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# Decontamination of chemical and biological warfare agents with a single multi-functional material

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ABSTRACT

We report the synthesis of new polymers based on a dimethylacrylamide-methacrylate (DMAA-MA) co-polymer backbone that support both chemical and biological agent decontamination. Polyurethanes containing the redox enzymes glucose oxidase and horseradish peroxidase can convert halide ions into active halogens and exert striking bactericidal activity against gram positive and gram negative bacteria. New materials combining those biopolymers with a family of *N*-alkyl 4-pyridinium aldoxime (4-PAM) halide-acrylate co-polymers offer both nucleophilic activity for the detoxification of organophosphorus nerve agents and internal sources of halide ions for generation of biocidal activity. Generation of free bromine and iodine was observed in the combined material resulting in bactericidal activity of the enzymatically formed free halogens that caused complete kill of *E. coli* (>6 log units reduction) within 1 h at 37 °C. Detoxification of diisopropylfluorophosphate (DFP) by the polyDMAA MA-4-PAM iodide component was dose-dependent reaching 85% within 30 min. A subset of 4-PAM-halide co-polymers was designed to serve as a controlled release reservoir for *N*-hydroxyethyl 4-PAM (HE 4-PAM) molecules that reactivate nerve agent-inhibited acetylcholinesterase (AChE). Release rates for HE 4-PAM were consistent with hydrolysis of the HE 4-PAM from the polymer backbone. The HE 4-PAM that was released from the polymer reactivated DFP-inhibited AChE at a similar rate to the oxime antidote 4-PAM.



## 1. Introduction

Weapons of mass destruction pose almost unimaginable threats to our society. In response to these threats, scientists have long searched for environmentally benign approaches to decontamination of both biological and chemical agents. Since terrorists are unlikely to announce what type of agent has been deployed, the ideal approach is the development of broad spectrum decontaminants that are simple to use, active against both chemical and biological agents, and do not destroy the environment into which they are deployed. This has proven to be a vexing problem. For almost two decades we have been developing materials that can detect and decontaminate chemical and biological agents [1–6], but we have recently sought to bring together multiple approaches into one material. Such a material would rapidly destroy biological and nerve agents upon hydration. A schematic of how such a multi-functional biomaterial could generate biocidal halogens while releasing nerve agent detoxifying agents that could be used for decontamination or therapy is shown in Scheme 1. The minimum ingredients necessary to activate broad spectrum chem–bio defense are the multi-functional polymer–enzyme biomaterial [7], glucose and water. The ready availability of glucose and water in blood and bodily fluids would allow the biomaterial, as described herein, to be used internally or as a wound dressing. In the case of a wound dressing, the material would have the capacity to kill bacteria, viruses and even spores. In addition, the multi-functional material could also release oximes in a controlled way to combat the effects of cholinesterase inhibitors.

The ability of halogens to kill a wide variety of microorganisms including antibiotic-resistant bacteria, viruses, and fungi has been known for centuries [8,9]. For instance, multiple isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), a major nosocomial pathogen, were susceptible to povidone iodine within 30 min at a minimal biocidal concentration of 512 ppm [10]. Polyquaternary ammonium compounds spear-headed by positively charged quaternary ammonium units also exhibit remarkably broad bactericidal activity both in solution and when delivered

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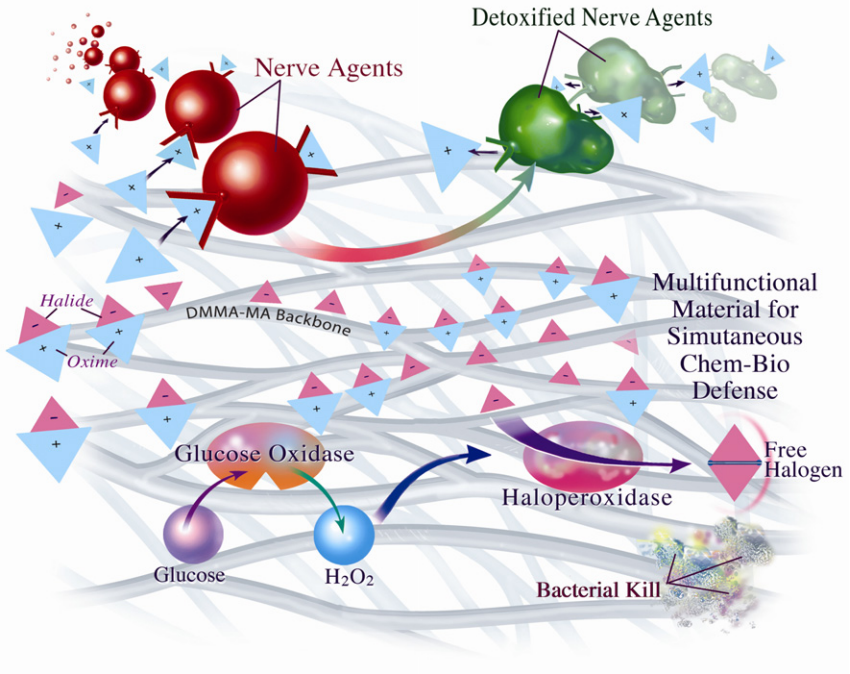



**Scheme 1.** Schematic Depiction of a multi-functional polymer that can detoxify chemical and biological warfare agents.

from a surface [5,11]. Interestingly, quaternary pyridinium aldoximes (e.g. 2-PAM, 4-PAM, and toxogonin) are efficient reactivators of cholinesterases (ChE) inhibited by organophosphate (OP) toxins and are currently used as antidotes against OP pesticides and nerve agent poisoning [12–14]. Further, the direct interaction of pyridinium aldoximes with toxic OPs results in slow detoxification of the OP agent [15]. This slow detoxification activity was enhanced in polymers carrying nano-magnetoparticles complexed with oxime groups which displayed catalytic degradation of the nerve agent analog diisopropylfluorophosphate (DFP) [16,17]. The addition of positively charged groups was shown to further enhance the reaction rates of pyridinium oximes against toxic OPs [18] through a "charge effect" that likely increased the nucleophilic activity of the oxime group and resulted in reaction rates higher than calculated from Bronsted's law [19]. It follows that PAM-halide polymers could provide multiple, positively charged pyridinium groups thereby enhancing nucleophilic activity toward OPs as in the upper path of Scheme 1. Further, the negatively charged halide counter ions ($I^-$ and $Br^-$) of the pyridinium aldoxime moiety could serve as the substrate for in-situ redox enzyme-catalyzed generation of biocidal halogens [6,9] as depicted in the lower path of Scheme 1.

We have previously shown that when glucose oxidase (GOX) and horseradish peroxidase (HRP) were electrospun into a non-reactive polyurethane fiber mesh, the generation of free iodine by the tandem redox reactions in the presence of sodium iodide and glucose killed gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria [6]. Modifying that matrix to include a polymer containing multiple positively charged aromatic nucleophiles (such as 4-PAM) with halides as counter ions could provide both a substrate for enzymatically-generated free halogen and a detoxification activity toward nerve agents. In this combined polymer matrix both positively charged nucleophiles and their negatively charged halide counter ions would serve as complementary components in a multi-functional material for decontamination of microorganisms and toxic chemicals. Further, the nerve agent detoxification component of the composite could also be a delivery vehicle for PAM-based therapeutics by conjugating the 4-PAM groups to the polymer backbone via hydrolytically metastable ester bonds. Hydrolysis of the ester bond would release 4-PAM derivatives which could then reactivate OP-inhibited AChE. We describe herein how such materials are true broad spectrum decontaminants both on surfaces and in liquid environments.

