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Triggered and Tunable Hydrogen Sulfide Release from Photo-Generated Thiobenzaldehydes

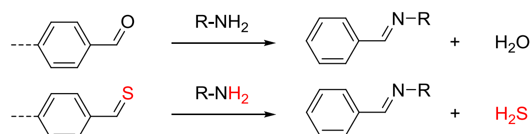
Zeyun Xiao,[a] Thomas Bonnard,[b] Aida Shakouri-Motlagh,[a] Ross A. L. Wylie,[a] Joe Collins,[a] Jonathan White,[c] Daniel E. Heath,[a] Christoph E. Hagemeyer,[b] and Luke A. Connal*[a]

Abstract: Hydrogen sulfide (H_2S) has been identified as an important cell signaling mediator and has a number of biological functions such as vascular smooth muscle relaxation, neurotransmission and regulation of inflammation. Here we develop a facile and versatile approach for H_2S production initiated by light irradiation and controlled by reaction with an amine or an amino acid. The donor is synthesized in a one-pot reaction and simple crystallization leads to ~90% yield. The synthetic strategy we have devised is scalable and versatile; we demonstrate that the H_2S donors can be expressed in number of different molecular and macromolecular forms including crystalline small molecules, water soluble polymers, polystyrene films, and hydrogels. The polystyrene film and hydrogel based H_2S donors have been used as cell culture scaffolds. The water soluble polymer based H_2S donor has been applied in photo controlled inhibition of P-Selectin expression on human platelet and subsequently regulation of platelet aggregation. This study provides the simplest controllable H_2S source to study its biological functions. The materials developed also present new therapeutic platforms to deliver H_2S as there is no accumulation of toxic byproducts, the donor materials from polystyrene films and hydrogels can be readily removed after releasing H_2S.

Introduction

Hydrogen sulfide (H_2S), while considered as a broad-spectrum poison, has recently been recognized as a signalling molecule that modulates neuronal transmission, relaxes smooth muscle and regulates the release of insulin.[1-2] It is produced endogenously in small amounts by various mammalian tissues and plays essential roles in many diseases.[3-7] H_2S is also recognized as a potentially protecting factor against cardiovascular disease.[6-7]

As the mechanism underlying the function of H_2S is still not clear, there is strong interest to develop controllable H_2S donors to study its biological functions.[8] Meanwhile, the use of H_2S as a therapeutic molecule also requires delicate H_2S delivering platforms. Direct usage of H_2S on large mammals has resulted in less-than-ideal results, and can be difficult to control the exact concentration.[9] While many indirect donors currently do exist, they all suffer from various disadvantages. Inorganic salts, such as Na_2S and NaHS, are lacking controllability and are not suitable for mimicking biological H_2S release, which is thought to be relatively slow and continuous.[10] Therefore, controllable H_2S donors are required for detailed investigation of the physiological functions of H_2S and for potential therapeutic applications.[11-13] The use of light as a trigger is of particular interest since its characteristics can be remotely and accurately controlled, quickly switched and easily focused into specific areas.[14] There have been reports on photo controllable H_2S donors.[10, 15-18] However, these donor molecules take a number of steps for synthesis and are generally not water soluble. Furthermore, the byproducts may react with the donor, diminishing H_2S production and exasperating its already low yield (for example, 30 μM H_2S produced from 500 μM of donor).[14] The accumulation of toxic by-products and donors material is another serious health concern. Furthermore, the reported photo controllable H_2S donors are solely controlled by UV light which is obviously insufficient when UV induced fluorescence microscopy is used to study its functions. As H_2S is highly toxic and volatile, it would be of great benefit to provide a second control factor.



Scheme 1. The reaction of benzaldehyde with an amine gives off H_2O while the reaction of thiobenzaldehyde with an amine releases H_2S.

