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Polymeric persulfide prodrugs: Mitigating oxidative stress through controlled delivery of reactive sulfur species

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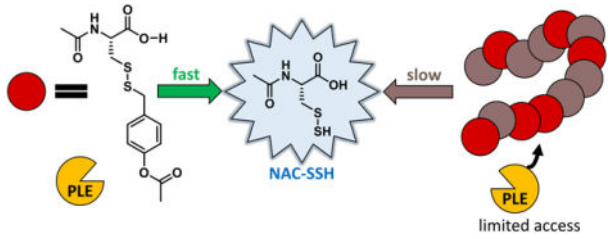
Abstract

Related biologically to the known gasotransmitter hydrogen sulfide (H_2S), persulfides (R–SSH) have recently been recognized as native signaling compounds and redox regulators in their own right. Reported here is the synthesis, characterization, and *in vitro* evaluation of a small molecule persulfide donor and its polymeric counterpart, both of which release *N*-acetyl cysteine persulfide (NAC-SSH) in response to esterases. The donors, termed EDP-NAC and poly(EDP-NAC), underwent controlled decomposition in response to porcine liver esterase, resulting in pseudo-first-order release half-lives of 1.6 h ± 0.3 h and 36.0 h ± 0.6 h, respectively. In cell experiments, slow-releasing poly(EDP-NAC) rescued H9C2 cardiomyocytes more effectively than EDP-NAC when cells were treated with 5-fluorouricil (5-FU), which induces sustained production of ROS. Neither EDP-NAC nor poly(EDP-NAC) rescued MCF-7 breast cancer cells from 5-FU-induced oxidative stress, suggesting that polymeric persulfide donors could be used as adjuvants to reduce the deleterious cardiotoxic effects of many chemotherapeutics.

Graphical Abstract



Introduction

Macromolecular prodrug systems offer a means to modulate the chemical, physical, and pharmacokinetic properties of a drug without extensively changing its chemical nature.[1] This drug delivery strategy can be particularly helpful for the delivery of drugs that are rapidly cleared, have narrow therapeutic indices, or generate adverse side effects. Both

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Supporting Information

- Experimental details, protocols for synthesis, and additional experiments including ^{1}H NMR kinetics, SEC and LC traces, and DLS experiments in Figures S1–S26.

physical encapsulation or covalent attachment of a drug to a polymer can improve circulation time and extend release rates to maintain therapeutic efficacy without reaching supratherapeutic concentrations.[2–4] Due to their ability to modulate these important pharmacological properties of drugs, several polymer prodrug systems have exhibited therapeutic efficacy in a clinical setting. For example, in 1999, Duncan and Kopecek developed the first polymer-doxorubicin conjugates, which were studied in phase I and II clinical trials.[5] Despite this progress in delivery of conventional drugs, much less attention has been paid to polymers for delivery of reactive signaling molecules, which can have profound biological effects.[6–9] Due to their instability, reactive signaling molecules, such as reactive sulfur species (RSS), stand to benefit from polymer prodrug delivery even more than stable small molecule drugs, but such prodrugs are almost entirely unexplored.

Sulfur takes on oxidations states ranging from +2 to −6 in the body, with many types of RSS implicated in the (patho)physiological pathways that exist within oxidation states between the extremes.[10] Interest in the biological roles of RSS has increased dramatically following the 1996 discovery that hydrogen sulfide (H_2S) serves as an endogenous neuromodulator.[11] In the time since this initial report, many different types of H_2S-releasing compounds (termed H_2S donors) have been synthesized to aid in probing its signaling pathways and to realize its therapeutic potential. Thus far, H_2S donors with a variety of triggers have been developed, including water,[12–14] nucleophiles,[15–16] light,[17–18] and enzymes.[19–22] More recently, donors of related RSS, including carbonyl sulfide (COS),[23–24] sulfur dioxide (SO_2),[25–28] and persulfides (also called perthiols, R–SSH)[29–32] have also been developed. All of these donors increase our understanding of the roles that RSS play in a biological context, and some may hold therapeutic value in the form of prodrugs and drug conjugates.[6, 33]

In particular, persulfides are now of interest because recent advances in redox biology indicate that native persulfides carry out vital physiological functions as signaling products of H_2S.[34–37] Persulfides contain an internal sulfur atom with an oxidation state of 0 and are endogenously constructed *via* disulfide exchange reactions between H_2S and biologically relevant disulfides mediated by cystathionine gamma lyase (CSE) and cystathionine β-synthase (CBS). Due to the high abundance of their corresponding thiols, the most common persulfides in eukaryote systems are cysteine persulfide (Cys–SSH) and glutathione persulfide (G–SSH).[38] The physiological importance of persulfides is likely due to their enhanced nucleophilicity (*via* the alpha effect) relative to thiols.[39] This increase in nucleophilicity has multiple proposed effects on protein activity, where S-atom transfer from Cys–SSH or G–SSH generates persulfidated Cys residues on proteins.[40] For example, persulfidated proteins are generally more reactive than their native (i.e., unpersulfidated) analogs. Another outcome of protein persulfidation is protection of enzyme thiols from irreversible oxidation, where the terminal sulfur atom of a persulfide reacts with oxidants more readily than the internal sulfur atom.[41–42] Breaking the S–S bond in a reductive step then regenerates the protein thiol, so the terminal sulfur atom acts as a sacrificial oxidant in the process. Thus, with multiple mechanisms to enhance or protect enzyme activity, persulfides appear to be important players in the reactive species interactome.[43]

