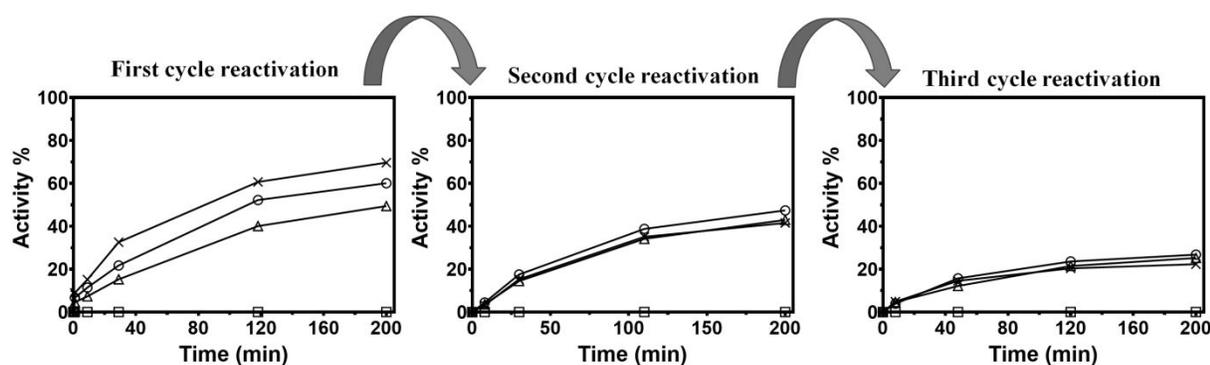
The degree of reactivation was determined relative to the respective initial activity of native BChE or a given BChE-PDMAA-OX conjugate in the assay. After complete inactivation by EMP-MeCyC, the combination of intra- and intermolecular reactivation was proportional to the oxime content of the polymer for the BChE-PDMAA-IO conjugates (Figure 5A). BChE-PDMAA-PO conjugates also recovered significant activity, but with a less pronounced dependence on oxime content (Figure 5B). Prolonging the reactivation

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4 time to 24 hours at pH 8.0, for the highest oxime content conjugates, resulted in dramatic
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7 levels of reactivation (84% for BChE-PDMAA-IO and 55% for BChE-PDMAA-PO) (**Figure**
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10 **S16**).

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14 We next challenged the performance of imidazolium-oxime containing BChE-PDMAA-IO
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17 conjugates as catalytic bioscavengers against VX using the fluorogenic analogue EMP-
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21 MeCyC. The enzyme-polymer conjugates were inactivated with a 10-fold molar excess
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25 of EMP-MeCyC in PB buffer at pH 6.0, then, after ultrafiltration, we followed the
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29 reactivation at pH 8.0 for 200 min. At the end of the reactivation cycle we re-adjusted the
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33 pH to 6.0 and again challenged the conjugates with another 10-fold molar excess of EMP-
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37 MeCyC. The entire process was repeated three times to determine whether the
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We next challenged the performance of imidazolium-oxime containing BChE-PDMAA-IO conjugates as catalytic bioscavengers against VX using the fluorogenic analogue EMP-MeCyC. The enzyme-polymer conjugates were inactivated with a 10-fold molar excess of EMP-MeCyC in PB buffer at pH 6.0, then, after ultrafiltration, we followed the reactivation at pH 8.0 for 200 min. At the end of the reactivation cycle we re-adjusted the pH to 6.0 and again challenged the conjugates with another 10-fold molar excess of EMP-MeCyC. The entire process was repeated three times to determine whether the conjugates could self-reactivate after multiple challenges. In the second and third reactivation cycles, significant activity was recovered for all of the conjugates. We did observe a gradual decrease in the maximal reactivation levels at each consecutive cycle for BChE-PDMAA-IO₁₇₂ conjugate, from 70% to 45% and 20% maximal activity recovery during the first, second and third inhibition/reactivation cycle, respectively (**Figure 6**). This