## 2. Materials and methods

### 2.1. Materials

Horseradish peroxidase (HRP) (1500 U/mg) and *Aspergillus niger* glucose oxidase (GOX) (100 U/mg), butyrylcholinesterase (BChE) (horse serum, 1690 U/mg), acetylcholinesterase AChE (electric eel 250 U/mg), iodine, bromine, sodium iodide, sodium bromide, partially saponified (87%) polyvinyl alcohol (PVA), hexafluoroisopropanol (HFIP) and glucose were purchased from Sigma St Louis MO. Medical grade polyurethane ChronoFlex AR is a product of AdvanSource Biomaterials Corp, Wilmington, MA.

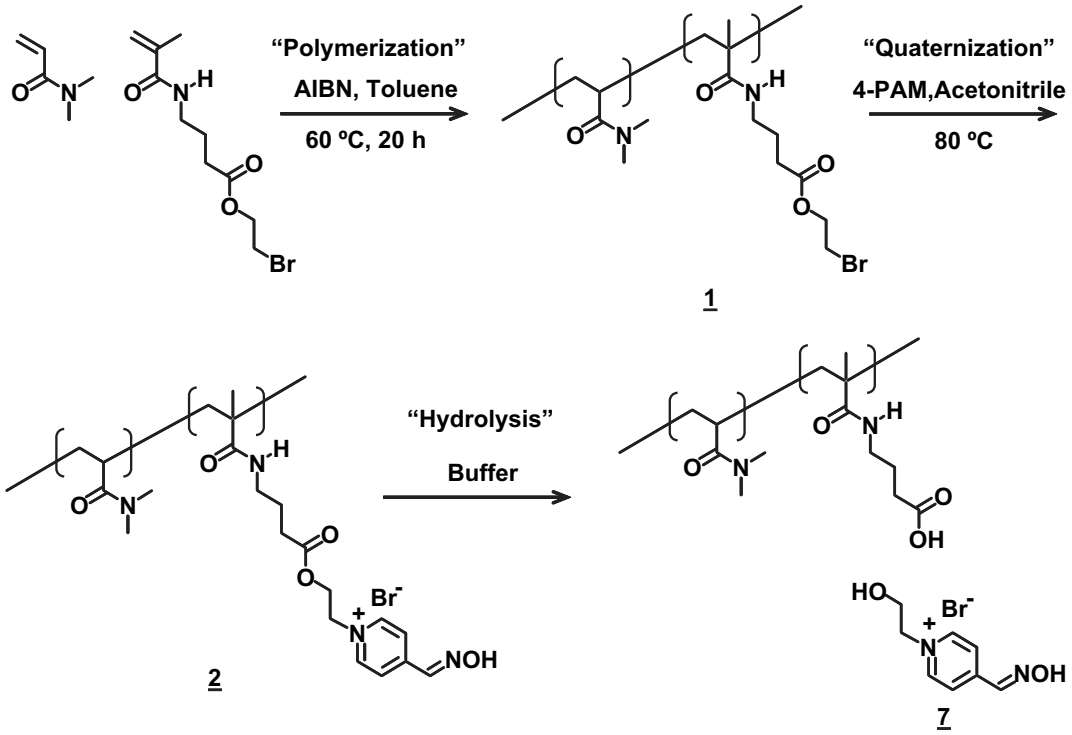
γ-Aminobutyric acid, *p*-toluenesulfonic acid monohydrate (TosOH), methacryloyl chloride, 2-bromoethanol, *N,N,N*-triethylamine (TEA), *N,N*-dimethylacrylamide (DMAA), 2,2′-Azobis(2-methylpropionitrile) (AIBN), 4-pyridine aldoxime (4-PAM), 3-iodopropanol, 4-hydroxybenzophenone, pyridine, *N,N*-dimethylethylamine (DMEA), 3-bromopropanol, toluene, diethyl ether, 2-propanol, ethanol, dichloromethane ($CH_2Cl_2$) and acetonitrile were purchased from Sigma–Aldrich Chemical Co (St Louis MO).

### 2.2. Synthesis of multi-functional decontamination polymers

Polymers were prepared from a dimethylacrylamide (DMAA) – methacrylate (MA) co-polymer backbone that contained repeating quaternary 4-pyridinium aldoxime (4-PAM) covalently attached via halo-propionyl side chains. Detailed synthetic data are provided in supplementary materials.

Scheme 2 describes the strategy for synthesis of DMAA MA-3-Propionyl-ethyl 4-PAM bromide (polymer **2**). The precursor polymer DMAA-MA-3-propionyl ethyl bromide **1** was synthesized by radical polymerization from the corresponding monomers. Following the co-polymerization of DMAA with MA-propionyl bromide ester monomers, the bromo-ethyl-propionyl side chains of polymer **1** were reacted with 4-pyridine aldoxime to form the quaternary 4-PAM polymer **2**. The quaternary polymer product **2** contains *N*-hydroxyethyl 4-pyridinium aldoxime units (**7**, Scheme 2) tethered to the polymer backbone through ester bonds. The ester bonds connecting **7**, (*N*-HE-4-PAM, Scheme 2) to the polymer backbone are prone to spontaneous or enzyme-induced hydrolysis rendering polymer 2 a macromolecular carrier for sustained drug delivery (see release of **7** in Scheme 2).

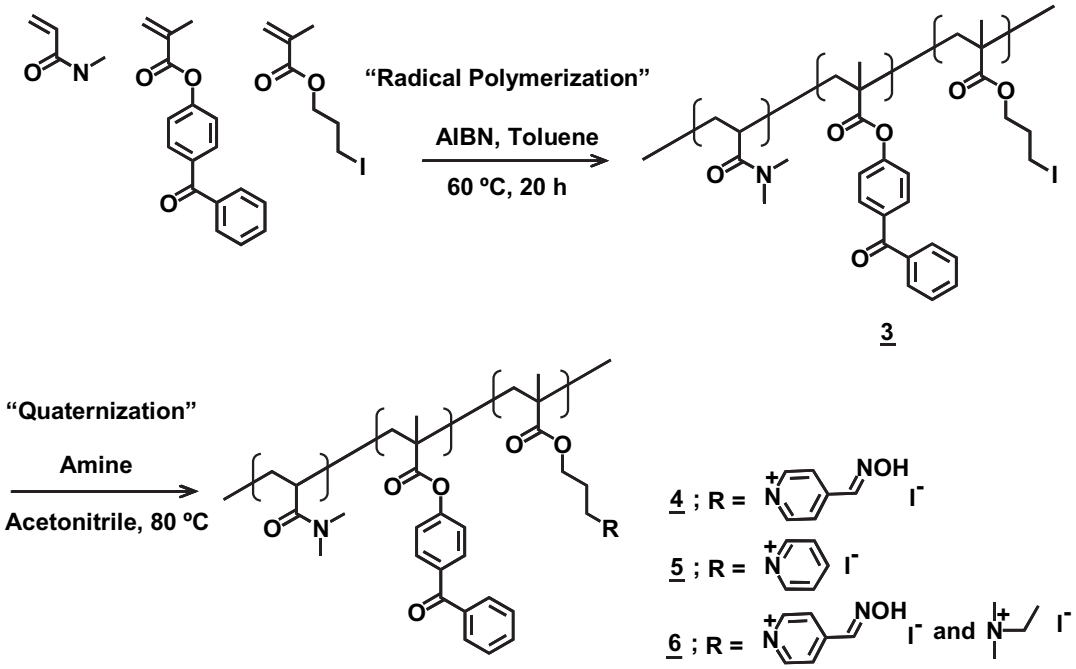
Scheme 3 describes the synthetic pathway to four co-polymer structures. First, a non-quaternary co-polymer backbone containing propyl iodide side chains and benzophenone DMAA MA-propyl iodide – MA-propyl 4-pyridinium aldoxime MA-




