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Poly(PS-*b*-DMA) Micelles for Reactive Oxygen Species Triggered Drug Release

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Abstract

A new micelle drug carrier that consists of a diblock polymer of propylene sulfide (PS) and N,N-dimethylacrylamide (poly(PS₇₄-*b*-DMA₃₁₀)) has been synthesized and characterized for site-specific release of hydrophobic drugs to sites of inflammation. Propylene sulfide was first polymerized using a thioacyl group transfer (TAGT) method with the RAFT chain transfer agent (CTA) 4-cyano-4-(ethylsulfanylthiocarbonylsulfanyl) pentanoic acid (CEP), and the resultant poly(PS₇₄-CEP) macro-CTA was used to polymerize a second polymer block of DMA using reversible addition-fragmentation chain transfer (RAFT). The formation of the poly(PS₇₄-*b*-DMA₃₁₀) diblock polymer was confirmed by ¹H NMR spectra and gel permeation chromatography (GPC). poly(PS₇₄-*b*-DMA₃₁₀) formed 100 nm micelles in aqueous media as confirmed by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Micelles loaded with the model drugs Nile red and DiO were used to demonstrate the ROS-dependent drug release mechanism of these micelles following treatment with hydrogen peroxide (H₂O₂), 3-morpholininosydnonimine (SIN-1), and peroxyxynitrite. These oxidants were found to oxidize the micelle PPS core, making it more hydrophilic and triggering micelle disassembly and cargo release. Delivery of poly(PS₇₄-*b*-DMA₃₁₀) micelles dual-loaded with the Förster Resonance Energy Transfer (FRET) fluorophore pair DiI and DiO was used to prove that endogenous oxidants generated by lipopolysaccharide (LPS)-treated RAW 264.7 macrophages significantly increased release of nanocarrier contents relative to macrophages that were not activated. In vitro studies also demonstrated that the poly(PS₇₄-*b*-DMA₃₁₀) micelles were cytocompatible across a broad range of concentrations. These combined data suggest that the poly(PS₇₄-*b*-DMA₃₁₀) micelles synthesized using a combination of TAGT and RAFT have significant potential for site-specific drug delivery to tissues with high levels of oxidative stress.

Keywords

Poly(propylene sulfide) (PPS); Reversible addition fragmentation chain transfer (RAFT) polymerization; Reactive oxygen species (ROS); Inflammation; Smart polymer micelles; Targeted drug delivery

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1.0 Introduction

Rheumatoid arthritis, neurodegenerative diseases, atherosclerosis, diabetes, and many cancers are among the pathologies characterized by hyper-activation of enzymes (i.e. NADPH oxidase, iNOS, etc.) that create high concentrations of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, singlet oxygen, superoxide anions, nitric oxide, nitroxyl, and nitrogen dioxide [1]. Nitric oxide and superoxide anion can also react with each other to generate other more potent reactive oxygen and nitrogen species such as peroxynitrite [2]. Excessive levels of these ROS, known as oxidative stress, can cause DNA mutations and can alter function of proteins, leading to apoptosis or other aberrant cell activities than can cause or exacerbate disease [1-3].

Because of the role of imbalanced ROS activity in the etiology of numerous diseases, delivery platforms that enable targeted release of antioxidants or other drugs at sites of high ROS activity have the potential for high therapeutic impact [4-8]. Stimuli-responsive, “smart” polymer-based micelles have attracted considerable attention for drug delivery because they can stably package their cargo until disassembly and drug release is triggered in response to changes in environmental signals such as temperature [9-11], pH [12], oxidation [13-15], light [16, 17], or other specific molecules [18]. The aim of the current study was to investigate a new smart micelle for targeted drug release in regions of high ROS activity.

Sulfur(II)-containing materials undergo a phase transition from a hydrophobic to hydrophilic state under oxidative environments [19], with poly(propylene sulfide) (PPS) being an example synthetic polymer known to exhibit oxidation responsiveness [14]. Specifically, hydrophobic PPS has a two-stage transition to more hydrophilic poly(propylene sulfoxide) and ultimately poly(propylene sulphone) upon oxidation [14]. Block copolymers of PEG and PPS have been explored by Hubbell et al for engineering of polymersomes for vaccine delivery and micelles for encapsulation of hydrophobic drugs [20, 21]. PEG-b-PPS micelles were shown to enable sustained, slow release of the hydrophobic drug cyclosporin A, but, to our knowledge, these or similar polymers have not been rigorously investigated for triggered release of hydrophobic drug cargo in response to different ROS. Furthermore, a thioacyl group transfer (TAGT) method [22] was utilized here that enables pairing of the anionic ring opening polymerization of PPS with the RAFT polymerization of the hydrophilic polymer block. RAFT is amenable to use with a wide variety of monomers [23], and here, DMA was chosen for the corona forming polymer block due to its solubility in water and low toxicity [24, 25]. The focus of the current study was on an amphiphilic diblock copolymer of poly(PS₇₄-b-DMA₃₁₀). This diblock polymer self-assembled into micelles, and its oxidation, disassembly, and cargo release was thoroughly characterized under H₂O₂, 3-Morpholininosydnonimine (SIN-1), peroxynitrite, and endogenous ROS generated by lipopolysaccharide (LPS)-activated macrophages.