Herein we report a simple and scalable synthesis of extremely efficient H_2S donors controlled by two exogenous factors: light and the presence of an amine group. This two-pronged approach gives access to increased control of the system and thus fine control of the rate of H_2S release. The side products of the new H_2S donor are biocompatible. The versatility of our synthetic strategy also enables a range of materials to be prepared: small organic molecules, hydrophilic and hydrophobic copolymers and hydrogels. We further assessed the suitability of these novel materials to provide precise photo controlled intracellular delivery of H_2S and potent anti-platelet therapy.

Results and Discussion

Thioaldehydes are the analogs of aldehydes in which the oxygen atom (O) is replaced with a sulfur atom (S) (Scheme 1). Thioaldehydes have been studied for over 40 years in areas of atmospheric, synthetic organic, and biological chemistry.[19] Recent trapping of thioaldehydes with amines led to a series of surface modification applications by Barner-Kowollik[20] and

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flocculation application by our group.[21] These highly reactive chemical intermediates quickly form imines in the presence of amines in a very similar chemistry to aldehydes and amines, instead of water as the byproduct, H_2S is released. However, the release of H_2S has so far been overlooked. In this work we employed a photomasked thiobenzaldeyde as a versatile strategy for H_2S release upon a light trigger and subsequent trapping with an amine. In this approach, benzyl bromide or *p*-xylene dibromide first reacted with thiourea and then treated with bromoacetophenone under basic phase transfer condition to generate the thiobenzaldehyde precursor **1** in 93% yield (Figure 1a).[21] This facile synthesis was performed in a one-pot reaction manner and purification of the products was achieved by crystallization from ethyl acetate (the X-ray structures are shown in Figure S1). The thiobenzaldehyde precursor is extremely stable in the crystalline forms as no change in color or NMR spectra was observed after one year of refrigerated storage.

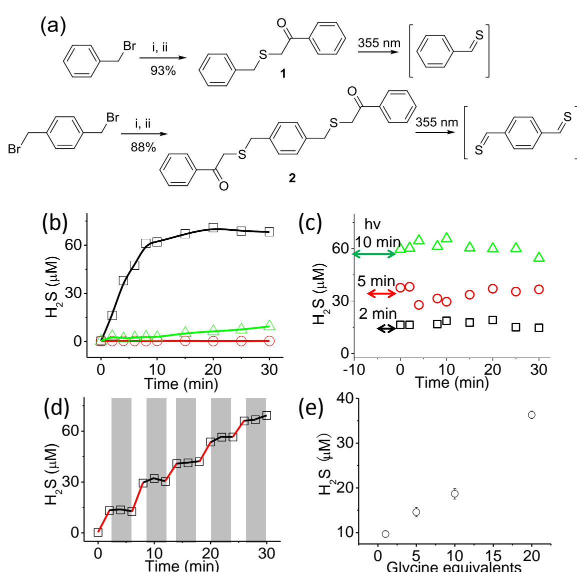

Figure 1. H_2S release from small molecular donor. (a) One pot synthesis of the thiobenzaldehyde precursors **1** and **2** and the resulting thiobenzaldehydes after UV irradiation. i, thiourea, ethanol, reflux; ii, tetrabutylammonium bromide, NaOH, bromoacetophenone, 60 ℃. (b) Time-dependent H_2S concentration under continuous UV light. Red circle, compound **1** and *tert*-butyl amine without irradiation; green triangle, compound **1** under irradiation without amine; black square, compound **1** and *tert*-butyl amine under irradiation. (c) Time-dependent H_2S concentration (from compound **1** and *tert*-butyl amine) after 2 minutes (black square), 5 minutes (red circle), and 10 minutes (green triangle) UV irradiation. (d) Time-dependent H_2S concentration (from compound **1** and *tert*-butyl amine) with 2 minutes UV irradiation and 4 minutes dark. (e) H_2S concentration after 10 minutes of UV irradiation in the presence of 1, 5, 10 and 20 equivalents of glycine. The initial concentration of **1** is 133 μM.