**Scheme 2.** Synthesis of non-quaternary co-polymer **1** DMAA MA propionyl *N*-hydroxyethyl and the quaternary polymer DMAA MA propionyl *N*-hydroxyethyl 4-PAM bromide **2**. The release of *N*-hydroxyethyl 4-PAM bromide **7** from polymer **2** by hydrolysis of the ester bond is described in the final step.

benzophenone **3** was prepared by radical polymerization. Subsequently, the 3-iodopropyl side chains on the polymer were reacted with 4-pyridine aldoxime to form the 4-PAM quaternary polymer **4**. The introduction of benzophenone (at 3.2 mol%) into the polymer chains of **3** and **4** was performed in order to obtain photo-activated cross-linking of the soluble polymer with polyurethane after electrospinning. This synthetic approach yielded polymer with 78.2% of DMAA, 13.7% of 4-PAM iodide, 1.5% of propyl iodide (that did not react with 4-pyridine aldoxime), and 3.2% benzophenone subunits as evaluated by $^{1}$H NMR analysis (see Supplementary material Scheme S1).

Scheme 3 also describes the synthesis of a polyquaternary amine consisting of the positively-charged *N*-hydroxypropyl-pyridinium moiety **5**. Since the pyridinium moiety in this polymer lacks any active nucleophile, this polymer was primarily prepared as a reference polymer to distinguish the role of the nucleophilic oxime group in the 4-PAM co-polymer for scavenging OP compounds (e.g. DFP).




**Scheme 3.** Synthesis of quaternary co-polymers: poly-DMAA MA-propyl iodide MA-benzophenone (BP) **3**, poly-DMAA MA-propyl iodide MA *N*-hydroxypropyl 4-PAM MA-benzophenone **4**, poly-DMAA MA-propyl iodide MA *N*-hydroxypropyl MA-pyridinium iodide MA-benzophenone **5**, and DMAA MA-*N*-ethyl *N*, *N*-dimethylammoinum propyl iodide MA *N*-hydroxypropyl 4-PAM MA-benzophenone **6**.

In order to increase the density of positively charged quaternary nitrogen (and that of the associated halide counter ion) polymer **6**, poly-DMAA MA-propyl 4-PAM iodide MA-propyl-dimethyl-ethyl ammonium iodide MA-benzophenone, was prepared (Scheme 3). $^1$H NMR analysis (Supplementary information) determined that polymer **6** contained 15.4% w/w iodide.

### 2.3. Measurement

$^1$H NMR spectra were recorded on a Bruker Avance (300 MHz) spectrometer in DMSO-$d_6$ and $CDCl_3$. Routine FT-IR spectra were obtained with ATI Mattson Infinity series FTIR spectrometer. Melting points (mp) were measured with a Laboratory Devices Mel-Temp. Number average molecular weights ($M_n$) and the distributions ($M_w/M_n$) were estimated by gel permeation chromatography (GPC) on a Waters 600E Series with a data processor, equipped with three polystyrene columns (Waters styragel HR1, HR2 and HR4), using DMF with LiBr (50 mM) as an eluent at a flow rate 1.0 mL/min, polymethylmethacrylate calibration, and a refractive index (RI) detector.

### 2.4. Determination of polymer $pK_a$

The $pK_a$'s were determined by a spectrophotometric method. At each specified pH the ratio of absorbance of oximate anion (at 345 nm) to that of oxime group (at 280 nm) was measured. The $OD_{345}/OD_{280}$ ratio equals the $[Ox^-]/[OxH]$ ratio at a given pH. The pH values were plotted against $\log[Ox^-/OxH]$ and the $pK_a$ was calculated from the intercept of the linear plot with the ordinate using the Henderson–Hasselbalch equation Eq. (1):

$$pH = pK_a + \log[Ox^-]/[OxH] \quad (1)$$

The UV/visible spectra of 4-PAM bromide polymers were scanned at various pH's (ten solutions with pH's ranging between 7.4 and 10.3 in 50 mM Tris–HCl buffer using 0.05 mg/mL polymer concentration).

### 2.5. Release of N-hydroxyethyl-4-pyridinium aldoxime from polymer 2

Release of *N*-hydroxyethyl 4-pyridinium aldoxime bromide from polymer **2** was measured at 37 °C in phosphate buffer at pH 6.2, 7.4 and 8.2. 30 mg of polymer **2** was dissolved in 3 mL of deionized water. 500 μL of the polymer solution was injected into dialysis cassettes with a molecular weight cutoff of 2000 (ThermoScientific, Rockford, IL). The dialysis cassette was placed in a beaker containing 75 mL of buffer and shaken at 37 °C. Absorption of the dialysates at 280 and 340 nm were recorded over time using a Lamba 2 spectrometer (Perkin Elmer).

### 2.6. Enzymatic free iodine formation

The rate of free iodine formation, catalyzed by enzymatic oxidation, was measured by the addition of the enzymes GOX and HRP (10 μL of 1 mg/mL, each), NaI solution (20 μL, 0.04 M), 0.1 mL polyvinyl alcohol (PVA, 1% w/v in water), and 0.1 mL of 50 mM glucose into 50 mM phosphate buffer pH 6.2. The increase in absorbance at 490 nm arising from PVA-$I_2$ complex formation was measured continuously [6]. The rate of increase in the concentration of iodine was also measured by replacing the enzyme solutions with solid samples (1–30 mg) of electrospun fiber in which the enzymes GOX and HRP were entrapped in the polyurethane ChronoFlex AR.

### 2.7. Electrospinning of polyurethane fibers with and without enzymes

ChronoFlex AR (CF) and the polymers **1**, **2**, (Scheme 2) and **4** (Scheme 3) were dissolved in hexafluoroisopropanol (HFIP) at 5% w/v to form the materials CF-**1**, CF-**2**, and CF-**4**. Electrospining of CF-co-polymers fibers was performed by streaming an HFIP solution of the mixed polymers from a 1 mL syringe using a syringe pump (Aladdin Programmable Syringe Pump – AL1000) at a rate of 1 mL/h. The polymer solution then entered a stainless steel capillary (20 gauge, 2 cm long). Fibers were collected on aluminium foil at a 30 kV voltage drop (+18 kV at the capillary and −12 kV at the collection plate) for 30 min. When GOX and HRP were incorporated into the electrospinning process the resultant materials were CF-**1**-GOX-HRP, CF-**2**-GOX-HRP, and CF-**4**-GOX-HRP. The enzymes were dissolved at 10 mg/mL in phosphate buffer pH 6.2 and placed in a 1 mL syringe and driven at a rate of 0.33 mL/h in parallel to the polymer solution flowing at 1 mL/h. The tubing from the syringes was connected at a Y-junction immediately ahead of the metal capillary. The end of the capillary was 20 cm above the collection plate.

### 2.8. Scanning electron microscopy (SEM)

Small samples of the fiber mats were cut and sputter coated with gold particles. Samples were analyzed in a JSM6330F scanning electron microscope at magnifications varying from 300×–1500×. The entire surface was scanned to view the uniformity of fibers.