2.0 Materials and Methods

2.1 Materials

Propylene sulfide (PS), N,N-dimethylacrylamide (DMA), ethanethiol, carbon disulfide (CS₂), sodium hydride (NaH), 4,4'-Azobis(4-cyanovaleric acid) Tetraphenylphosphonium chloride (TPPCI), 2,2'-azobis(isobutyronitrile) (AIBN), Nile red, DiO (3,3'-di-octadecyloxacarbocyanine perchlorate), DiI (1,1'-di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), and hydrogen peroxide (H₂O₂) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). SIN-1 was purchased from Invitrogen (San Diego, CA, USA) as packages of 1 mg plastic vials. Peroxynitrite was purchased from EMD Millipore (Billerica, MA, USA). 4-Cyano-4-

(ethylsulfanyltiocarbonyl)sulfanylpentanoic acid (CEP) was synthesized following the previously reported procedure [26] (^1H NMR (400 MHz CDCl_3) δ : 1.35 (t, $-\text{S}-\text{CH}_2-\text{CH}_3$); δ 1.85 (s, $-\text{C}(\text{CN})-\text{CH}_3$); δ 2.4-2.67 (m, $-\text{CH}_2-\text{CH}_2-$); δ 3.42 (q, $-\text{S}-\text{CH}_2-\text{CH}_3$)). Propylene sulfide was dried and distilled over calcium hydride (CaH_2) before use, and *N,N*-dimethylacrylamide (DMA) was purified by distillation under reduced pressure just before polymerization. 1-Methyl-2-pyrrolidone (NMP) was dried and distilled over CaH_2 .

2.2 Polymer synthesis and characterization

2.2.1 Synthesis of PPS macro CTA poly(PS_{74} -CEP)—Poly(propylene sulfide) was synthesized using the RAFT chain transfer agent CEP through the TAGT polymerization method [22]. Briefly, PS (0.30 mL, 3.8 mmol), CEP (24.9 mg, 0.095 mmol), TPPCI (7.1 mg, 0.019 mmol), and NMP (0.95 mL) were placed into a flame-dried ampoule equipped with a three-way stopcock and degassed for 30 min with three freeze-pump-thaw cycles. The ampoule was immersed in an oil bath at 50°C for 20 h, and afterwards, cooled in a liquid nitrogen bath. The crude polymerization mixture was precipitated twice from NMP into hexane, which is a nonsolvent for PPS. The resulting polymer was dissolved into chloroform and precipitated into tenfold excess of cold methanol to better remove the TPPCI catalyst [22]. The product was dried at 60°C under vacuum to yield a yellow viscous oil (0.15 g) (poly(PS_{74} -CEP)), $M_n = 5,800$ g/mol, PDI = 1.32).

2.2.2 Synthesis of poly(PS_{74} -b-DMA $_{310}$) diblock polymer—RAFT chain extension of poly(PS_{74} -CEP) macro-CTA was done to prepare poly(PS_{74} -b-DMA $_{310}$). Poly(PS_{74} -CEP) (0.58 g, 1 mmol), DMA (4.0 mL, 403 mmol), AIBN (0.8 mg, 0.05 mmol), and dioxane (10.52 mL) were placed in a dry ampoule, and the solution was degassed by purging with nitrogen for 30 minutes. The polymerization was conducted at 60°C for 3 h and then quenched by cooling the reaction vessel in an ice bath and exposure to air. The final polymer (poly(PS_{74} -b-DMA $_{310}$), M_n 36,700 g/mol, PDI=1.29) was purified by precipitation into diethyl ether.

2.2.3 Polymer characterization— ^1H NMR spectra were recorded in CDCl_3 and D_2O with a Bruker 400 MHz spectrometer. For molecular weight determination, gel permeation chromatography (GPC) was performed using dimethylformamide (DMF) + 0.1 M LiBr mobile phase at 60°C through three serial Tosoh Biosciences TSKGel Alpha columns (Tokyo, Japan). A Shimadzu RID-10A refractive index detector and a Wyatt miniDAWN Treos multi-angle light scattering detector was used to calculate absolute molecular weight based on dn/dc values experimentally determined through offline injections into the RI detector.