Here we aimed to develop polymer persulfide donors, with the goal of extending their release half-life compared with small molecule persulfide donors and evaluate the biological effects resulting from sustained release. No polymeric persulfide donors have been reported, but currently available small molecule persulfide donors release persulfides in response to a variety of stimuli, including hydrogen peroxide (H_2O_2), esterase activity, pH changes, and addition of F^-.[29–32, 44] For example, Wang and coworkers reported an esterase-sensitive persulfide donor that utilized a 1,2-elimination reaction to release its persulfide payload (Scheme 1a), showing protective effects in myocardial ischemia/reperfusion injury.[31] The same group also recently reported the synthesis and characterization of a G–SSH donor using a trimethyl lock caging group (Scheme 1b).[45] Our lab recently developed a persulfide donor that responds to H_2O_2, utilizing a 1,6-elimination reaction to release *N*-acetyl cysteine persulfide (NAC–SSH), which rescued H9C2 cardiomyocytes from H_2O_2-induced oxidative stress as effectively or more effectively than a number of H_2S donors.[29] In spite of this recent progress in the development of persulfide donors, none thus far are capable of providing sustained persulfide release under specific release conditions. Persulfide prodrugs with longer half-lives could be more effective in mitigating chronic disease states (such as inflammation) than fast donors because fewer administrations would be required for the same level of therapeutic effects. Therefore, we hypothesized that a polymeric persulfide prodrug could provide longer lasting persulfide release and alleviate sustained oxidative stress more effectively than an equivalent small molecule by sterically shielding the cleavable bond from a triggering enzyme.

Results and Discussion

Prodrug design

We envisioned that the ideal persulfide prodrug would be inert under physiological conditions but undergo controlled decomposition in response to a stimulus, resulting in the breakdown of any caging groups (or linkers) and release of the persulfide payload (Scheme 1c). To avoid premature release, the persulfide payload should be caged using a protecting group that is stable in biological media but responds to a stimulus to release the persulfide under desired conditions. Therefore, we envisioned that a benzyl linker capable of undergoing 1,6-elimination (sometimes called self-immolation)[46] would provide a means of protecting the persulfide, yet reveal it under conditions governed by the functionality installed at the *para* position. Several prodrug systems have utilized this linker to effectively release RSS, including COS and H_2S prodrugs and our previously reported H_2O_2-responsive persulfide prodrug.[22, 24, 29] Ester functionalities are responsive to biologically ubiquitous esterases and are easy to install on the benzyl linker, and release half-lives can be dramatically increased simply by installing bulkier esters.[47] Therefore, we set out to synthesize a modular, esterase-responsive persulfide prodrug with the potential for conjugation to a polymer backbone.

Prodrug Synthesis and Confirmation of Persulfide Release with FDNB

Given these design parameters, we first synthesized a small molecule persulfide donor, which we termed EDP-NAC (Ester Disulfide-Prodrug *N*-Acetyl Cysteine). EDP-NAC was synthesized in four simple steps with an overall yield of 44% (Scheme 2). First, *p*-cresol was

acetylated with acetic anhydride in the presence of Na_2CO_3 as a proton scavenger. This product, *p*-tolyl acetate (**1**), was then brominated using *N*-bromosuccinimide (NBS) as a bromine source and azobisisobutyronitrile (AIBN) as a radical initiator. The resulting benzyl bromide (**2**) was then converted into a thiol using thiourea as a nucleophile, followed by addition of *N*-butylamine to cleave the resulting thiouronium intermediate, generating *p*-(mercaptomethyl)phenyl acetate (**3**). Finally, reaction of thiol **3** with activated *N*-acetyl cysteine disulfide (NAC-pyDS) afforded EDP-NAC. EDP-NAC is water soluble up to low millimolar concentrations and dually tunable at both the ester and disulfide.

Persulfide-release from EDP-NAC was then evaluated using fluorodinitrobenzene (FDNB), which has been shown to efficiently trap persulfides.[48] For all persulfide release experiments, we triggered release using porcine liver esterase (PLE), a representative esterase from this broad class of ubiquitous ester-hydrolyzing enzymes.[49] A one-dram vial was charged with a solution of EDP-NAC, FDNB, and water to give a clear, yellow solution. An aliquot was removed for a t=0 timepoint, then PLE was added to the reaction mixture, and additional aliquots were withdrawn at pre-determined time points for analysis by analytical HPLC (Figure 1, S18). At t=0, a single peak was observed at an elution time of 3.8 min, corresponding to EDP-NAC. This peak decreased in intensity over time, and a new peak appeared at 4.9 min elution time over the course of an hour. This new peak showed the same elution time as authentic dinitrobenzene *N*-acetylcysteine disulfide (DNB-NAC), confirming that it was the product of the trapping reaction between FDNB and NAC-SSH (DNB-NAC, Figure S19). These results highlight the clean conversion from EDP-NAC to NAC persulfide.