### 2.9. Bactericidal activity of fibers

Electrospun fiber materials were immersed in 2 mL sterile phosphate buffer (pH 6.2). Depending on the experimental conditions, solutions contained sodium iodide (0.8 mM) and/or glucose (5 mM). For fibers electrospun without enzymes, GOX and HRP were added to the buffer solution (0.01 mg/mL final concentration). *E. coli* or *S. aureus* was added to the reaction mixture to a final concentration of $3–7 \times 10^7$ or $2–5 \times 10^5$ cells/ml, respectively. Reaction mixtures were shaken for either 0.5 h or 1 h at 37 °C. The reaction was stopped by serial dilution into 0.3 mM phosphate buffer (pH 7.2) followed by seeding on nutrient agar plates and overnight incubation at 37 °C to determine the number of surviving cells.

### 2.10. Detoxification of DFP by polymers

Polymers, either soluble or incorporated in polyurethane fibers by electrospinning, were incubated in the presence of DFP (5 μM) in phosphate buffer (pH 7.5). DFP detoxification by polymer **4** was also tested in HEPES buffer (pH 8.1 or 9.0). Samples of the hydrolyzed DFP (20 μL) were added to horse serum BChE (2 U/ml, 230 μL) for 3 min at 22 °C. The inhibited BChE was sampled (30 μL) into a 1 mL cuvette containing Ellman's reagent DTNB and acetylthiocholine (ATC, 0.3 mM). Residual BChE activity was determined by measuring the change in $OD_{412}$ [20]. The rate of detoxification of DFP was calculated from the temporal increase in BChE activity caused by degradation of DFP by the 4-PAM polymer.

### 2.11. Reactivation of AChE

Electric eel AChE (0.5 mg/mL in 50 mM phosphate, 0.1% BSA, pH 7.5) was diluted in phosphate/BSA buffer to provide appropriate starting activity. AChE (20-fold dilution of AChE stock) was reacted with 10 μM DFP for 1 h at 22 °C, and further diluted 50 fold into a cuvette containing a mixture of ATC and DTNB in phosphate buffer (pH 7.50) [20]. The degree of AChE inhibition by DFP prior to reactivation was between 97 and 98%. The DFP-inhibited AChE (diisopropyl phosphoryl–AChE) was diluted 1:50 and incubated for specified time intervals with *N*-hydroxyethyl 4-pyridinium aldoxime bromide (HE 4-PAM, **7**, Scheme 2) or 4-PAM iodide at 0.4 mM. The diisopropyl phosphoryl–AChE conjugate was also incubated in phosphate buffer (pH 7.5) without oxime to determine the spontaneous reactivation rate. Aliquots of diisopropyl phosphoryl–AChE from the oxime solution or phosphate buffer were taken at various time intervals and AChE activity was determined [20].

## 3. Results and discussion

### 3.1. Synthesis of multi-functional polymers

Our initial hypothesis was that a polymer containing multiple quaternary oxime groups would have significantly higher rates of OP nerve agent detoxification than the cognate oxime monomer. We therefore designed a poly-oxime that included quaternization of the pyridine nitrogen of the oxime to the cationic form of the molecule to take advantage of the enhanced OP reaction rate that results from an increase in nucleophilic activity of aromatic nucleophiles when exposed to high densities of positive charges [18]. The polyquaternary amine nature of the polymer (Scheme 2, polymer **2**) suggested that, in addition to its activity against OP agents, it would also have antimicrobial activity [11].

Another aspect of the polymer design was that the pendant 4-PAM units were connected to the polymer chain through ester linkages. Hydrolysis of the ester bonds was designed to release *N*-hydroxyethyl 4-pyridinium aldoxime (Scheme 2, compound **7**) that could act as a reactivator of OP-inhibited acetylcholinesterase (AChE). In addition, the halide counter ions ($Br^-$ or $I^-$) of the quaternary amine groups were seen as "built-in" substrates for enzymatic redox conversion to active free halogens which are highly effective antimicrobials [6]. Because **2** was water soluble, derivatives were synthesized with pendant benzophenone groups (Scheme 3 polymers **3–6**) which could be used to "immobilize" the water soluble polymers to electrospun polyurethane fibers via photo-activation [21]. Additionally, a polymer with an increased concentration of quaternary amine groups and consequently with increased halide content (Scheme 3, polymer **6**) was prepared to enhance the halogen production. NMR analysis was used to confirm the composition of the polymers. The synthetic schemes, NMR

assignments, and NMR traces (Fig. S4) are presented in the supplementary material.

### 3.2. Determination of pKa of 4-PAM-containing polymers

The nucleophilic activity of 4-PAM is derived from the presence of an oxime group on the pyrdinium ring [12–14]. The oxime group R–CH = N–OH is in equilibrium with its negatively charged oximate anion, with their relative concentrations dependent on the oxime $pK_a$ and the pH of the solution. During detoxification of OP compounds, or reactivation of OP-inhibited AChE, the oximate anion is the active species that attacks the electrophilic phosphorus atom of toxic OPs such as diisopropylfluorophosphate (DFP) [13,14]. Since, an important factor that determines the nucleophilic potency of oximes in their reaction with various OPs is the $pK_a$, it was important to determine the $pK_a$ values of the newly synthesized 4-PAM polymers and compare them to the $pK_a$'s of their respective small molecule 4-PAM precursors.

The $pK_a$ determined for the 4-PAM bromide polymer **2**, 8.45 ± 0.02, was similar to the $pK_a$ of 8.20 ± 0.02 measured for its corresponding small molecule *N*-hydroxyethyl 4-PAM. The $pK_a$ of 4-PAM iodide polymer **4** was 8.00 ± 0.02 and the $pK_a$ of the corresponding *N*-hydroxypropyl 4-PAM bromide and iodide monomers were 8.23 and 8.36 respectively. All linear regression analyses resulted in an excellent fit to the Henderson–Hasselbalch equation ($R^2$ from 0.93 to 0.99, not shown). The $OD_{280}$ of polymer **4** was corrected for benzophenone (BP) absorbance ($OD_{280} = 0.100$) based on the UV spectrum absorbance of the DMAA-BP polymer **3**, which had an equivalent content of BP (3 mol %) but had no pyridinium.

The $pK_a$ values measured for the PAM polymers demonstrate a higher nucleophilic activity of the oxime toward OP nerve agents at pH values that were between 8 and 8.5 where the oximate anion is close to 50 mol%. At this range of $pK_a$'s the Bronsted plot (that describes the dependence of bimolecular rate constant on $pK_a$) loses its linearity and the bimolecular rate constant reaches its maximal saturating values. This behavior has been previously reported for oximes [15].

### 3.3. Release of N-hydroxyethyl 4-pyridinium aldoxime, 7, from polymer 2

The *N*-hydroxyethyl 4-PAM moiety was attached to the backbone of polymer **2** by an ester bond between the hydroxyethyl spacer and the carboxyl group of amino propionate linked to methyl acrylic acid (MA) (Scheme 2). This ester bond was designed to be hydrolytically unstable and able to release *N*-hydroxyethyl 4-PAM (HE 4-PAM, **7**) into the surrounding media (Fig. 1). Samples of **2** were placed in dialysis cassettes and the dialysate was sampled over time. A mixture of HE 4-PAM bromide **7** with the non-quaternary polymer **1** served as a control for the rate of release and transport of **7** from the inner volume of the dialysis cassette. The concentration of **7** in the dialysates was determined spectrophotometrically based on the molar extinction coefficient of **7** ($\epsilon_{280} = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$). The absorbance values of the dialysate solution were translated into molar concentrations of **7** at each time interval. The rate constants for release at pH 6.2, 7.4 and 8.2, calculated by linear regression of the initial rates, were 0.088, 0.094 and 0.116 $h^{-1}$ respectively. The pH-dependent variation in leaching rate was consistent with the reduced stability of ester bonds at basic pH. The rate constant for the release of free monomeric 4-PAM was 2.5 fold more rapid at 0.24 $h^{-1}$ indicating that the sustained release of compound **7** from polymer **2** was due to the ester hydrolysis.