2.3 Micelle preparation and characterization

To prepare micelle solutions, 10 mg of purified poly(PS_{74} -b-DMA $_{310}$) was dissolved in 250 μL of THF. PBS (10 mL, pH 7.4) was added dropwise into the THF polymer solution under vigorous stirring to dilute the sample to a final polymer concentration of 1 mg/mL. The solution was pushed through a $0.45\ \mu\text{m}$ syringe filter, and dynamic light scatter (DLS) measurements were done to measure hydrodynamic diameter using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, U.K) equipped with a 4 mW He-Ne laser operating at $\lambda = 632.8\ \text{nm}$. Transmission electron microscopy (TEM) samples were prepared by placing one drop of solution (micellar dispersion at 1.0 mg/mL concentration) onto copper grids (400 mesh, carbon coated, Ted Pella Inc. Redding, CA). The grids were dried overnight in a desiccator under vacuum before imaging with a Philips CM20 HR-TEM (Philips, EO, Netherlands).

2.4 Determination of critical micelle concentration (CMC)

The critical micelle concentration was assessed fluorescently using Nile red [27] and by identifying particle morphological changes with DLS. The Nile Red dye is hydrophobic and exhibits strong fluorescence in the presence of intact micelles but is poorly soluble and minimally fluorescent if released into an aqueous environment when micelles destabilize. Different dilutions were prepared from a 1 mg/mL stock solution to obtain micelle samples ranging in concentration from 0.0001 to 1 mg/mL. Then, 10 μ L of a 1 mg/mL Nile red stock solution in THF were added to 1 mL of each micelle sample and incubated overnight in the dark at room temperature. The next day, samples were filtered with a 0.45 μ m syringe filter and their Nile red fluorescence was measured in 96 well plates using a micro plate reader (Tecan Infinite 500, Tecan Group Ltd., Mannedorf, Switzerland) at an excitation wavelength of 535 $\pm$ 20 nm and an emission wavelength of 612 $\pm$ 25 nm. The CMC was defined, as previously described [28], as the intersection point on the plot of the Nile red fluorescence versus the copolymer concentration. For DLS measurements, a range of micelle concentrations were prepared by serial dilutions of a 1 mg/mL stock solution to obtain samples of concentration ranging from 0.001 to 1 mg/mL in PBS. DLS was then used to assess micelle hydrodynamic size and to determine the concentration at which the micelles exhibited morphological changes [29].

2.5 Measuring Micelle Loading with Nile Red and DiO

A fluorescence-based method was used to calculate drug loading and encapsulation efficiency of Nile red and DiO across a range of weight ratios from 6.25 – 200 μ g dye per mg poly(PS₇₄-b-DMA₃₁₀) based on a reported method [30]. After removal of any free dye through centrifugation, the loaded dyes were extracted from the micelles by diluting the samples into 90% DMF. Nile red (excitation at 535 $\pm$ 20 nm, emission 612 $\pm$ 25 nm) or DiO (excitation at 485 $\pm$ 20 nm, emission 535 $\pm$ 25 nm) content was quantified in the respective samples relative to fluorescence standard curves of the molecules measured in 90% DMF. Encapsulation efficiency (EE) was defined as the weight percent of the drug loaded versus what was added to the micelle solution, and drug loading (DL) was calculated as the weight percent of dye relative to polymer.

2.6 ROS-mediated release of Nile red and DiO

To assess ROS-dependent release from the poly(PS₇₄-b-DMA₃₁₀) micelles, Nile red and DiO were utilized as model small molecule drugs. To prepare 1% Nile red loaded micelle solution, 50 μ L of a 1 mg/mL Nile red stock solution in THF was added to 5 mL of micelle solution (1mg/mL). The residual THF was removed through rotary evaporation and the micelle solution incubated overnight in the dark at room temperature. The next day, samples were filtered using a 0.45 μ m syringe filter prior to use. Nile red-loaded micelles were exposed to a range of concentrations (0 to 3.3 volume %) of hydrogen peroxide. Fluorescence intensity of Nile red was monitored in a 96 well plate using a micro plate reader (Tecan Infinite 500) at an excitation wavelength of 535 $\pm$ 20 nm and an emission wavelength of 612 $\pm$ 25 nm. Release of the dye due to micelle oxidation and destabilization was assessed over time based on disappearance of Nile red fluorescence. The loss of fluorescence for each sample at each time point was determined by subtracting the fluorescent value from that of the sample prior to H₂O₂ addition, and the percent fluorescence remaining was determined by normalization to the same value (before addition of H₂O₂). This value for percent fluorescence remaining was subtracted from 100% and expressed as a percent release for each sample at each time point. An analogous experiment was completed to assess the H₂O₂-dependent release of DiO from micelles prepared using the same technique. A similar study was carried out to assay the release of Nile red from the micelles upon treatment with SIN-1, a molecule known to generate superoxide, nitric oxide, and peroxynitrite [31]. Nile red loaded micelles were treated with a range of SIN-1

concentrations (1 to 100 mM), and a fresh dose of the respective concentration of SIN-1 was added every 24h due to the short half life of SIN-1 [31]. Nile red release was quantified as described for H₂O₂ experiments. Because SIN-1 generates nitric oxide and superoxide that then react to form peroxynitrite, micelle release was also studied following direct peroxynitrite treatment. A range of final concentrations of peroxynitrite (1-100 μ M) were tested by addition of a basic peroxynitrite stock solution into buffered (PBS) solutions of Nile red-loaded micelles. The final pH of the samples was 7.4 after this addition, and fluorescent intensities were measured after 15 minutes to quantify release as described above. The full time course was not done with peroxynitrite because of its very short half-life at physiologic pH.