Thiobenzaldehyde precursor **1** exhibits maximum absorption at 327 nm (Figure S2). The absorption spectra is in good match with the emission spectrum of the fluorescent lamp (λ = 355 nm) which contributes to the high efficiencies of the photo reactions.[22] Fox *et. al* proposed mechanisms for the photo cleavage reaction.[22b] To confirm the light triggered thiobenzaldehyde formation and the trapping of the thiobenzaldehyde with amine, a model reaction in $CDCl_3$ with *tert*-butylamine as the trapping agent was performed under UV light (320-400 nm UV intensity 2.3 mW/cm^2). The photo reactions were completed within 10 minutes and the imine products were identified by ^1H NMR and ESI-MS (Figure S3-S6). Water does not hinder this reaction as the imine product was also obtained with d_6-DMSO/D_2O (v/v 3:1) as the solvent (Figure S7). More importantly, another product acetophenone is an excipient approved by the U.S. FDA.

As a ‘byproduct’, H_2S was released from the reaction of the thiobenzaldehyde with the amine. The generation of H_2S was first studied with the standard methylene blue method (Figure 1, S8-9).[15] The solution is stable and no H_2S release was detected. When the solution was subjected to UV irradiation at 355 nm, we observed a small amount of H_2S with a concentration of 9 μM after 30 minutes of irradiation (compound **1** concentration is 133 μM) (Figure 1b). While in the presence of *tert*-butyl amine, a significant enhancement of H_2S release was recorded. The H_2S concentration reaches 61 μM after 8 minutes of irradiation and then the concentration reaches a plateau with the highest H_2S concentration of 71 μM. This time-dependent H_2S release curve is in good accordance with the NMR study in which only 10 minutes is needed for the completion of the photo reaction and the imine formation. Without light irradiation, compound **1** in the presence of *tert*-butyl amine does not release any H_2S.

To further demonstrate the precise and fine control of this light initiated chemistry we investigated the light and dark kinetics of the reactions. After irradiating the samples for 2, 5, and 10 minutes, the H_2S concentrations were monitored in the dark for 30 minutes. A clear dependence of the final H_2S concentration on the irradiation time is observed (Figure 1c). After each specific irradiation time, the H_2S concentration maintained the same indicating that the photo reaction is the rate determining step while the imine formation is fast. We also demonstrate the temporal control of the photo induced release, whereby a stepwise increase in H_2S concentration is observed when irradiated for 2 minutes and then left in the dark for 4 minutes (Figure 1d). The time-dependent H_2S release profiles reveals that the H_2S release is highly controllable in the studied system.

To test the generality of the properties of the amine trapping agent to release H_2S, we substituted *tert*-butyl amine with amino acids. In this experiment, we used the simplest amino acid, glycine, to react with the photo generated thiobenzaldehyde. Enhanced H_2S production compared with no amine additive was also observed (Figure S10). The relatively lower H_2S production compared with using *tert*-butyl amine as the trapping reagent was attributed to the lower nucleophilicity of the amine group on glycine. To develop a dual control approach to release H_2S we investigated the effect of glycine concentration on final H_2S concentration. When using 1 equivalent of glycine, only very slight increase of H_2S concentration was observed. As the glycine concentration increased to 10 equivalents, the H_2S concentration after 10 minutes irradiation reached 19 μM. Further increase of the glycine to 20 equivalents led to H_2S concentration of 36 μM (Figure 1e). The glycine concentration dependent H_2S release suggests that H_2S production can be

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tuned with the second control factor, the amine. In addition, the success of the amino acid additives provides a more bio-compatible approach to this dual controllable H_2S releasing method.