The weight % of **7** bound to polymer **2** was 20.8% according to the $^1H$ NMR spectrum (see supplementary material). Based on this

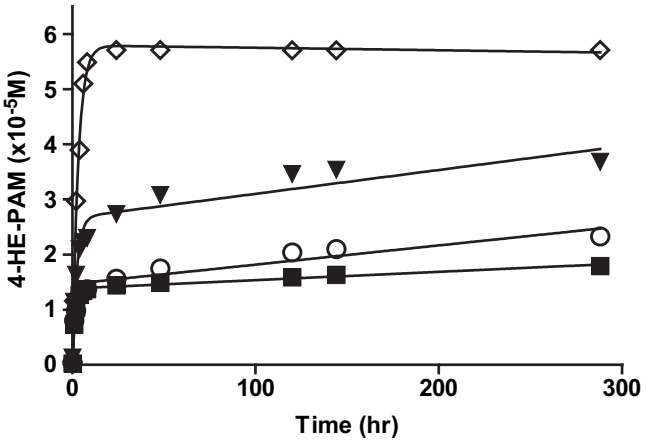



**Fig. 1.** 4-PAM release. **A**: Time course of *N*-hydroxyethyl 4-PAM (**7**) release from 4-PAM-bromide polymer **2** at different pH values. The rate of release of the polymer was compared to a mixture of **7** with polymer **1** (the non-quaternized precursor of polymer **2**). Empty diamonds: control (HE 4-PAM mixed with polymer 1 at pH 7.4), inverted full triangles: pH 8.2, empty circles: pH 7.4, filled squares pH 6.2. Total quantity of **7** is expressed as milligrams in 75 mL (bulk solution volume).

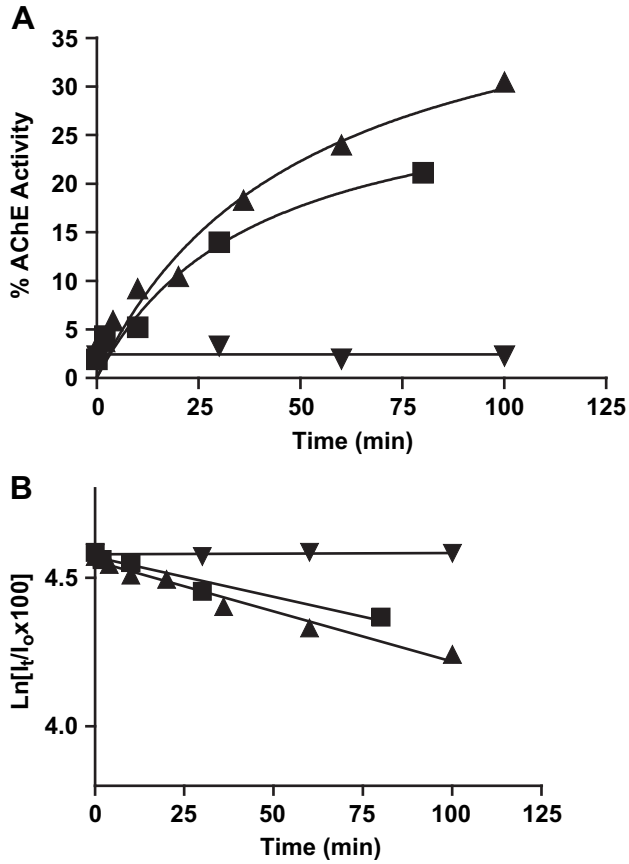



**Fig. 2.** Reactivation of AChE. **A**: Time-course of reactivation of DFP-inhibited AChE by HE 4-PAM (**7**) and 4-PAM iodide (0.4 mM oxime in 50 mM phosphate pH 7.4, 22 °C) Filled triangles: HE 4-PAM, filled squares: 4-PAM, filled inverted triangles: buffer. **B**: Rate of decrease in AChE inhibition during reactivation by oximes $I_o$ = AChE inhibition at $t = 0$. $I_t$ = AChE inhibition at time $t$ during reactivation (symbols are as described in Fig. 1A). Rate constants ($k_{obs} \times 10^{-3}\ min^{-1}$) for HE-4-PAM and 4-PAM were 3.4 and 2.7, respectively.

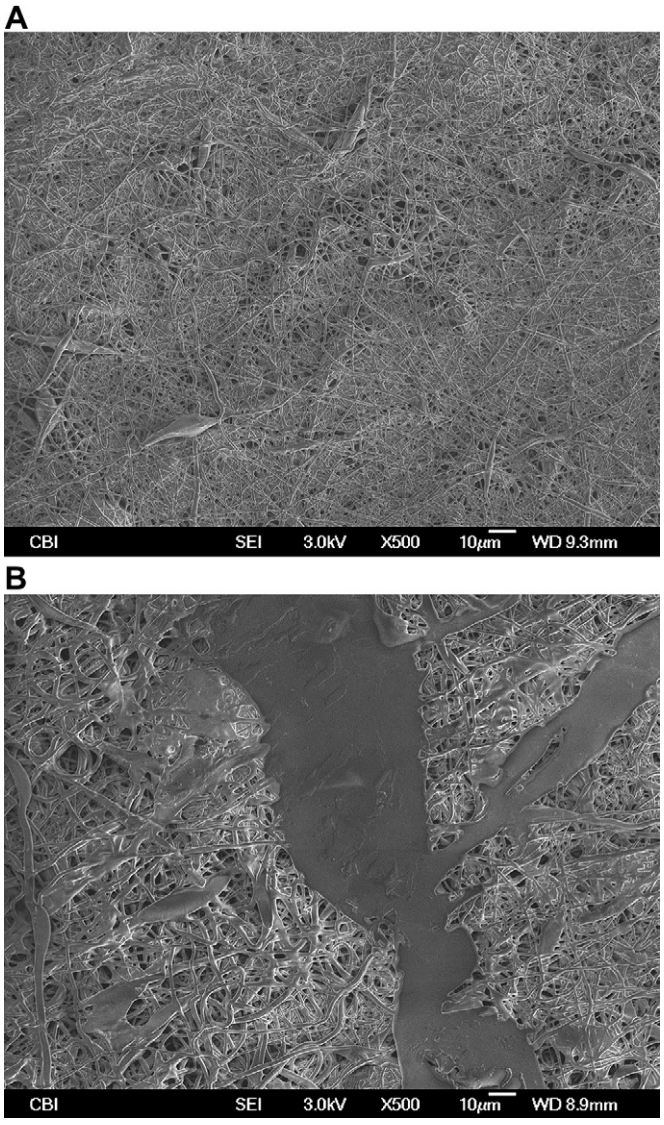



**Fig. 3.** SEM pictures of electrospun CF with the soluble polymer **4** before (A), and after (B), UV irradiation at 330–350 nm for 5 min.

