Polymers with dithiobenzoate endgroups constitutively release hydrogen sulfide upon exposure to cysteine and homocysteine

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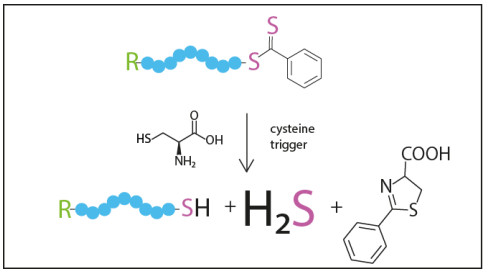
ABSTRACT: Dithioesters are well-established as efficient reversible addition fragmentation chain transfer (RAFT) agents. More recently, certain small molecule dithioesters have been reported to release the biological signalling molecule hydrogen sulfide (H_2S) upon exposure to cysteine. Herein, we examine the propensity of polymers synthesized using RAFT with a dithioester chain transfer agent to release H_2S via reaction of cysteine with constitutive dithioester end-groups. Homocysteine-triggered release of H_2S from these materials is also observed, with evidence suggesting that the mechanism is analogous to reaction with cysteine.



Polymerization using reversible addition fragmentation chain transfer (RAFT) has been widely utilised to prepare biomedical materials including drug delivery vehicles, biocompatible surface coatings and intrinsically bioactive substances.[1] The tight molecular weight control afforded by RAFT and its compatibility with a wide range of monomers makes it an attractive choice for these purposes.[2] Futhermore, polymers synthesized via RAFT are generally well tolerated at low concentrations.[3] Nevertheless, it has been postulated that cleavage of the thiocarbonylthio endgroups originating from the RAFT agent could contribute to biological toxicity.[2] This is usually overcome by removing the end-groups before biological application through a number of well characterised photo/chemical techniques.[4] As a consequence, comprehensive knowledge of the biological effects of many RAFT end-groups is still limited.

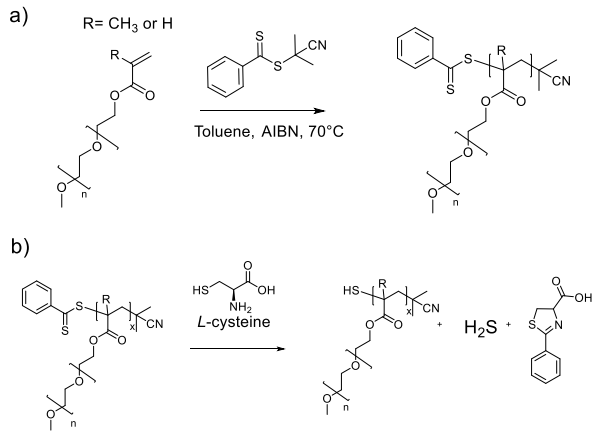
Herein, we investigated polymers with terminal dithiobenzoate moieties synthesized via RAFT as potential hydrogen sulfide (H_2S) donors. H_2S has recently been recognized as one of the three gasotransmitters, a small family of otherwise gaseous signalling molecules with a wide array of biochemical signalling functions.[5] Similarly to the other members of this family (nitric oxide (NO) and carbon monoxide (CO)), endogenous H_2S production underlies a plethora of important physiological functions.[6] This has prompted research groups to explore the therapeutic potential of H_2S donors across a wide range of applications including Parkinson's disease,[7] diabetes[8] and cardiovascular disease.[9]

The complex and often indiscriminate nature of H_2S activity has driven the requirement for sophisticated H_2S donors with well characterised biological activity. To this end, significant progress has been made in the spatio-temporally controlled release of H_2S, first with the replacement of classically used sulfide salts by controlled release small molecule donors[10-11] and, more recently, with the emergence of macromolecular donors.[12-14] Promisingly, it has been reported these macromolecular materials often provide more favourable pharmacological outcomes compared to their small molecule analogues. Given the aforementioned benefits of polymers synthesized by RAFT, it is no surprise that RAFT has been employed to prepare macromolecular H_2S donors. For instance, polymers synthesized via RAFT have been used as a scaffold to which H_2S releasing moieties are attached[15-23] or in other cases the polymer end-group has itself been modified to release H_2S.[24] Several groups, including our own, have previously employed dithiobenzoate RAFT agents to prepare polymers which release H_2S, with the resulting end-groups either modified or removed prior to investigation of the H_2S release.[21-24] However, there are some examples where the end groups have been left intact.[20]

Recently, it was observed that H_2S can be released from certain small molecule thionoesters[25] and dithiobenzoates[26] upon reaction with cysteine. A comprehensive analysis of H_2S release from a series of dithiobenzoate small molecules indicated that the rate of cysteine-mediated H_2S release is tuneable via structural and electronic modifications. Herein, we demonstrate that, when polymers are synthesized using a dithiobenzoate as the chain transfer agent, H_2S can be released from the native dithiobenzoate end-groups of the resulting polymers. Specifically, we demonstrate that the dithiobenzoate end capped poly(meth)acrylates react with both cysteine and homocysteine to yield thiol-terminated polymer, a dihydrothiazole- or a dihydrothiazine-containing small molecule and H_2S.