Convinced that the small molecule prodrug released NAC-SSH as intended, we set out to design a polymeric persulfide donor, termed poly(EDP-NAC) (Scheme 3). First, a block copolymer composed of 2-hydroxyethyl methacrylate and oligo(ethylene glycol) methacrylate, poly(HEMA-*co*-OEGMA), was synthesized using reversible addition-fragmentation chain-transfer (RAFT) polymerization. RAFT was chosen due to its ability to provide good control over the degree of polymerization and molar mass dispersity ($Đ_M$) of the resulting copolymers.[50] Next, poly(HEMA-*co*-OEGMA) (M_n = 10.4 kg/mol, $Đ_M$ = 1.28) was then coupled to EDP-NAC *via* EDC coupling at near 100% conversion to form poly(EDP-NAC) (M_n = 13.2 kg/mol, $Đ_M$ = 1.19). Comparative SEC traces of poly(HEMA-*co*-OEGMA) and poly(EDP-NAC) are included in Figure S22, showing a clear shift toward lower elution time after attachment of EDP-NAC. Additionally, poly(EDP-NAC) at 50% functionalization was synthesized using the same methods (Figure S14).

Evaluation of Persulfide Release Kinetics

With EDP-NAC and poly(EDP-NAC) in hand, we set out to compare their persulfide release kinetics. Because analytical HPLC is difficult with polymeric substrates, we used ^{1}H NMR spectroscopy to compare the half-lives between the small molecule and polymer prodrugs. First, EDP-NAC and poly(EDP-NAC) were each dissolved in DMSO-d_6 in an NMR tube, diluted with D_2O, and a t=0 timepoint was taken. Of note, poly(EDP-NAC) was much more water soluble than EDP-NAC, but a ratio of 1:4 DMSO-d_6:D_2O was used in both

experiments for sake of consistency. Next, PLE was injected into each NMR tube, and spectra were collected at subsequent timepoints as shown in Figure 2.

By comparing ^{1}H NMR spectra over time for both EDP-NAC and poly(EDP-NAC), pseudo-first-order persulfide release half-lives were determined. Figure 2B shows stacked spectra for EDP-NAC treated with PLE. Before the addition of PLE (t=0 h), two aromatic signals corresponding to EDP-NAC were observed at 7.0 and 7.3 ppm (a and b, respectively). After addition of PLE (t=0.4 h), two new sets of signals appeared in the aromatic region (6.7 and 7.1 ppm, a' and b' respectively). These signals were attributed to the formation of *p*-hydroxybenzyl alcohol (pHBA), as confirmed by comparison to an authentic standard. No other aromatic signals were present in the ^{1}H NMR spectra, indicating clean conversion of EDP-NAC to pHBA. With these results, we measured a pseudo-first-order release half-life ($t_{1/2}$) of 1.6 h ± 0.3 h. Using a similar technique with dimethyl sulfone as an internal standard (Figure S20), the $t_{1/2}$ of 100% functionalized poly(EDP-NAC) was measured to be 36.0 h ± 0.6 h, while the $t_{1/2}$ of 50% functionalized poly(EDP-NAC) was measured to be 26 ± 3 h. A kinetics plot comparing EDP-NAC to 100% functionalized poly(EDP-NAC) under the same release conditions is shown in Figure 2C. Only 100% functionalized poly(EDP-NAC) was utilized in *in vitro* studies due to its longer half-life.

In Vitro Evaluation of EDP-NAC and poly(EDP-NAC) Against ROS-induced Toxicity

Persulfides, naturally-occurring biological reductants, possess the capacity to quench reactive oxygen species (ROS), and thus reduce inflammation. Thus, we set out to investigate the ability of EDP-NAC and poly(EDP-NAC) to quench ROS *in vitro*. First, however, we evaluated cell viability of EDP-NAC and poly(EDP-NAC). H9C2 cardiomyocytes were treated with EDP-NAC and incubated for 4 h before measuring viability. Both EDP-NAC and poly(EDP-NAC) exhibited no toxicity under these conditions, maintaining high cell viability up to concentrations of 400 μM prodrug (Figure S25A and B).

H_2O_2 is one of the most common naturally occurring ROS. We expected EDP-NAC, with its short persulfide release half-life, to be effective in combating toxicity induced by direct addition of H_2O_2 to cells. In contrast, 5-fluorouracil (5-FU) is a common cancer drug that produces ROS slowly over time; in fact, it is typically administered *via* bolus injection on a weekly schedule due to its sustained production of ROS over the course of multiple days.[51] Additionally, dosing of 5-FU in cancer patients is largely limited by off-target toxicity due to its narrow therapeutic window. For healthy cells exposed to 5-FU, we envisioned that poly(EDP-NAC) might be effective in mitigating ROS-induced toxicity due to its ability to produce persulfides in a sustained manner. Therefore, given the large discrepancies in half-life, we hypothesized that EDP-NAC would be better suited to quench ROS on a shorter timescale (direct H_2O_2 addition), while poly(EDP-NAC) would perform better in response to a sustained release of ROS (generated as a result of treatment with 5-FU) due to its extended persulfide release half-life.