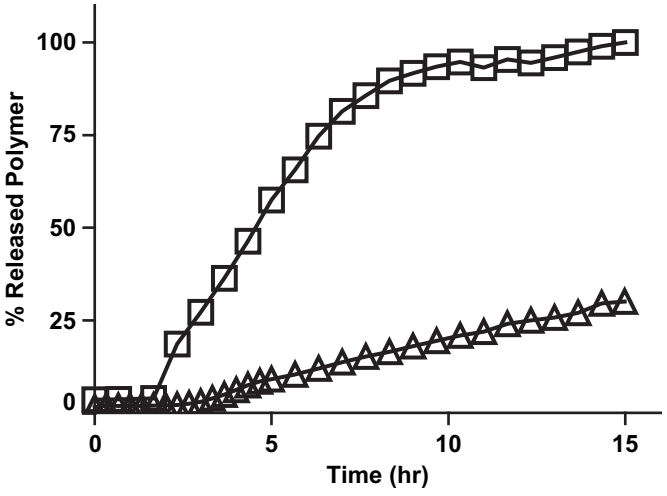



**Fig. 4.** Leaching of polymer from electrospun fibers. **A**: Time-course of polymer **4** leaching from CF-**4** fibers empty squares no UV, empty triangles UV irradiated fibers.

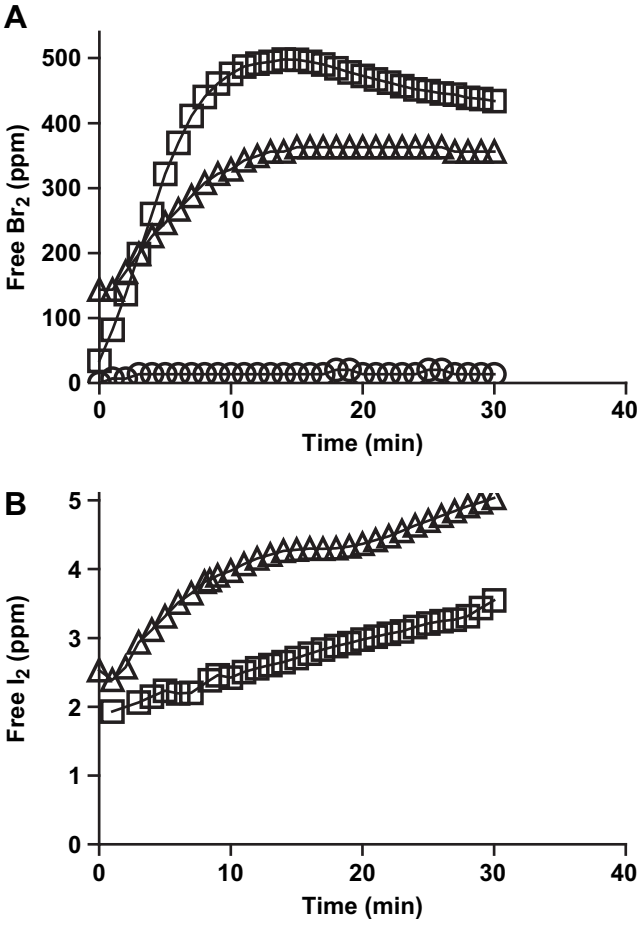



**Fig. 5.** Enzymatic generation of free bromine and iodine by electrospun polymers **2** and **4** with CF polyurethane. **A**: Time-course of free bromine generation by GOX, HRP and polymer **2** incorporated by electrospinning into ChronoFlex. Empty squares: 49.8 mg, empty triangles 20.7, open circles 18.5 mg without redox enzymes. **B**: Time-course of free iodine generation by GOX, HRP and polymer **4** incorporated by electrospinning into ChronoFlex. Empty triangles Non-UV treated fibers, empty squares: UV irradiated fiber matrix. UV irradiation was performed at 330–350 nm with 13 cm distance for 5 min.

weight % of **7** in polymer **2**, the maximum concentration of **7** that could be released from polymer **2** was $5.6 \times 10^{-5}$M. The peak concentration of **7** released to the external buffer (after 288 h at pH 8.2) was $4.6 \times 10^{-5}$M which corresponds to 82% of the theoretical value.

### 3.4. Reactivation of DFP-inhibited AChE by HE 4-PAM

Since the sustained release of **7** from polymer **2** was intended to provide a functional oxime reactivator of DFP-inhibited AChE, it was necessary to demonstrate that **7** was indeed an AChE reactivator. A sample of AChE was inhibited by DFP (10 μM) to create a stable diisopropyl phosphoryl–AChE conjugate with 97% inhibition of the initial enzymatic activity of AChE. The inhibited AChE was incubated with either **7** or 4-PAM iodide (0.4 mM each). The rates of reactivation of DFP-inhibited AChE by **7** and 4-PAM were calculated from the decrease of AChE relative inhibition with time (Fig. 2). The $k_{obs}$ for **7** and 4-PAM (0.4 mM) were $2.7 \times 10^{-3}$ and $3.4 \times 10^{-3}$ $min^{-1}$ respectively, and were similar to those previously reported for 4-PAM with DFP-inhibited AChE [14]. The reactivation levels obtained after 24 h were 70% of the initial AChE activity. The 30% activity loss was likely due to a known aging process that

**Table 1**
Bactericidal activity of 4-PAM polymers electrospun with polyurethane CF fibers against *E. coli*.

| Sample | Polymer in solution | Electrospun fibers[a] | Enzyme(s) in solution | Glucose (mM) | NaI (mM) | Number of Surviving Cells[b] |
|---|---|---|---|---|---|---|
| 1 | **1** | – | – | 5 | 0 | $4.4 \pm 1.4 \times 10^6$ |
| 2 | **1** | – | GOX | 5 | 0 | $3.8 \pm 0.1 \times 10^6$ |
| 3 | – | CF-**1**-GOX-HRP | – | 5 | 0 | $9.1 \pm 0.7 \times 10^6$ |
| 4 | – | CF-**2**-GOX-HRP | – | 5 | 0 | 0 |
| 5 | – | CF-**2**-GOX-HRP | – | 0 | 0 | $6.0 \pm 2.0 \times 10^6$ |
| 6 | – | CF-**4**-GOX-HRP | – | 5 | 0 | 0 |
| 7 | – | CF-**4**-GOX-HRP UV irradiated[c] | – | 5 | 0 | 0 |
| 8 | – | CF-**4** | GOX/HRP | 5 | 0 | 0 |
| 9 | – | CF-**4** UV irradiated[c] | GOX/HRP | 5 | 0 | 0 |
| 10 | – | CF-**6** | GOX/HRP | 0 | 0 | $2.0 \pm 0.1 \times 10^6$ |
| 11 | – | CF-**6** | GOX/HRP | 5 | 0 | 0 |
| 12 | – | – | GOX/HRP | 5 | 0.8 | 0 |

[a] Fiber weights were 12–15 mg.
[b] Bactericidal activity was measured after 60 min incubation in 2 mL phosphate buffer pH 6.2, at 37 °C containing $9.1 \pm 0.7 \times 10^6$ *E. coli* for samples 1–9 and $2.0 \pm 0.1 \times 10^6$ for samples 10–12.
[c] Fibers were UV irradiated at 330–360 nm for 20 min prior to antimicrobial testing.

produces a dealkylated, non-reactivatable diisopropyl phosphoryl-AChE conjugate [14]. Spontaneous reactivation of DFP-inhibited AChE in buffer was less than 2% after 24 h (Fig. 2A).