To investigate these reactions, poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) and poly[oligo(ethylene glycol) methyl ether acrylate] (POEGA) were first synthesized using 2-cyano-prop-2-yl benzodithioate (CPDB) as a RAFT agent (Scheme 1a). Approximately 50% monomer conversion was achieved in each case by reacting at 70°C for 6 h and 21 h respectively. The resulting polymers were isolated by precipitation into petroleum spirit and characterised via ^{1}H NMR spectroscopy (Figures S1 and S2). Estimation of molecular weights by ^{1}H NMR yielded values of 8400 g mol^{-1} and 6600 g mol^{-1}, respectively. Additionally, the signals associated with the aromatic dithiobenzoate group were clearly visible in the spectra indicating that the dithiobenzoate functionality was preserved during the polymerization and purification (as expected). Detailed synthetic protocols are provided in the supplementary information.

To examine whether these dithiobenzoate-terminated polymers synthesized via RAFT were able to release H_2S upon exposure to various potential triggers, the synthesized POEGA and POEGMA were exposed to 10 equivalents of L-cysteine, homocysteine, *N*-acetylcysteine and glutathione, and the liberation of H_2S was analysed using an H_2S-selective amperometric probe. Both POEGA (Figure 1)



Scheme 1 - a) Synthesis of dithiobenzoate terminated POEGMA and POEGA using RAFT. b) Expected reaction products after exposure to cysteine based on literature report of small molecule dithiobenzoate H_2S donors.[26]

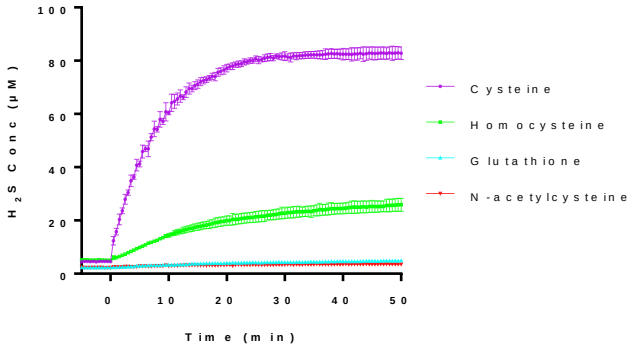


Figure 1 - H_2S release from POEGA (200 μM) in pH 7.4 PBS measured by an amperometric microsensor after polymer exposure to 10 equivalents of stimulus. Data are mean ± s.d., n=3.

and POEGMA (Figure S3) displayed similar H_2S release behaviour, with both L-cysteine and homocysteine proving to be effective stimuli for the liberation of H_2S. In the case of L-cysteine, the H_2S concentration peaked approximately 30 min after the addition of the trigger molecule. H_2S release was also confirmed upon POEGA exposure to an approximate intracellular cysteine concentration (200 μM), thus verifying the physiological relevance of this phenomenon (Figure S4). Homocysteine triggered H_2S release was, on the other hand, substantially lower than for cysteine, with a quarter of the amount released over the first 50 mins of testing. Importantly, negligible release was observed when both *N*-acetylcysteine and glutathione were employed as the trigger, which is consistent with the mechanism of H_2S being analogous to that observed for small molecule dithioesters (Scheme 1b).[26] Specifically, the release requires a trigger molecule having both thiol- and amino- substituents positioned to facilitate intramolecular cyclization, yielding the intermediate **3a** from which H_2S is emitted (Figure 2a).

To investigate the kinetics and mechanism of H_2S release from the dithiobenzoate terminated polymers, ^{1}H NMR spectra were recorded for 24 h following introduction of either cysteine or homocysteine (~10 equiv.) to a solution of polymer in D_2O (20 mg/mL (Figure 2b and S5). Addition of both cysteine and homocysteine resulted in consumption of the dithiobenzoate end-groups over the ensuing 24 h, accompanied by the formation of new peaks in the aromatic region arising from the expected dihydrothiazole- and dihydrothiazine-containing small molecule products (**4a** and **4b**, respectively). For comparison purposes, the dihydrothiazole small molecule **4a** was also synthesised separately by reaction of benzonitrile and cysteine via a thioimidate intermediate (Figure S6 and S7). Based on these spectra, it is clear that H_2S production correlates with consumption of the dithiobenzoate end group and formation of the anticipated dihydrothiazole by-product(**4a**). Moreover, cell viability experiments revealed this molecule is well tolerated in HEK293 cells up to 100 μM (Figure S8).