To investigate the antioxidative properties of the prodrugs, H9C2 cardiomyocytes were exposed to H_2O_2 (100 μM) concomitantly with EDP-NAC or poly(EDP-NAC) for 1 h incubation time (Figure 3A). Cells receiving no persulfide donor (i.e., H_2O_2 only), were only

45% viable after H_2O_2 treatment. In contrast, EDP-NAC increased viability to 59% at 100 μM and 78% at 200 μM, demonstrating the ability of EDP-NAC to rescue cells from the hazardous effects of H_2O_2. Poly(EDP-NAC) was not able to rescue cells as well under these conditions—treatment with 100 μM and 200 μM led to statistically smaller increases in viability over the H_2O_2-only control, reaching 51% and 57% viability, respectively. Therefore, consistent with our hypothesis, EDP-NAC was more effective in mitigating H_2O_2-induced oxidative stress than poly(EDP-NAC). Similar experiments were performed with KO_2 as a donor of superoxide, another type of endogenous reactive oxygen species, and the same trends were observed (Figure S24A).

Next, EDP-NAC and poly(EDP-NAC) were evaluated for their efficacy in rescuing H9C2 cells from 5-FU exposure. EDP-NAC and poly(EDP-NAC) were each co-administered with 5-FU (200 μM) for 4 h incubation time. Cells receiving no prodrug were only 51% viable after treatment with 5-FU. Co-treatment with poly(EDP-NAC) (200 μM) resulted in nearly 100% H9C2 viability under this timescale, whereas co-treatment with EDP-NAC (200 μM) only resulted in 65% viability. (Figure 3B) These findings are consistent with the extended half-life of poly(EDP-NAC) providing an appropriate amount of available persulfide to combat ROS produced slowly by 5-FU, whereas EDP-NAC is consumed at a rate that is most likely too fast to combat sustained ROS production.

Ideally, antioxidative effects would only be observed in non-cancer cells for EDP-NAC (or poly(EDP-NAC)) to be considered usable for co-administration in chemotherapy. Therefore, we investigated the ability of EDP-NAC and poly(EDP-NAC) to rescue MCF-7 breast cancer cells against 5-FU exposure. First, general toxicity assays were performed by treating MCF-7 cells with each prodrug for 4 h. No decrease in viability was observed up to 200 μM (Figure S25D). Next, EDP-NAC and poly(EDP-NAC) were each co-administered with 5-FU (200 μM) in MCF-7 cancer cells and incubated for 4 h. Neither EDP-NAC nor poly(EDP-NAC) rescued MCF-7 cells from 5-FU exposure, as viability was consistently around 50% in all treatment groups compared with an untreated control group (Figure 3C). Additionally, we performed an identical MCF-7 toxicity assay for 24 h treatment time and observed similar outcomes, with viabilities near 40% for all treatment groups (Figure S24B). We hypothesize that the inability of EDP-NAC and poly(EDP-NAC) to rescue MCF-7 cells from 5-FU exposure results from a shift in redox balance of the cancer cells towards a more reducing environment, which contrasts with their naturally higher oxidative cell environment.[52]

Taken together, these cell experiments show that EDP-NAC was more effective in mitigating ROS-induced toxicity in healthy H9C2 cardiomyocytes by H_2O_2, an immediately available form of ROS. However, poly(EDP-NAC) was more effective in mitigating toxicity in H9C2 cells treated with 5-FU, a sustained ROS-inducer. Neither EDP-NAC nor poly(EDP-NAC) rescued MCF-7 breast cancer cells from 5-FU exposure, suggesting that persulfide donors like EDP-NAC and poly(EDP-NAC) could be useful in mitigating cardiotoxicity without hindering anticancer activity of oncology medications.