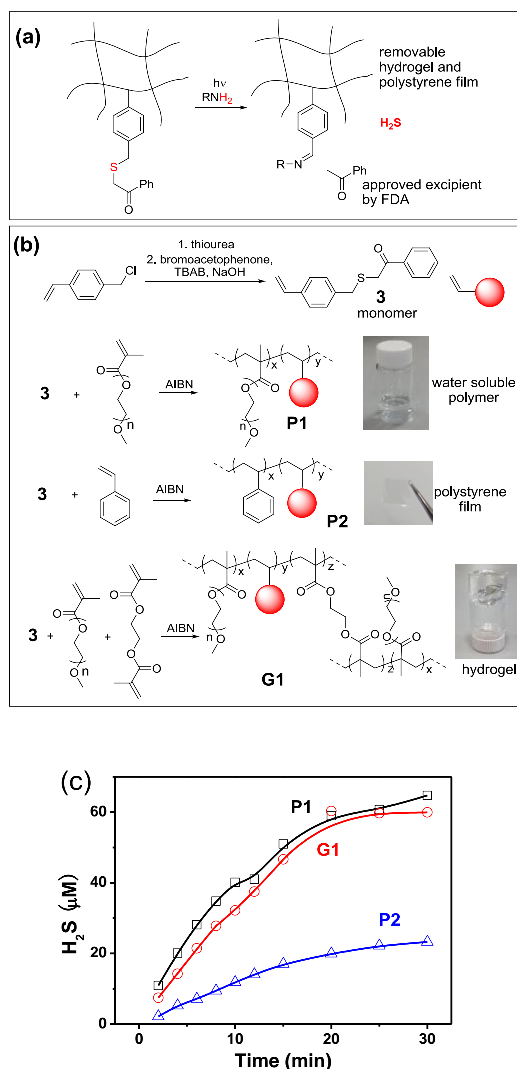

Figure 2. H_2S release from polymer donors. (a) Schematic diagram of the toxic byproduct free route for H_2S release. (b) Synthesis of the monomer **3**, **P1**, **G1,** and **P2**. Insets show the water solution of **P1**, a film of **P2** and the hydrogel of **G1**. (c) Release of H_2S from **P1** (black square), **G1** (red circle) and **P2** (blue triangle) in the presence of *tert*-butylamine under UV light. The concentration for **P1** and **G1** are 0.27 mg/mL, the concentration of **P2** is 2.7 mg/mL. The methylene blue assays were performed in pure PBS buffer with **P1** dissolved and, **G1** and **P2** suspended in the solution.

We also investigated the H_2S donor potential of compound **2** (Figure S11), which generates 1.2 moles of H_2S per mole of the compound **2**. To the best of our knowledge, this represents the most efficient organic H_2S donor by far.

The facile synthesis of the small molecule based H_2S donors spurs us to develop monomer **3** in a similar way as compound **1** in 89% yield (Figure 2). PEGMA was chosen for copolymerization because of its water solubility and demonstrated biocompatibility.[25-26] Copolymerization of monomer **3** and PEGMA (mass ratio, **3**/PEGMA 1:10) with the initiator AIBN gave a water soluble polymer (M_n 23.3 kDa, *Đ* 2.06) (Figure S12-S14). Irradiation of the polymer under UV light in the presence of amine also released H_2S as determined by the methylene blue method. Compared with the small molecule H_2S donor, the polymer-based H_2S donor releases H_2S more gradually as the plateau H_2S concentration is reached after approximately 20 minutes of irradiation (Figure 2c).

Light responsive hydrogels have found applications in drug delivery, tissue engineering, immunoassays, and biosensors. [27, 28] However, light controlled H_2S releasing hydrogels have not been reported. Based on the water soluble polymer H_2S donor, we further developed hydrogels capable of releasing H_2S under control of light and amine. Here, ethylene glycol dimethacrylate (EGDMA) was used as the crosslinker for the hydrogel formation. Copolymerization of monomer **3**, PEGMA and EGDMA (mass ratio **3**/PEGMA/EGDMA 1:10:1) provided a gel **G1** (Figure 2b). Similar to polymer **P1**, the hydrogel showed more gradual H_2S release (Figure 2c). The hydrogel represents the first example of photo responsive H_2S releasing hydrogel. It is also worth noting that the hydrogel can be removed after releasing H_2S and the acetophenone formed has low solubility and toxicity.