In light of the above results it was clear that **7** was indeed a reactivator of OP-inhibited AChE. While peak levels of hydrolytically released **7** were obtained after 288 h, it is anticipated that the rate of hydrolysis could be enhanced *in vivo* by the activity of endogenous esterases further increasing the therapeutic utility of polymer **2**.

### 3.5. Electrospinning of soluble polymers with polyurethane

Electrospinning of polymers offers a versatile method to produce non-woven mats composed of nanofibrous materials. Incorporation of enzymes into polymers through electrospinning provides desirable features for biocatalysis such as large surface areas for enzyme display, enhanced mass transfer rates of substrate to the enzyme active site, and an enzyme-polymer matrix suitable for multiple challenges [22]. Additionally, electrospinning water soluble materials with water insoluble materials can immobilize the soluble material within a stable matrix as a surface coating [23].

To test the ability of our polymers to act as surface decontaminating agents, the water soluble polymers **2** and **4** (5% w/v) were electrospun with the water insoluble medical grade polyurethane ChronoFlex AR (CF), generating the composite materials CF-**2** and CF-**4**. The electrospinning resulted in homogenous, elastomeric,

**Table 2**
Bactericidal activity of polymer **6** electrospun with CF polyurethane, HRP and GOX against *S. aureus*.

| Sample | Electrospun fibers[a] | Enzyme(s) in solution | Glucose (mM) | NaI (mM) | Number of Surviving Cells[b] |
|---|---|---|---|---|---|
| 1 | CF-GOX-HRP | – | 5 | 0 | $1.2 \pm 0.1 \times 10^4$ |
| 2 | CF | – | 5 | 0.8 | $2.3 \pm 1.4 \times 10^5$ |
| 3 | CF-**6** | – | 5 | 0 | $9.8 \times 10^3$ |
| 4 | CF-**6**-GOX/HRP | – | 5 | 0 | 0 |
| 5 | – | GOX/HRP | 5 | 0.8 | 0 |

[a] Fiber weights were 12–15 mg.
[b] Bactericidal activity was measured after 60 min incubation in 2 mL phosphate buffer pH 6.2, at 37 °C containing $2.5 \times 10^5$ *S. aureus*.

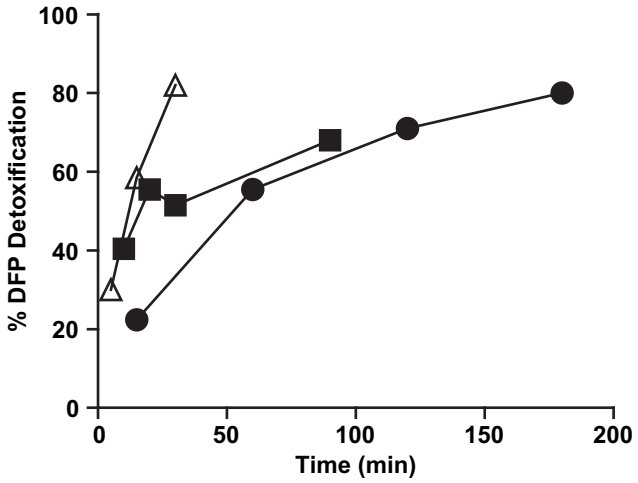



**Fig. 6.** Detoxification of DFP by polymer **2**. Time-course of DFP detoxification by polymer **2** dissolved in phosphate buffer (pH 7.5) at 5 (●), 8 (■), and 15 (△) mg/ml. Samples were taken at indicated times and assayed for DFP detoxification.

fiber mats. These electrospun fiber mats were assayed for DFP detoxification capacity, free iodine and bromine generation, and bactericidal activity.

Electrospinning does not create covalent linkages between non-reactive polymer chains such as polyurethanes and acrylates. This means that the water soluble acrylates are entrapped within the polyurethane and can still leach out from the matrix. To limit this leaching, four polymers were designed to contain a benzophenone moiety to allow UV-induced cross-linking of the soluble polymer to polyurethane (Scheme 3, [21]). Photo-activated cross-linking was performed using UV irradiation on polymer mat samples wetted with acetone. Fig. 3 shows the scanning electron microscopy (SEM) picture of electrospun CF-**4** fibers before UV irradiation (Fig. 3A) and after 5 min UV irradiation (Fig. 3B). The fibers of the electrospun polymer in Fig. 3A were spread evenly whereas gaps in the fiber morphology were observed following UV irradiation (Fig. 3B).

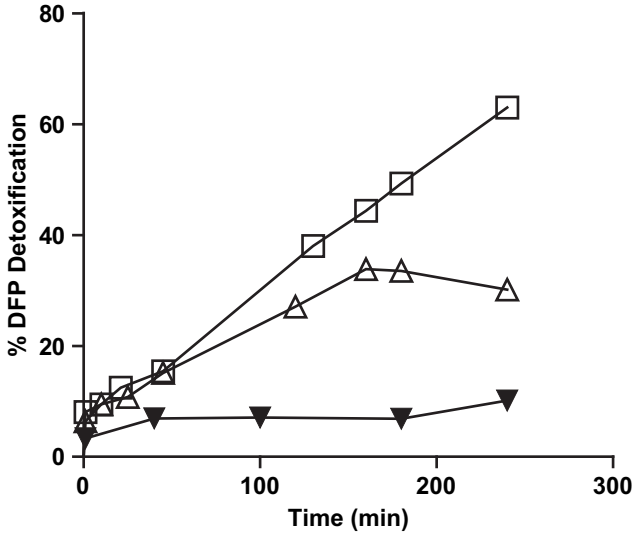



**Fig. 7.** Detoxification of DFP by polymer **4**. The rate of degradation of DFP ($5 \times 10^{-6}$ M) was measured for: 15 mg/mL solution of polymer **4** dissolved in phosphate buffer (pH 7.5); 15 mg/mL solution of polymer **4** dissolved in phosphate buffer with GOX. HRP (0.01 mg/mL each) and glucose (5 mM) added to the buffer solution (△); or phosphate buffer alone (▼). 20 μL aliquots were sampled at specified times and incubated with BChE for 3 min. BChE activity was measured by Ellman assay [20].

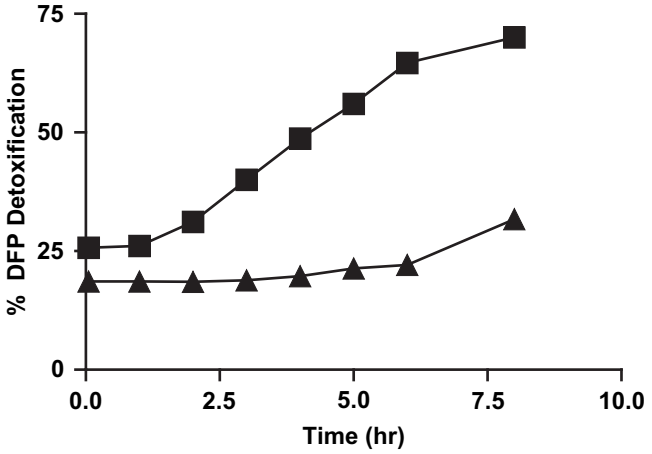



**Fig. 8.** Time-course of DFP detoxification by insoluble fibers of polymer **4** electrospun with ChronoFlex polyurethane. A 15 mg electrospun fiber sample of CF-**4** was immersed in a test tube with $5 \times 10^{-6}$ M DFP in phosphate buffer (50 mM, pH 7.5). Filled squares: CF-**4** fibers, filled triangles: buffer. Samples were drawn at the indicated times and incubated for 3 min with BChE. BChE activity was measured by the Ellman assay [20].