Conclusions

In summary, a polymeric persulfide prodrug, poly(EDP-NAC), was synthesized for the first time. Persulfide release, as characterized by ^{1}H NMR spectroscopy and analytical HPLC, was over an order of magnitude slower in poly(EDP-NAC) than a small molecule analog (EDP-NAC). In cell studies, EDP-NAC rescued H9C2 cardiomyocytes from H_2O_2 exposure more effectively than poly(EDP-NAC), while poly(EDP-NAC) rescued H9C2 cells exposed to 5-FU more effectively than EDP-NAC. Neither EDP-NAC nor poly(EDP-NAC) mitigated 5-FU-induced toxicity in MCF-7 breast cancer cells, suggesting that persulfide prodrugs may be appropriate for coadministration with chemotherapeutics to minimize off-target toxicity. This work highlights the importance of matching the release half-life of persulfide prodrugs with the appropriate disease condition, placing polymeric persulfide donors in a promising position to combat chronic disease states due to their extended release profiles and potentially extended circulation times in comparison to small molecule analogs. We envision that similar polymeric persulfide prodrug systems may be developed to afford a wide variety of materials for end-use applications including gels, coatings, and functional biomaterials that are properly tuned to combat specific disease indications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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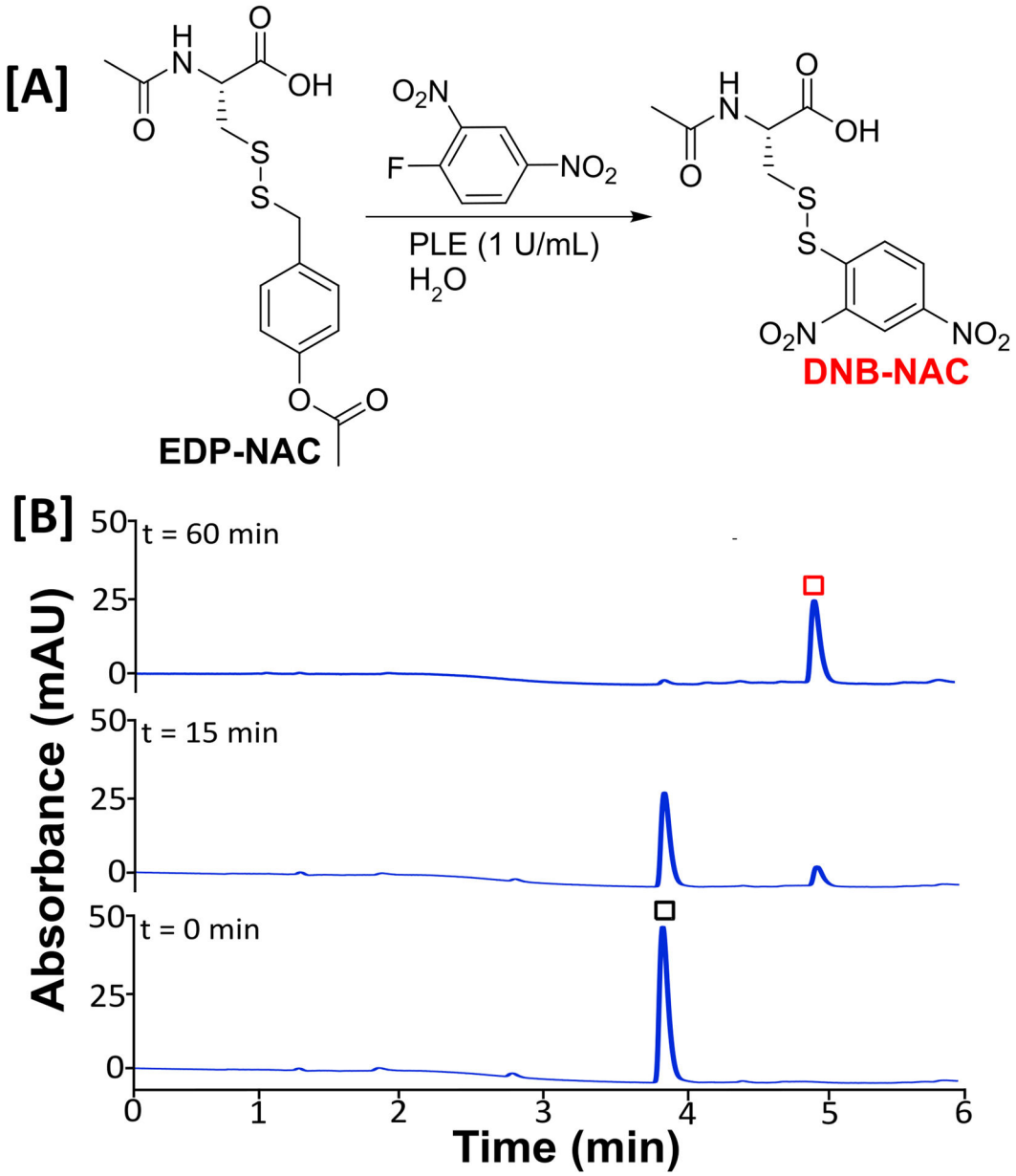


Figure 1: Persulfide trapping from EDP-NAC via reaction with FDNB.

[A] Scheme depicting persulfide trapping using FDNB; [B] Analytical HPLC traces showing the loss of EDP-NAC and the production of persulfide adduct DNB-NAC over time. The peak eluting at 3.8 min (black square) corresponds to EDP-NAC while the peak at 4.9 min (red square) corresponds to the persulfide adduct DNB-NAC. Monitoring wavelength was 282 nm.