Additionally, HPLC/LCMS experiments were conducted to further elucidate the mechanism of H_2S release from the

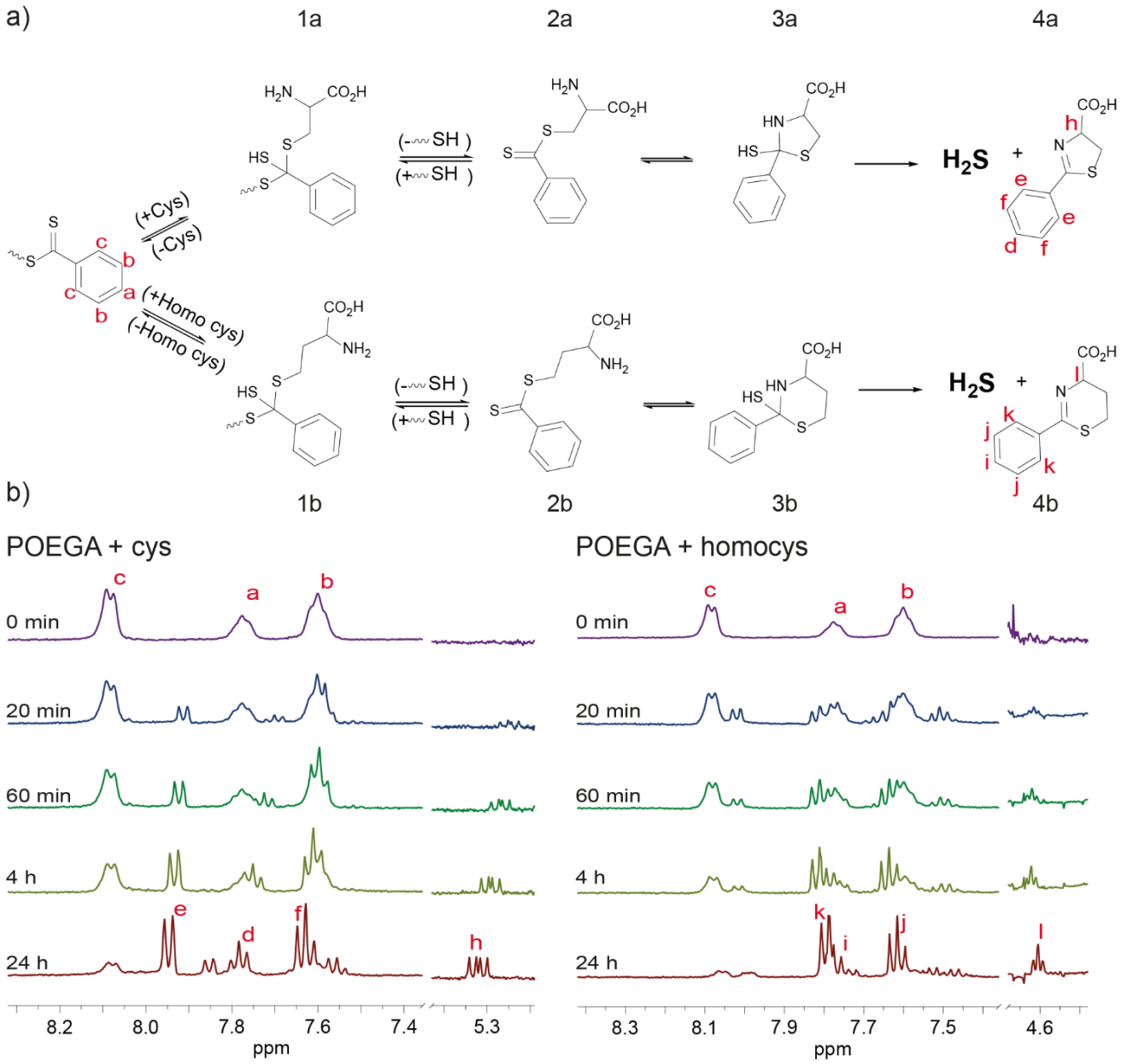


Figure 2 – a) Expected mechanism for H_2S release from dithiobenzoate polymer end-groups when triggered by cysteine or homocysteine. b) ^{1}H NMR analysis of POEGA after the addition of 10 equivalents of cysteine (left) or homocysteine (right) as a function of time.