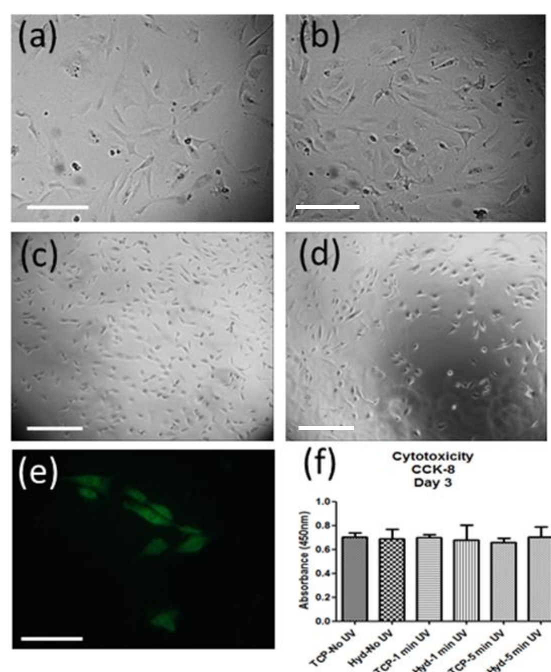

Figure 3. Polymer based cell culture media capable of delivering H_2S. 3T3 fibroblast cell culture with hydrogel **G1** before (a) and after (b) UV irradiation and cell culture with polystyrene film of **P2** before (c) and after (d) UV irradiation, (a, b, scale bar: 200 μm; c, d, scale bar: 300 μm). (e), Fluorescence microscope image of the cells uptake H_2S from polystyrene film and incubated with fluorescence probe, **WSP-1** (scale bar: 200 μm). (f) Cytotoxicity assay without UV or irradiated for 1 min and 5 min (TCP: Tissue culture plastic alone; Hyd: with hydrogels).

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Polystyrene based materials are the gold standard for tissue culture plates because they have excellent optical clarity, are easy to mould and sterilize and cells grow readily on these surfaces. For the purpose of making advanced cell culture interfaces with H_2S releasing properties, monomer **3** was copolymerized with styrene (mass ratio, **3**/styrene 1:100) to form polymer **P2**. Polymer **P2** was characterized with NMR, GPC (M_n 78.8 kDa, *Đ* 1.53) (Figure S15-S16). As expected, **P2** forms a transparent film (Figure 2b) which was used for cell culture and H_2S release.

The H_2S releasing polymer **P2** and hydrogel **G1** were used as cell culture surfaces as convenient ways to deliver H_2S into live cells. Initially the cytotoxicity of the materials was measured using 3T3 fibroblast cells. Cells grew well on both surfaces (Figure 3a-3d) and the H_2S donor materials showed excellent biocompatibility to the 3T3 fibroblast cells as evidenced by measurement of the metabolic activity of the cells for 3 days during culture compared to tissue culture plastic controls (measured as absorbance intensity at 450 nm) (Figure 3f and S19).

We then validated the donors' H_2S delivery into live cells. As high concentration of H_2S and long exposure of UV light are toxic to cells, we irradiated the cells cultured on the donor materials for up to 5 min in the presence of fetal calf serum. At such conditions, it was found that the released H_2S concentration is not toxic to cells. To visualize the H_2S delivery into live cells we used a fluorescent probe **WSP-1** (Figure S17).[23] 3T3 fibroblast cells, after irradiation were incubated with **WSP-1** for 30 min. The cells exhibited strong green fluorescence indicating the presence of H_2S inside the cells (Figure 3e). In the control experiments where no H_2S donor materials were present or no UV irradiation was performed, no green fluorescence was observed in the cells (Figure S20-21).