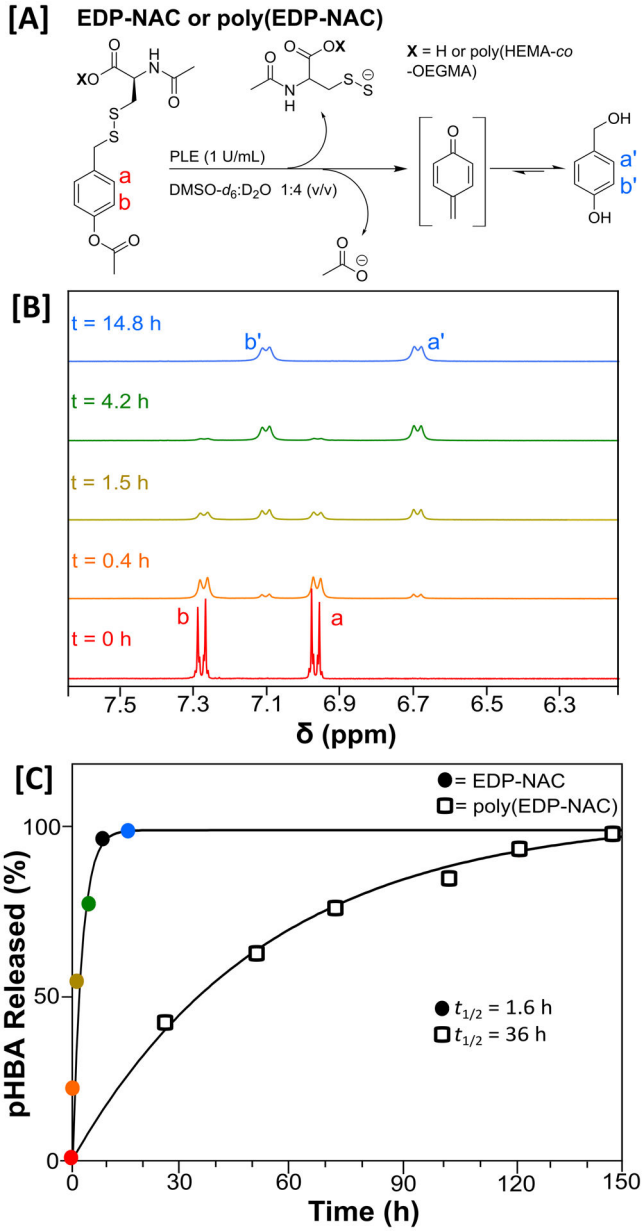


Figure 2: ^{1}H NMR analysis of pHBA release kinetics.

[A] Proposed mechanism of pHBA release from EDP-NAC and poly(EDP-NAC); [B] ^{1}H NMR traces depicting disappearance of EDP-NAC and the appearance of pHBA; [C] Comparative kinetics plots of EDP-NAC and poly(EDP-NAC). Release half-lives were measured to be 1.6 h ± 0.3 h and 36.0 h ± 0.6 h for EDP-NAC and poly(EDP-NAC), respectively.

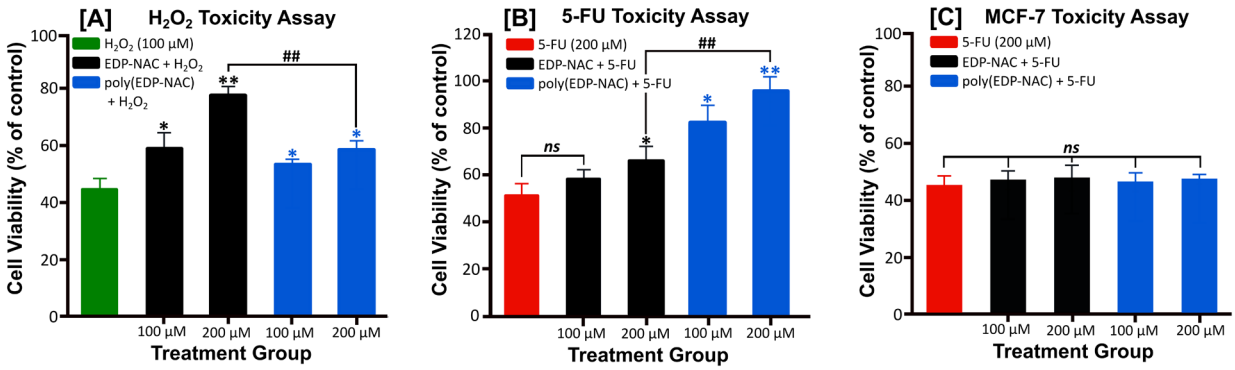
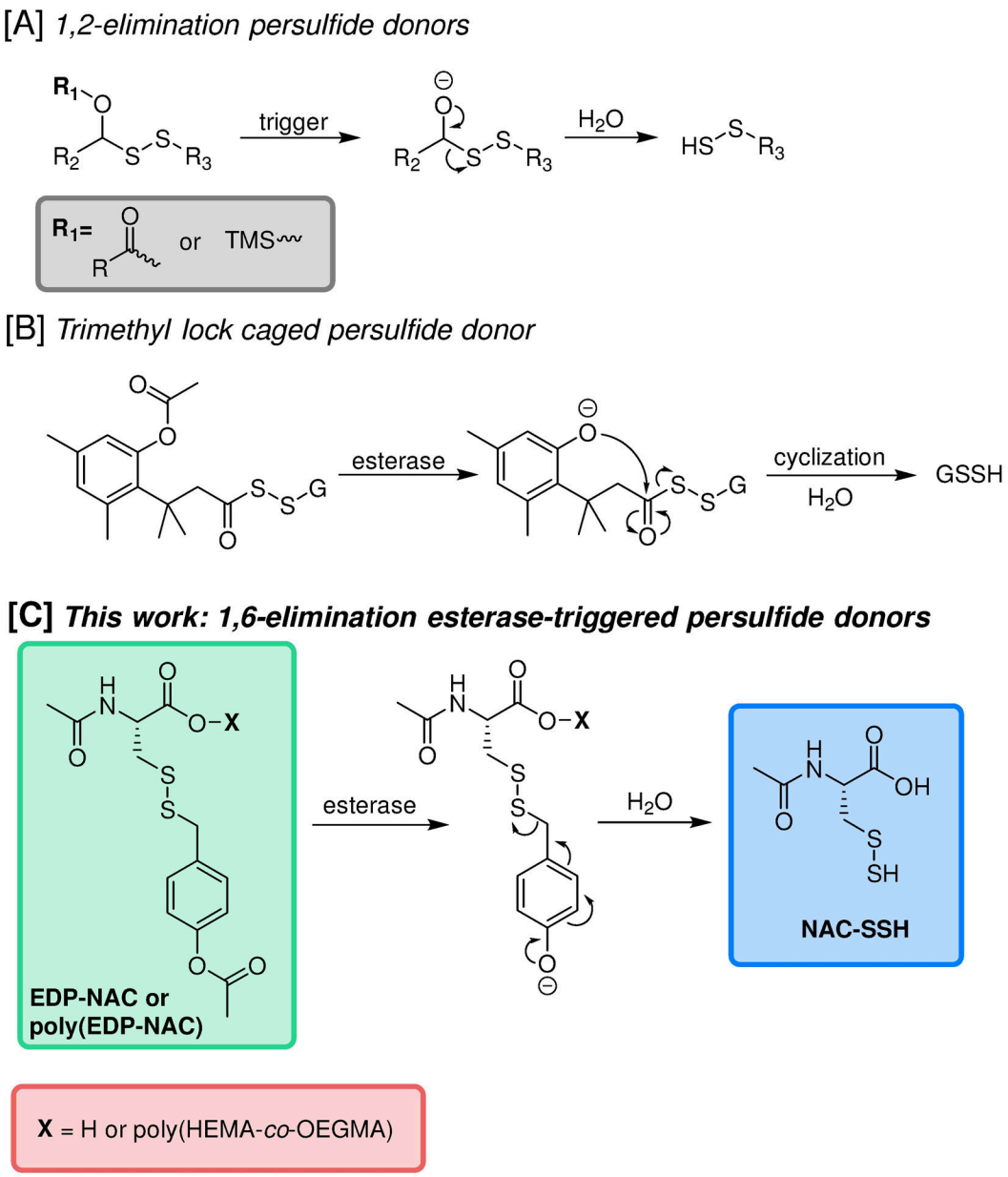