2.7 FRET-based Imaging of Micelle Release by Activated Macrophages

Micelle release of the Förster Resonance Energy Transfer (FRET) dye pair DiO and DiI was used as a readout for model drug release *in vitro* [32]. To prepare FRET micelles, 10 μ l of DiO solution (1 mg/ml solution in THF) and 10 μ l of DiI solution (1 mg/ml solution in THF) were added into 1 ml of micelle solution (1 mg/ml). The THF was then removed by rotary evaporation and the samples were left overnight in the dark. To remove any unloaded dye, the micelle solution was diluted four fold in sterile DI water and reconcentrated using a centrifuge filtration tube with 10,000 MW cutoff for 30 minutes. This process was repeated four times. Control micelles loaded with just DiO or DiI were prepared in the same manner as FRET micelles. Generation of the FRET effect within the dual loaded micelles was confirmed using a Jobin Yvon/Horiba Fluorolog-3 FL3-111 Spectrophotofluorometer at an excitation wavelength of 484 nm.

To image micelle release mediated by endogenously-produced ROS *in vitro*, RAW 264.7 mouse macrophages were seeded in phenol-red free DMEM at 5,000 cells/well onto multi-chambered #1 borosilicate cover-glass slides (Fisher Scientific) and allowed to adhere overnight. After 24 hours, the media was replaced with phenol-red free DMEM supplemented with 100 ng/ml lipopolysaccharide (LPS) or vehicle control. After 24 hours, LPS-activated and control cells were treated with vehicle control or with DiO/DiI FRET micelle solutions to achieve a final concentration to 1.67 μ g/ml for each dye. Images were acquired 24h after micelle treatment with a Zeiss LSM 510 META confocal microscope using a 63 $\times$ oil immersion lens equipped with a 488 nm Argon laser with 505-550 band-pass filters for green emission and 560-615 band-pass filter for red (FRET) emission. The exposure time, gain, and all other microscope settings were held constant during fluorescent imaging of all treatment groups. Image J was used to quantitatively assess cellular fluorescence. Cells were also seeded onto 96 well plates and treated as explained above to confirm quantitative FRET data using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA,) plate reader (see additional details in Figure S-8).

2.8 Cell Viability Assay

RAW 264.7 macrophages (RAWs) were plated at 8,000 cells/well in a 96-well plate and incubated at 37 °C in Alpha MEM supplemented with 10% FBS and 1% penicillin-streptomycin. After 24h, the medium was replaced with fresh medium containing a range of polymer micelle concentrations (62.5-1000 μ g/ml). The cells were then incubated at 37 °C for 24 h or 48 h. At the respective endpoints, the cells were lysed and analyzed for intracellular LDH with a Cytotoxicity Detection Kit (Roche Applied Science) as previously reported [33]. With this assay, a colorimetric readout (absorbance at 492 nm with reference at 595 nm) is acquired that represents relative cell number. Each group was assayed in triplicate, and LDH quantities in the micelle treatment groups were normalized to samples receiving no treatment (NT).

3. Results and Discussion

3.1 Synthesis and characterization of poly(PS₇₄-b-DMA₃₁₀)

The AB diblock copolymer poly(PS₇₄-b-DMA₃₁₀) was successfully prepared using the TAGT and RAFT methods described (Scheme 1). The final diblock copolymer poly(PS₇₄-b-DMA₃₁₀) displayed relatively low polydispersity ($M_w/M_n = 1.29$) and showed the expected decrease in the elution time following chain extension from poly(PS₇₄-CEP) (Figure 1).

Successful diblock polymerization was also confirmed by ¹H NMR spectra of the poly(PS₇₄-CEP) macro-CTA and the poly(PS₇₄-b-DMA₃₁₀) diblock copolymer (Figure 2). The ¹H NMR spectra of the resulting poly(PS₇₄-b-DMA₃₁₀) taken in CDCl₃, a solvent in which micelles do not form, displayed characteristic signals attributed to both PPS and PDMA. The use of RAFT for polymerization of the corona-forming polymer block provides synthetic flexibility because it enables the possibility of using other monomers or even random copolymer compositions (i.e., for incorporation of pendant groups that can be used to attach cell-specific targeting moieties within the micelle corona).