To determine the mobility of the 4-PAM polymers within the electrospun matrices, CF-**4** and UV crosslinked CF-**4** fibers (5 mg) were placed in 1 mL of 50 mM phosphate buffer (pH 6.2). The absorbance of the pyridinium aldoxime group at 280 nm was measured continuously for 15 h at 22 °C (Fig. 4). Leaching was not observed in both sets of fibers within the first 2 h with virtually all of the polymer **4** leached from the non-crosslinked fibers within 8 h (Fig. 4). While there was leaching of polymer **4** from the crosslinked fibers the leaching rate was 6.8 fold lower than that of the non-crosslinked fibers.

### 3.6. Enzymatic generation of free iodine and bromine by multi-functional polymers

Polymers **1**, **2**, and **4** were mixed with CF polyurethane and electrospun together with the redox enzymes GOX and HRP to produce fibers of CF-**1**-GOX-HRP, CF-**2**-GOX-HRP and CF-**4**-GOX-HRP. Homogenous mats of electrospun fibers were used to assess bactericidal activity and DFP detoxification.

PAM-halide polymers, **2** and **4** were shown to contain halide anions at 10–12% w/v as determined by $^{1}$H NMR analysis (see supplementary material). Weighed amounts of fibers were immersed in cuvettes containing phosphate buffer. Free halogen generation was initiated by the addition of glucose (5 mM to approximate its physiological concentration) into the buffer containing the immersed fibers and the rate of halogen evolution was detected by a chromogenic reaction with PVA. Based on calibration curves prepared for bromine and iodine, bromine was generated at 500–1000 ppm and iodine at 4–5 ppm steady state concentrations (Fig. 5A and B respectively). These levels are significantly above the minimal inhibitory concentrations (MIC) of iodine and bromine for bactericidal activity against *S. aureus* (0.1 and 28 ppm, respectively) and *E. coli* (0.6 and 4.3 ppm, respectively) indicating that the materials would be effective antimicrobials [6].

### 3.7. Bactericidal activity of soluble and electrospun polymers

Polymer **2** and its non-quaternary precursor polymer **1** were electrospun with CF, GOX, and HRP. Samples of the electrospun fibers were incubated with bacteria in the presence of glucose (5 mM). The CF-**2**-HRP-GOX sample killed >6 logs of *E. coli* within 1 h at 37 °C (Table 1). As expected, since the non-quaternary polymer **1** carries with it no halide counter ions, *E. coli* cells incubated with CF-**1**-GOX-HRP survived. CF-**4**,-GOX-HRP fibers together with NaI and glucose in solution also killed all of *E. coli* within 1 h (Table 1). The benzophenone in CF-**4**-HRP-GOX fibers did not affect the bactericidal activity, even after cross-linking the fibers by UV irradiation, suggesting that the enzyme activity was not affected by either the UV light or the cross-linking reaction. Polymer **6** included dimethyl ethyl propyl ammonium moieties in addition to the 4-PAM groups providing additional halide ions to increase the rate of enzyme-catalyzed generation of halogen. CF-**6** fiber (with GOX, HRP, and glucose in solution) was an excellent biocide against both *E. coli* and *S. aureus* (Tables 1 and 2). Interestingly, a significant bactericidal activity of polymer **6** was observed with *S. aureus* even without the redox enzymes (reduction of 2 log units, Table 2). This is in keeping with the known antimicrobial activity of high density positively charged quaternary ammonium polymers [11] as found in polymer **6** when compared to the lower density of quaternary amine groups in polymer **4**.

### 3.8. Detoxification of DFP by multi-functional polymers

The reaction of DFP with quaternary oximes proceeds by nucleophilic attack of the oximate anion on the electrophilic phosphorus atom creating an unstable phosphoryl-oxime product [15]. The direct reaction of oximes with OP compounds in solution is usually very slow [15]. Based on the positive charge effect that can enhance the nucleophilicity of aromatic oximes [18] it was of interest to study the reaction rate of the polyquaternary oxime polymers with DFP. DFP detoxification as a function of pH was measured by incubating 5 μM DFP with either soluble polymers **2** or **4**, or electrospun fibers of those polymers within polyurethane in phosphate buffer. Analysis of the residual concentration of DFP was performed by measuring the residual activity of butyrylcholinesterase (BChE) which had been treated with DFP for 3 min at a final concentration of $4 \times 10^{-7}$ M.

The rate of DFP detoxification by soluble polymer **2** was dose-dependent and reached 80% DFP degradation within 30 min at 15 mg/mL[2] (Fig. 6). The same level of degradation was obtained after 3 h with polymer **2** at 5 mg/mL (Fig. 6) indicating dose-dependence of DFP detoxification by the oxime polymer. The degree of DFP detoxification by soluble polymer **4** reached 35% after 3 h (Fig. 7). Interestingly, addition of GOX/HRP and glucose to this solution enhanced DFP hydrolysis by soluble polymer **4**–60% within 4 h (Fig. 7). It was likely that the rate enhancement was achieved by an oxidation mechanism mediated by the free halogen or the peroxide produced by GOX [24].

With CF-**4** 80% DFP detoxification was achieved after 10 h (Fig. 8). Although the rate of DFP detoxification by the electrospun material was slower than soluble polymer **4** it was significantly faster than phosphate buffer alone (Fig. 8 and S1). The rate of CF-**4** induced detoxification could be improved by simply reformulating the component ratios in the electrospinning.

The importance of the nucleophilic activity of the oximate anion was observed by performing the DFP degradation at pH 6.2. DFP was only marginally degraded (10%) by polymer **4** after 4 h (Fig. S2). Clearly, the oximate anion was responsible for detoxification since its nucleophilic activity increased under more basic conditions (pH = 7.5). DFP detoxification was also performed at pH 7.5 with pyridinium iodide polymer **5** that contained no oxime groups. The extent of DFP detoxification by polymer **5** was less than 10% after 4 h (Fig. S3), showing again that the oxime group was responsible for the detoxification activity of polymer **4**.

## 4. Conclusions

We have designed, synthesized, and characterized a multifunctional material for decontamination of biological and chemical warfare agents based on oxime-polymer decontamination of OP nerve agents and enzymatic generation of reactive halogens as a bactericide. Detoxification of DFP was achieved and the polymeric oxime exhibited enhanced activity toward DFP compared to small molecule oximes. DFP detoxification by the PAM polymers was pH-dependent indicating that the oximate anion was the reactive moiety in attacking the electrophilic phosphorus atom in DFP releasing the fluoride anion. The redox enzymes GOX and HRP in conjunction with oxime-halide polymers, either soluble or electrospun as one integrated matrix, killed *E. coli* and *S. aureus* efficiently. The introduction of additional positively charged aliphatic quaternary ammonium groups (polymer **6**) caused a parallel increase in halide content which provided a higher substrate level for HRP. A partial non-enzymatic biocidal activity was observed with polymer **6** (2 log units *S. aureus*) stemming from the increase in density of quaternary ammonium groups. Polymers containing the AChE reactivator compound, **7**, conjugated to the polymer backbone via an ester linkage were synthesized. The oxime reactivator **7** was shown to be released from the polymer and to reactivate DFP-inhibited AChE. The materials described decontaminate both chemical and biological agents without cross interference with either process. Indeed the activity primarily directed at the biological agents may even enhance the chemical decontamination.

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## Appendix. Supplementary data

Supplementary data associated with this article can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.02.004.

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