polymer end-groups. In the case of cysteine triggered H_2S release, calibration with the synthesised dihydrothiazole **4a** (Figure S9) was used to estimate that ~90% of the polymer end-groups were reacted after 1 h. This differs somewhat from the ^{1}H NMR data, most likely due to the difference in reaction solvent/conditions (pH 7.4 PBS vs D_2O). Furthermore, LCMS analysis after 1 h of the cysteine-triggered reaction revealed only the dihydrothiazole was present: neither intermediate **2a** nor **3a** was observed (Figure S10 and S11). In contrast, in the case of homocysteine-triggered release, the final product **4b** was observed alongside an intermediate which was determined to be **2b** based on both its mass and fragmentation products (Figure S12). This suggests that the lower release of H_2S in the presence of homocysteine may be attributable to slower intramolecular cyclisation of **2b** relative to **2a**. Specifically, it is thought that the initial nucleophilic attack by cysteine and homocysteine to form tetrahedral intermediates **1a** and **1b** respectively occur at a similar rate. This is evidenced by the similar reduction in the ^{1}H NMR polymer end-group peaks across the first 24 h. The difference in the rate of H_2S generation from cysteine-triggered release compared to homocysteine is likely due to the rate of one of the other intermediate steps. This difference is highlighted in the LCMS experiments by the sustained presence of intermediate **2b** after 1 hour in the case of homocysteine triggered H_2S release, whereas intermediate **2a** is not observed for cysteine-mediated release. Cerda *et al* determined that the initial addition of cysteine to the dithioester to generate **1a**

is the rate-determining step for cysteine-triggered H_2S release.[26] However, in the case of homocysteine triggered-release the intramolecular cyclization via nucleophilic attack of the pendant amine to form **3b** is sufficiently slow to account for both the slower H_2S release and persistence of intermediate **2b** at longer reaction times.

Since exposure of phenyl dithiobenzoate to L-cysteine was previously shown to liberate thiophenol as a second by-product,[26] we anticipated that polymeric thiol should be produced when dithiobenzoate-terminated polymers are exposed to L-cysteine. To examine this possibility, the polymer product after reaction was investigated using gel permeation chromatography (Figure 3). After reaction between POEGA and cysteine, the molecular weight distribution broadened significantly, consistent with the expected liberation of thiol terminated polymer and subsequent dimerization to form a disulphide with double the initial molecular weight. To confirm that this broadening was indeed due to disulphide formation, the product was treated with the reducing agent tris(2-carboxyethyl)phosphine in the presence of oligo(ethylene glycol) methyl ether acrylate 480 to 'catch' the liberated thiol via a Michael addition. The results demonstrate that both the Đ is restored to the original value and the MW returns to a value congruous with thiol-ene Michael addition. Interestingly, when POEGMA is reacted similarly (Figure s13) disulfide coupling is not observed, which may be due to the effect of back biting to form a thiolactone chain end[27] or higher stability of the methacrylyl thiol.

In summary, we have shown that native dithiobenzoate polymer end-groups produced during RAFT polymerization release H_2S when exposed to either cysteine or homocysteine. Such polymers may hold untapped promise as either a standalone H_2S donor or as reactive pharmaceutical excipients for co-delivery of H_2S. The simple preparation of these materials via RAFT polymerisation and the innate nature of this groups lends itself to the development of complex H_2S donating polymeric architectures without the need for post polymerization modifications. However, considering the dichotomous nature of H_2S, the findings of this work equally serve as a warning to researchers employing dithioester end-group polymers for biological applications, as the incidental release of H_2S may trigger unwanted (and hitherto unexplored) biochemical effects. As such, we would counsel caution in the bio-application of these materials without first removing the dithiobenzoate end-group.

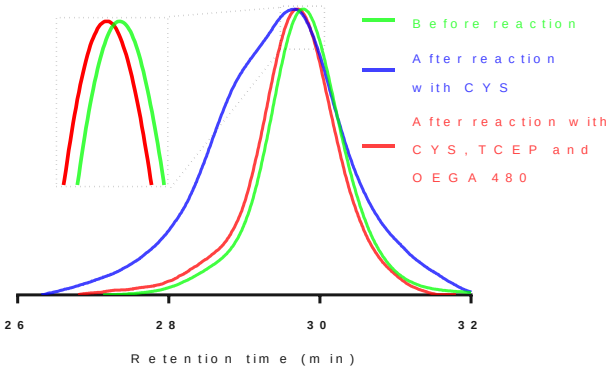


Figure 3- Gel permeation chromatography analysis of POEGA reaction product with cysteine showing (i) initial monomodal distribution; (ii) thiol formation and concomitant dimerization; and (iii) recovery of monomodal distribution following reduction and subsequent Michael addition with $OEGA_{480}$.

ASSOCIATED CONTENT

Supporting Information. Description of materials, synthetic protocols, analytical techniques, ^1H and ^{13}C NMR spectra, HPLC and LCMS results, GPC data for POEGMA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Any additional relevant notes should be placed here.

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ABBREVIATIONS

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