3.2 Micelle preparation and characterization

Formation of poly(PS₇₄-b-DMA₃₁₀) micelles with a PPS core and a PDMA corona were successfully prepared by a solvent evaporation method using THF. Verification that the poly(PS₇₄-b-DMA₃₁₀) existed in water as micelles stabilized by a hydrophilic poly(DMA) block was done by recording ¹H NMR spectra in CDCl₃ and D₂O (Figure S-1). The ¹H NMR spectrum of the diblock copolymer in CDCl₃ showed all peaks associated with both the PPS and PDMA blocks (Figure S-1a). Under these conditions, both blocks are solvated and show free segmental motion, which is consistent with a molecularly dissolved unimeric polymer. In contrast, ¹H NMR of poly(PS₇₄-b-DMA₃₁₀) in D₂O (Figure S1-b) showed suppression of the PPS peaks (note suppression of peak 'a' in Figure S-1b D₂O spectrum versus S-1a CDCl₃ spectrum) and resembled the spectrum for a homopolymer of PDMA ($M_n=20$ KDa) taken in D₂O (Figure S-1d). These combined results indicate that, in aqueous conditions, the poly(PS₇₄-b-DMA₃₁₀) assembles into micelles with the PPS polymer block confined in the micelle core away from the D₂O solvent, and thus unable to generate ¹H NMR signal.

¹H NMR spectra of micelles were also compared in D₂O before and after H₂O₂ treatment to verify that ROS could oxidize the PPS in the micelle core and increase its water solubility. For reference, ¹H NMR spectra were first recorded for a PPS homopolymer in D₂O before and after treatment with H₂O₂ (Figure S-1e, S-1f). The appearance of a new peak at 1.53 ppm in the ¹H NMR spectra of poly(PS₇₄-b-DMA₃₁₀) in D₂O following treatment with H₂O₂ is a characteristic shift [14] indicating that, in the micelle form, the H₂O₂ was still able to oxidize the PPS from a sulfide into a more hydrophilic sulfone (Figure S-1c). The shifting of the PPS peaks (CH₂ and CH protons) from 2.5-3.0 ppm to 3.2-4.2 ppm (circled and labeled 'b+c') was also observed (Figure S-1c) and further confirmed PPS conversion into a more hydrophilic sulfone, which also matched observations for PPS homopolymer H₂O₂ oxidation by us (Figure S-1e, S-1f) and others [14].

Physical characterization of the micelles was also done using DLS and TEM. The average hydrodynamic diameter of the poly(PS₇₄-b-DMA₃₁₀) micelles was measured by DLS to be 99 nm (Figure 3a). This size, along with the spherical morphology of the micelles, was confirmed using TEM (Figure 3b). These micelles fall in a desirable size range for nanotherapeutics and may be well-suited for tumor targeting through the enhanced permeation and retention (EPR) effect [34].

DLS and TEM were also utilized to confirm the H_2O_2 -responsive disassembly of the micelles. Figure 3a shows the change in hydrodynamic diameter of micelles before and after H_2O_2 (3.3 volume % for 24h) treatment. The average size of micelles was found to change from 99 nm to 5 nm after treatment with H_2O_2 , and this behavior was also supported by absence of visible micelles in TEM images of H_2O_2 treated micelle samples (Figure 3c). These data indicate that the poly(PS_{74} -b- DMA_{310}) micelles may be useful for site-specific cargo delivery to sites with high ROS through environmentally-triggered micelle disassembly.

3.3 Determination of critical micelle concentration (CMC)

The critical micelle concentration (CMC) was determined using Nile red as a fluorescent probe [35]. Nile red is hydrophobic and does not fluoresce in an aqueous environment but is strongly fluorescent when it partitions itself into the hydrophobic core of intact micelles. The fluorescence intensity of Nile red in poly(PS_{74} -b- DMA_{310}) aqueous solutions of various concentrations were recorded, and the maximum fluorescence intensity of the Nile red emission is shown in Figure 4 as a function of micelle concentration. The CMC of the diblock copolymer was determined using the crossing point of the plot and was estimated to be 0.09 mg/mL. The CMC value of the micelles was also estimated using a DLS-based dilution method [29]. Table 1 shows the hydrodynamic diameter (D_h) of poly(PS_{74} -b- DMA_{310}) in PBS from concentrations ranging 1.0 mg/mL to 0.001 mg/mL, and the full DLS spectra for these samples are also shown in Figure S-2. The hydrodynamic diameter of micelles was stable down to 0.1 mg/mL, and dilution to concentrations below this value caused the micelles to become increasingly unstable, resulting in the formation of aggregates. This indicated that the CMC value fell in the range from 0.05-0.1 mg/mL, which was in agreement with the Nile red based assay.

3.4 Assessment of Micelle Loading with Nile red and DiO

The drug loading and encapsulation efficiency poly(PS_{74} -b- DMA_{310}) micelles were determined using both Nile red and DiO. For the drug to polymer ratios tested, the maximum drug loading content and encapsulation efficiency of Nile red were calculated to be 1.76 wt % (Figure S-3a) and 63.1% (Figure S-3b), respectively. The values were found consistent with the previously reported literature [30]. DiO also showed similar maximum drug loading content (1.48 wt%, (Figure S-3c)) and encapsulation efficiency (63%, (Figure S-3d)).