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3 gradual decline in maximal reactivation level may stem from slow aging of inhibited
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7 conjugate active site. The distance of each oxime from the active site of the enzyme would
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10 likely influence the ability of a particular oxime to reactivate BChE.⁸⁰ Location-specific
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13 reactivation, and how it couples with cholinesterase aging, is beyond the scope of this
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17 manuscript but will be an important area for future study. The significant reactivation
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20 levels with inhibited conjugate after three inhibition/reactivation cycles were consistent,
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23 however, with catalytic reactivation of OPNA-inhibited BChE by matrix-conjugated oximes.
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28 In totality, these data demonstrated that covalent modification with imidazolium-oxime
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31 containing copolymers on the surface of BChE could catalytically decontaminate VX,
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35 even when the number of oximes per BChE tetramer molecule was as low as 28.
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51 **Figure 6.** Inactivation and reactivation cycles of EMP-MeCyC-inhibited BChE-PDMAA-IO
52 conjugates. Fork: BChE-PDMAA-IO₁₇₂; Circle: BChE-PDMAA-IO₈₄; Triangle: BChE-
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3 PDMAA-IO₂₈; Square: BChE. Results are presented as mean values ± standard deviation
4 (n = 3).
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11 CONCLUSIONS

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15 BChE-PDMAA-OX conjugates, synthesized by *in situ* ATRP and click chemistry with
16 imidazolium-oxime and pyridinium-oxime side chains, were remarkable self-reactivators
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19 after repeated challenges with organophosphate inhibitors. The catalytic activity of these
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23 functional BChE-PDMAA-OX conjugates was dependent on the number of oximes per
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27 BChE molecule. The conjugates exhibited both inter- and intra-molecular protection
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33 against fluorescent analogues of VX and cyclosarin and functional enzyme activity in the
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36 presence of these OPs was prolonged by the conjugated polymer-oximes. After complete
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40 inactivation by EMP-MeCyC and CMP-MeCyC at a low pH (6.0), the conjugates
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44 recovered up to 84% and 55% activity after self-activation pH (8.0) within 5-6 hours.
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47 Imidazolium-oxime containing conjugates achieved three cycles of inhibition and auto-
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51 reactivation, demonstrating high-performance decontamination bio-scavenging.
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4 ASSOCIATED CONTENT
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8 Supporting Information for this article can be found online.
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11 Complete dataset, working curves, synthesis schemes, UV-VIS absorbance spectrum,
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13 additional GPC data, Fluorescence assay, activity assay, inhibition assay and
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5
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9

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20 21 **Conflict of Interest** 22

23
24 AR and KM have founded a company that is commercializing protein-ATRP.
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34
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49 human BChE isolated from human plasma.
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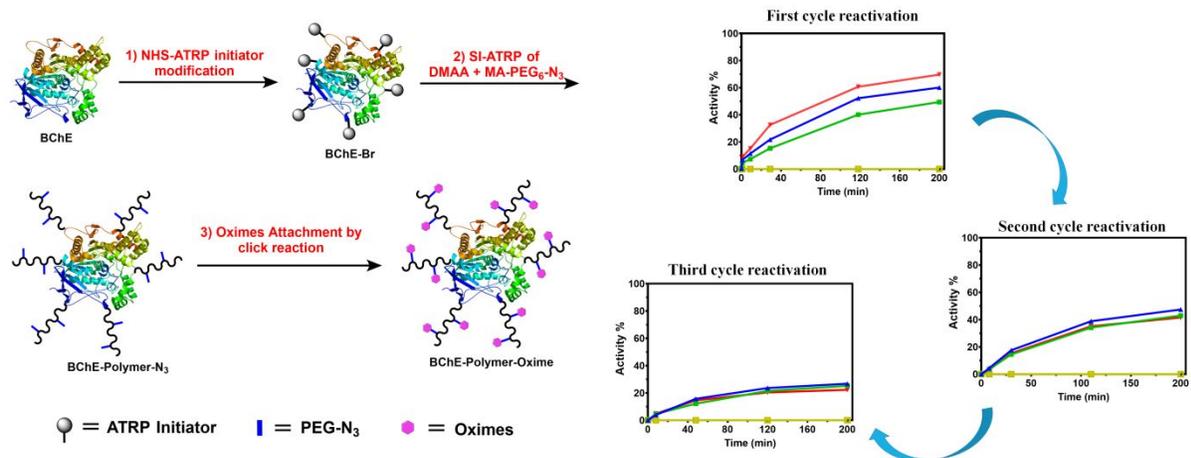
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TOC:



Butyrylcholinesterase (BChE)-polymer-oxime conjugates were designed and synthesized by in-situ growth of azide containing copolymers and azide-alkyne “click” chemistry.

BChE-polymer-oxime conjugates self-reactivate at up to 85%.