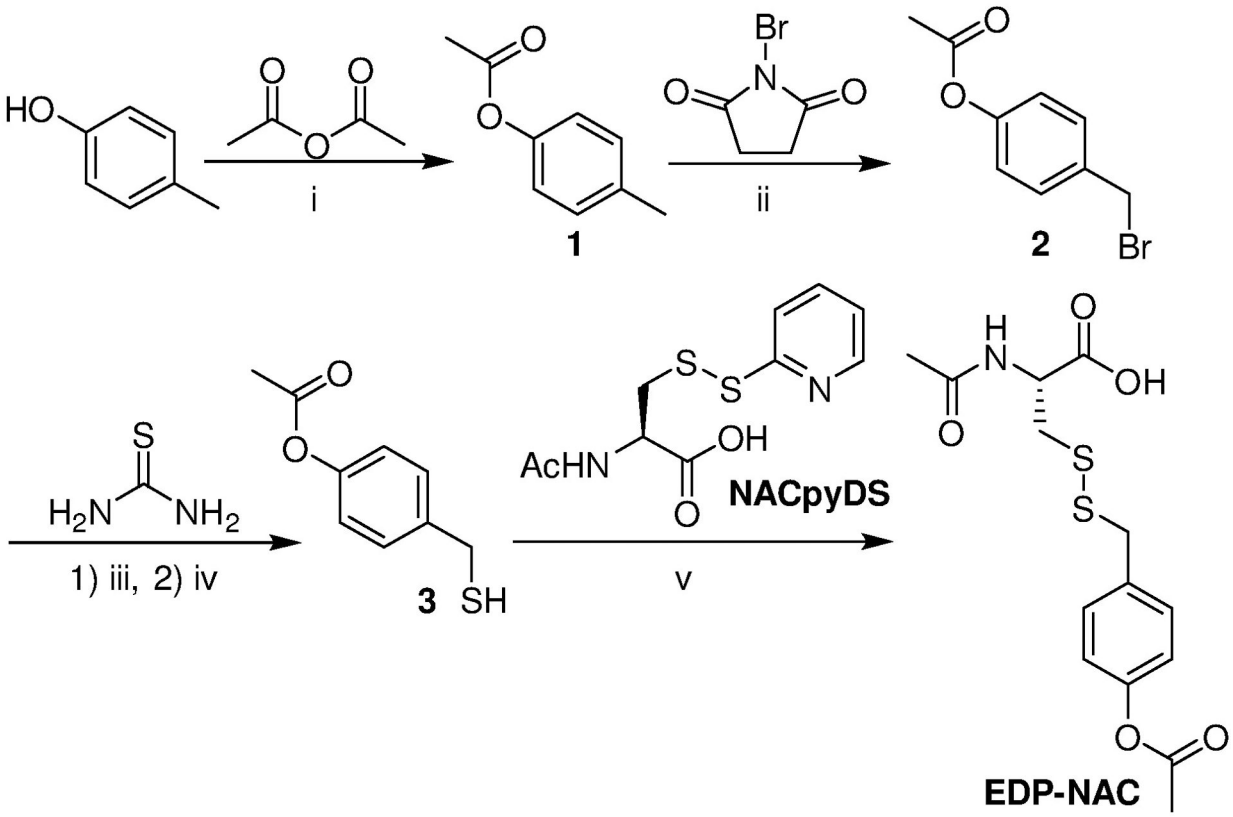
Figure 3: Cell viability of H9C2 (panels A and B) or MCF-7 cells (panel C) treated with EDP-NAC or poly(EDP-NAC).