3.5 ROS dependent release of Nile red

Nile red was chosen as a model hydrophobic drug for the investigation of drug release [27] in response to H_2O_2 , which was used to mimic the presence of pathophysiologic oxidative stress [36]. Figure 5 shows *in vitro* release kinetics of Nile red mediated by different concentrations of H_2O_2 . The fluorescence intensity of Nile red-loaded micelles treated with H_2O_2 was found to decrease over time, and the rate of this decrease correlated to the concentration of H_2O_2 present. The loss of Nile red fluorescence intensity under oxidative environments can be explained by the conversion of the PPS block from a hydrophobic sulfide to more hydrophilic sulfone. This triggers disassembly of the micelle into unimeric polymers and releases the Nile red into the more polar aqueous environment where its fluorescence is no longer apparent. The fluorescence intensity of the negative control samples that were not treated with H_2O_2 remained relatively constant over the timeframe studied, indicating that the micelles were stable in the absence of ROS. This observation suggests that the poly(PS_{74} -b- DMA_{310}) micelles would have little nonspecific drug “leak” *in vivo* prior to targeted disassembly and drug release at sites of oxidative stress.

To verify that the decrease in fluorescence intensity of Nile red during the release experiments was due to micelle release and not H₂O₂ degradation of the dye itself, the free Nile red molecule was dissolved in a mixture of THF/water (15/85) and treated with a range of concentrations of H₂O₂. The fluorescence intensity was found to remain constant over the period of 170 h, confirming that loss of signal was due to dye released from the micelles due to destabilization of the micelle structure (Figure S-4). Furthermore, because subsequent cell experiments were done using the dyes DiO and DiI, an additional experiment was done to confirm that H₂O₂ dose dependent micelle release of these model drugs was similar to the release profile for Nile red-loaded micelles (Figure S-5).

To assess the responsiveness of the poly(PS₇₄-b-DMA₃₁₀) micelles to other ROS, the *in vitro* release kinetics of Nile red were measured over a period of 92 h following treatment with a range of concentrations (1-100 mM) of the peroxynitrite generator SIN-1 (Figure S-6). The rate of release of Nile red from the micelles was found to be dependent on the concentration of SIN-1. To confirm that release was mediated by peroxynitrite, Nile red release in response to treatment with peroxynitrite alone (1-100 μ M) was also tested. Use of exogenous peroxynitrite to model endogenous production is difficult to mimic *in vitro* because it has a very short ($\sim$ 1 s) half-life at physiological pH. However, it was found that 20% Nile Red release was triggered from micelles after a single treatment with 100 μ M peroxynitrite (Figure S-7), further validating the multi-faceted response of the (PS₇₄-b-DMA₃₁₀) micelles to various ROS species. These results indicate that poly(PS₇₄-b-DMA₃₁₀) may be broadly applicable for *in vivo* delivery to pathological sites with high oxidative stress.

Direct comparison of hydrogen peroxide or peroxynitrite concentrations *in vivo* versus those used in the cell-free experiments reported here are difficult because cells at sites of inflammation *in vivo* continuously generate ROS (many of which have very short half-lives), whereas, for practical reasons, bolus dose delivery is utilized in cell free studies. It has been postulated, for example, that much higher concentrations of exogenous peroxynitrite (half-life <1 s at physiologic pH) may be required to achieve biologic responses similar to those produced by much lower concentrations of continuously produced endogenous peroxynitrite *in vivo* [37]. Here, we have compared cell-free bolus doses to the total quantity of H₂O₂ or peroxynitrite that would be generated within a given tissue volume during a defined timeframe. These calculations are based on an average (spherical) macrophage diameter of 21 μ m [38] and published values of 0.63 - 1.258 nmol/(10⁶ cells)/min and 0.11 nmol/(10⁶ cells)/min for activated macrophage production of H₂O₂ and peroxynitrite, respectively [39-41]. Using these values, one can estimate that, over a brief 6 hour period, a concentration of 47-93 mM H₂O₂ or 8 mM peroxynitrite would be accumulated locally *in vivo* if there was no diffusional loss or degradation of these species. Note that the intermediate H₂O₂ concentration of 0.33 vol% tested in the Nile red release study corresponds to 97 mM and correlates well to the higher end values in this calculation, while the calculated value for peroxynitrite exceeds the highest dose (100 μ M) tested in the peroxynitrite Nile red release study by 80-fold. It is estimated that the peroxynitrite generation rate of SIN-1 is 1 μ M/min/(mM SIN-1), and an approximately 1 mM dose has been used to mimic pathological conditions on cultured cells [42]. Over 6 hours, the 1 mM SIN-1 would generate a concentration of 360 μ M peroxynitrite under the no-loss assumption, which is 3.6-fold higher than the highest dose tested in our peroxynitrite bolus delivery experiment (Figure S-7).