H_2S was reported to have anti-thrombotic properties and to regulate thrombogenesis by interference with platelet activation and adhesion molecule-mediated aggregation.[7b, 24a] With the versatile H_2S donors in hand, we further studied the photo controlled P-Selectin expression and platelet aggregation effects *in vitro*. To obtain reliable flow cytometry analysis results and fluorescence signals, water soluble polymer based H_2S donor **P1** is employed in the studies. P-Selectin functions as a cell adhesion molecule on the surfaces of activated endothelial cells as well as platelets and plays a pivotal role in thrombosis. After UV irradiation, adenosine diphosphate (ADP) stimulated platelets supplemented with H_2S donor polymer (3 mg/mL) and glycine (0.84 mg/mL) showed significantly reduced P-Selectin expression as revealed by flow cytometry analysis, indicating the inhibition of platelet function (Figure 4a and 4b). These findings are consistent with previous studies with the common H_2S donor GYY4137.[7b, 24a] Importantly, our donor material provides a photo controllable manner to study this effect.

We further studied the aggregation of human platelet-rich plasma (PRP) samples supplemented with 0.84 mg/mL glycine (platelet-poor plasma, PPP supplemented with 0.84 mg/mL glycine used as blank) in the presence of the photo controllable H_2S donor (Figure 4c and 4d). The aggregation was significantly lower in PRP samples supplemented with 3 mg/mL polymer based H_2S donor (versus PBS buffer) and pre-exposed to UV light (versus no exposure). It should be noted that a mild anti-aggregation effect is seen for controls (with polymer and no UV) for both the flow cytometry and the platelet aggregation experiments. Both of these techniques rely on a light source and hence a small amount of H_2S is released under these conditions giving rise to a mild effect.