[A]: H9C2 H_2O_2 toxicity assay. H9C2 cells were treated with 100 μM H_2O_2 only (green bar) or co-treated with H_2O_2 and EDP-NAC (black bars) or H_2O_2 and poly(EDP-NAC) (blue bars) for 1 h exposure time. [B]: H9C2 5-FU toxicity assay. H9C2 cells were treated with 200 μM 5-FU only (red bar) or co-treated with 5-FU and EDP-NAC (black bars) or 5-FU and poly(EDP-NAC) (blue bars) for 4 h exposure time. [C]: MCF-7 5-FU toxicity assay. MCF-7 cells were treated with 5-FU only (red bar) or co-treated with 5-FU and EDP-NAC (black bars) or 5-FU and poly(EDP-NAC) (blue bars) for 4 h exposure time. Quantification of cell viability was conducted using Cell Counting Kit-8 (CCK-8). *P<0.05 and **P<0.01 for comparisons against H_2O_2-only or 5-FU-only control treatment groups. $^{\#\#}$P<0.01 for comparisons between EDP-NAC and poly(EDP-NAC) as indicated. Error bars indicate standard deviation of three separate experiments (n = 3, with 4–5 wells in each group in each experiment). Statistical analyses were conducted using a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls post-hoc test.



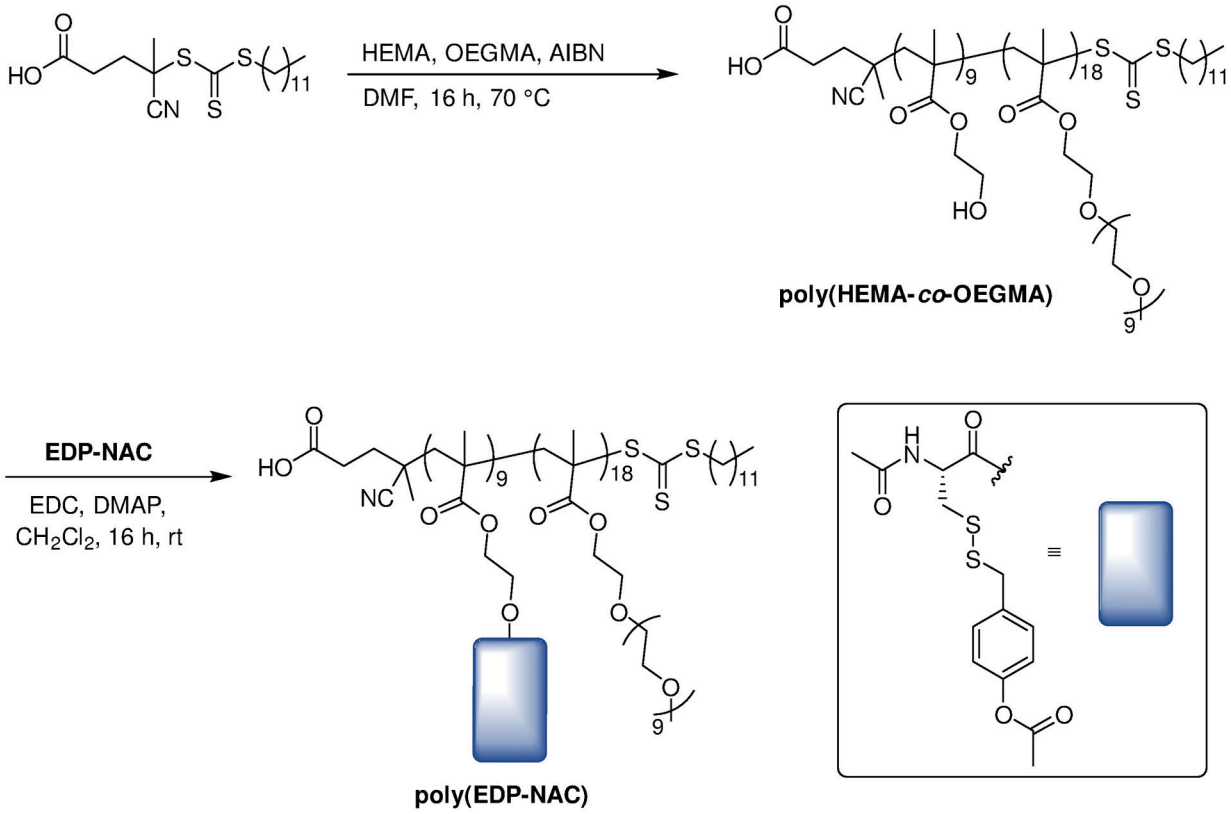
Scheme 1: Depiction of reported esterase-triggered persulfide donors and the small molecule and polymeric persulfide donors targeted here.

[A] 1,2-Elimination persulfide donors; [B] Trimethyl lock caged persulfide donor with pendant glutathione (GSH) disulfide; [C] Small molecule and polymeric esterase-responsive 1,6-elimination persulfide donors (this work).



Scheme 2: Synthesis of EDP-NAC.

Reaction conditions: i) Na_2CO_3, EtOAc, rt, 16 h; ii) AIBN, benzene, reflux, 16 h; iii) MeOH, rt, 16 h; iv) butylamine, $CHCl_3$, rt, 24 h; v) CH_2Cl_2, rt, 16 h



Scheme 3.

Synthetic route to poly(HEMA-*co*-OEGMA) and poly(EDP-NAC) copolymers