The preceding analysis suggests that the peroxynitrite and SIN-1 concentrations used in our drug release experiments span physiologically-relevant ranges, while the hydrogen peroxide concentrations may have been higher than what would be present in inflamed tissues. Because sites of inflammation *in vivo* consist of a complex milieu of different ROS and

reactive nitrogen species, it is potentially advantageous that poly(PS₇₄-b-DMA₃₁₀) micelles were responsive to multiple reactive species, and it is not necessarily a problem if the micelles do not sufficiently respond to H₂O₂ alone to trigger rapid release. The release behavior of the poly(PS₇₄-b-DMA₃₁₀) carriers may in fact be ideally tuned to remain stable “in transit” and slowly respond to achieve sustained release of cargo once a pathophysiological environment is encountered. Though more switch-like release from the nanocarriers may be ideal for some applications, it is conceivable that the sustained release profile that these nanocarriers display may be therapeutically advantageous in some scenarios, and in vivo testing will need to be done to better validate the behavior of these carriers for different pathologies.

3.6 FRET-based Imaging of Micelle Release by Activated Macrophages

Proof-of concept studies in LPS-activated macrophages indicated successful use of poly(PS₇₄-b-DMA₃₁₀) micelles for environmentally-targeted drug release in response to endogenously-produced ROS. To do so, release from micelles co-loaded with DiO and DiI was measured using FRET. A FRET signal (emission of DiI, a red fluorophore, upon excitation of DiO, a green fluorophore) occurs only when the two dyes are in close proximity, i.e., co-loaded into the micelle core. Fluorimetry measurements following excitation at 484 nm was utilized to confirm FRET signal in dual DiO/DiI-loaded Poly(PS₇₄-b-DMA₃₁₀) micelles (Figure 6a). Tenfold dilution of dual DiO/DiI-loaded micelles into DMF, an organic solvent that disrupts the micelle core, disrupted the FRET signal, but a similar tenfold dilution into PBS did not (Figure 6b), further confirming successful generation of FRET micelles.

ROS endogenously produced by LPS-activated macrophages were shown to trigger release of the DiO/DiI dual-loaded poly(PS₇₄-b-DMA₃₁₀) micelles *in vitro* using this FRET-based readout. Bacterial LPS was used to activate RAW macrophages since it models inflammatory disease states, activates ROS-producing enzymes, and stimulates production of ROS and peroxynitrite in macrophage [43-45]. Confocal microscopy imaging of control cells showed the presence of the FRET signal (quenched DiO/green, bright DiI/red) in control cells excited at 488 nm, suggesting cell internalization of the intact DiO/DiI dual-loaded micelles. In LPS-activated macrophages, a decrease in DiI/red signal and increase in DiO/green signal suggested that endogenously-produced ROS had triggered micelle disruption and intracellular dye release (Figure 7a). Quantitative analysis of the macrophage images confirmed this change in the fluorescence profile between LPS-treated and control cells (Figure 7b). Macroscopic plate reader data also indicated that there was a significant reduction in FRET signal for cells treated with LPS relative to vehicle controls ($p < .005$) (Figure S-8). These combined FRET microscopy and plate reader data strongly indicate that “inflamed” macrophages generate sufficient ROS to facilitate release of poly(PS₇₄-b-DMA₃₁₀) micelle cargo. This proof-of-principle experiment confirmed the potential feasibility in using this system for environmentally-targeted delivery of hydrophobic drugs to sites of inflammation.

3.7 Cell viability

The cytotoxicity of the poly(PS₇₄-b-DMA₃₁₀) micelles was evaluated in RAW 264.7 cells for a range of concentrations (0-1000 μ g/mL) using the LDH assay. This assay offers a simple way to measure LDH, a stable cytoplasmic enzyme present in most cells that provides an accurate marker for cell viability [46]. Figure 8 shows that viability of the RAW cells remained high in the presence of micelles across the entire range of concentrations tested after both 24h and 48h incubation times.

4. Conclusion

This report presents novel, ROS-responsive polymeric micelles that have the potential to be applied to preferentially release entrapped hydrophobic drug cargo under pro-inflammatory, oxidative environments. Thorough characterization of the “smart” poly(PS₇₄-b-DMA₃₁₀) micelles demonstrated responsiveness to H₂O₂, SIN-1, and peroxynitrite, indicating that multiple reactive species would contribute to cargo release at sites of inflammation. The reaction of PPS-containing polymers with SIN-1/peroxynitrite is a novel finding to our knowledge, and it is anticipated that response to oxidants other than H₂O₂ is important for accomplishing drug release under pathophysiologically-relevant conditions. The importance of this finding is further supported by the fact that other sulfides have been shown to react three times faster with peroxynitrite than to H₂O₂ [47]. To this end, it was found that LPS-activated macrophages, a model system known to robustly produce peroxynitrite [44], preferentially triggered cell-mediated micelle release of the FRET pair DiO and DiI. It was also shown that the micellar carrier did not cause any *in vitro* cytotoxicity, and it is anticipated that conversion of polypropylene sulfide to more water soluble forms (i.e., polypropylene sulfoxide) would result in safe removal of the micelle constituents from body after exposure to ROS rich environments. These combined data indicate that poly(PS₇₄-b-DMA₃₁₀) micelles provide a promising platform for targeted drug therapy at sites of oxidative stress and that this carrier may be especially good candidate for delivery to pathological sites with high peroxynitrite production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Confirmation of RAFT polymerization of a DMA second block from a poly(PS₇₄-CEP) macro-CTA. The GPC elugram showed a characteristic shift to a decreased elution time following RAFT polymerization of the larger molecular weight poly(PS₇₄-b-DMA₃₁₀) diblock from the poly(PS₇₄-CEP) macro-CTA.

Figure 2. ^1H NMR spectra of (a) poly($\text{PS}_{74}\text{-CEP}$) and (b) poly($\text{PS}_{74}\text{-b-DMA}_{310}$) in CDCl_3 show characteristic peaks for both PS and DMA, providing additional evidence of diblock polymer formation.

Figure 3. poly(PS₇₄-b-DMA₃₁₀) micelles disassembled following exposure to H₂O₂. (a) DLS measurement of poly(PS₇₄-b-DMA₃₁₀) demonstrated transition from nanoparticulate micelles to smaller polymer unimers following H₂O₂ treatment. TEM imaging (b) before and (c) after H₂O₂ treatment visually confirmed the presence of micelles that disassembled following oxidation. TEM scale bars = 100 nm.

Figure 4. Changes in the fluorescence intensity of Nile red (1.0×10^{-6} M) with different concentrations of poly(PS₇₄-b-DMA₃₁₀) indicated that the CMC was approximately 0.09 mg/mL.

Figure 5. H_2O_2 concentration-dependent release of the model drug Nile red from poly(PS_{74} -b-DMA $_{310}$) micelles. Figure legend indicates volume % of H_2O_2 in PBS ($n=3$).

Figure 6.

Demonstration of FRET effect in Poly(PS₇₄-b-DMA₃₁₀) micelles co-loaded with DiO and DiI. **(a)** Fluorescence emission spectra of PPS₇₄-b-PDMA₃₁₀ FRET micelles (1% DiI and 1% DiO), 1% DiO micelles, and 1% DiI micelles with 484-nm excitation. **(b)** Fluorescence spectra of micelles diluted tenfold into PBS or DMF. FRET signal is indicated by the quenching of emission at 510 nm and increase in emission at 580 nm in intact, dual-loaded micelle samples.

Figure 7.

Cell-mediated micelle release of FRET pair DiO and DiI by LPS-activated macrophages. (a) Representative FRET confocal microscopy images of RAW 264.7 cells delivered poly(PS₇₄-b-DMA₃₁₀) micelles co-loaded with DiI and DiO with and without pre-activation with LPS. Upon excitation at 485 nm, decreased red emission (560-615 nm) and increased green emission (505-550 nm) was visible in cells treated with LPS relative to controls, indicating that LPS-driven ROS generation triggered release of micelle cargo. Scale bars = 25 μ m. (b) Green and red fluorescent emission intensity integrated across the longest dimension of macrophages (n= 4) showed quantitative differences, with a significant drop in the red (FRET-based) signal in the cell populations treated with LPS. Standard error is indicated by the shaded region overlaying each data set.

Figure 8.

Poly(PS₇₄-b-DMA₃₁₀) cytocompatibility in RAW 264.7 macrophages was found to be high across the entire range of micelle concentrations tested at both 24 and 48 h. The cell viability was determined by LDH assay and each treatment group was normalized to no treatment (NT) controls (n = 3).

Scheme 1.

Table 1

DLS measurements of poly(PS₇₄-b-DMA₃₁₀) demonstrating that the polymer CMC falls below 0.1 mg/mL.

Polymer Concentration	Size (nm)
poly(PS ₇₄ -b-DMA ₃₁₀) - 1 mg/mL	99.45
poly(PS ₇₄ -b-DMA ₃₁₀) - 0.5 mg/mL	97.55
poly(PS ₇₄ -b-DMA ₃₁₀) - 0.1 mg/mL	99.28
poly(PS ₇₄ -b-DMA ₃₁₀) - 0.05 mg/mL	506.6
poly(PS ₇₄ -b-DMA ₃₁₀) - 0.01 mg/mL	175.00
poly(PS ₇₄ -b-DMA ₃₁₀) - 0.001 mg/mL	407.2