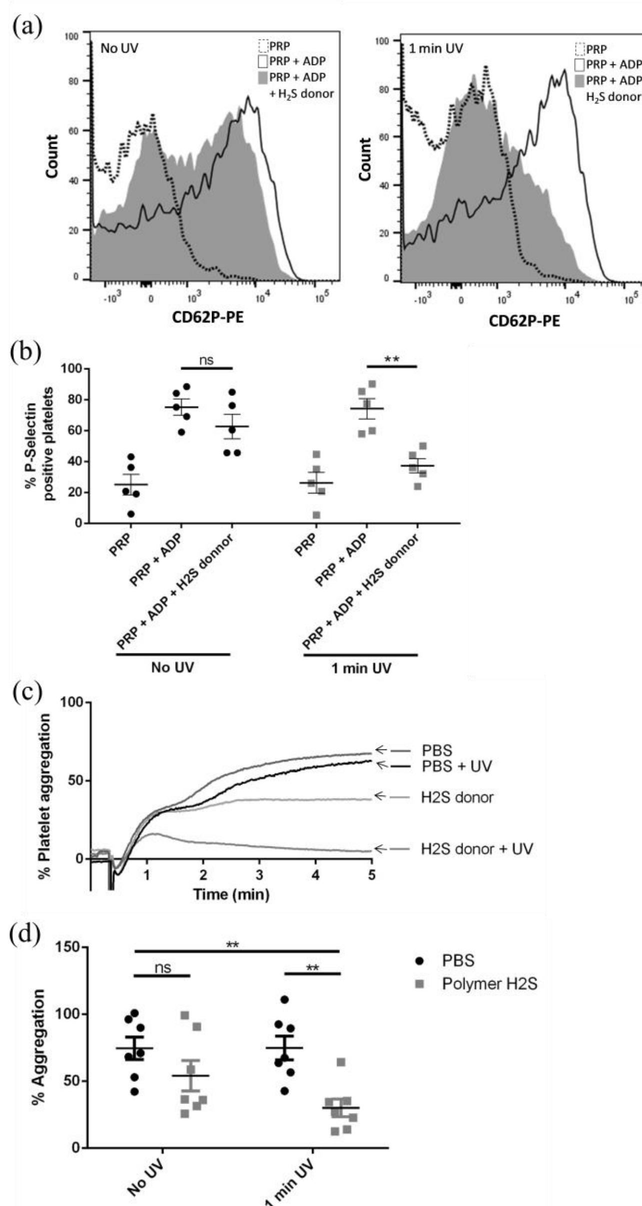

Figure 4. H_2S donor polymer inhibits P-Selectin expression and reduces platelet aggregation when exposed to UV light. (a) Representative histogram from flow cytometry analysis showing P-Selectin fluorescence signal measured on resting platelets (PRP), ADP stimulated platelets (PRP+ADP) or ADP stimulated platelets supplemented with H_2S donor polymer (3 mg/mL) after no UV exposure (left) or 1 minute UV exposure (right). (b) Quantification of the percentage of P-Selectin positive platelets (mean ± SEM, n=5, ns: non-

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significant, **p<0.01). (c) Typical aggregation trace. (d) Quantification of the aggregation studies (mean ± SEM, n=8, ns: non-significant, **p<0.01). The standard deviation (SD) figures are shown in Figure S22.

Conclusions

We developed a facile and versatile platform for triggered and controlled H_2S release from simple but overlooked chemistry. The H_2S donors have been synthesized in a one pot reaction. The H_2S release is controlled by light and an amine, which represents a new way for H_2S production. The versatile synthetic approach enabled us to engineer new forms of H_2S donor materials including crystalline small molecules, water soluble polymers, hydrogels and polystyrene films. The feasibility of using an amino acid to initiate H_2S release makes this system suitable for biological applications. The developed materials showed excellent biocompatibility and showed efficient light controlled H_2S delivery to living cells. The water soluble polymer based H_2S donor provided potent inhibition of platelet activation and aggregation. This approach has great interest to be adapted as a controlled delivery anti-thrombotic treatment as the precise spatial and temporal anti-platelet activity could solve the undesired bleeding side effect encountered with current anti-platelet drugs. The hydrogel and polystyrene materials also provide new therapeutic approaches to deliver H_2S as the accumulation of by-products and donor materials can be avoided.

Experimental Section

General. All commercially obtained reagents were used without further purification. Anhydrous solvents were prepared by drying HPLC-grade solvents using freshly activated molecular sieves. ^{1}H NMR spectroscopy was conducted on a Varian Unity 400 MHz spectrometer. Chemical shifts (δ) were reported relative to the solvent residue peak and are in ppm (δ_{CHCl3} = 7.26 ppm, $\delta_{d6\text{-}DMSO}$ = 2.50 ppm). UV irradiation was conducted with Philips 8W long wave (355 nm) UV lamp. Fourier transform infrared spectroscopy was performed on a Perkin Elmer Frontier FT-IR Spectrometer with a universal ATR sampling accessory. UV-Vis spectra were recorded on a Shimadzu UV-Vis Spectrophotometer. Mass spectra were recorded on an Agilent 6520 QTOF MS. Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer. Methylene blue assays were performed on a Thermo Scientific GENESYS 10 UV spectrophotometer. The GPC was equipped with a Shimadzu RID-10 refractometer (λ = 633 nm) using two Phenomenex Phenogel columns (5 μm bead size, 104 and 106 Å porosity) in series, operating at 70°C. DMF with 0.05 mol·L-1 LiBr (>99%, Aldrich) was used as the mobile phase. The polymer molecular weight was determined by comparison to polystyrene calibration curves. The UV intensity (at a distance of 0.5 cm to the lamp) is 2.3 mW/cm^2 in the spectral range of 320 nm to 400 nm as measured by Solarmeter Model 4.0 UVA Digital UV meter with GaAsP Photodiode. The cell image was obtained on Leica DMIL or Olympus IX81 and the fluorescent images were obtained on Olympus AX70.

Synthesis. Compound **1** was synthesized following a modified literature procedure.[29]

Compound 1. To a solution of thiourea (1.52 g, 20 mmol) in 95% ethanol (50 mL) was added benzylbromide (3.42 g, 20 mmol) slowly. The mixture was heated to reflux for 5 hrs. After cooling to room temperature, the solvent was removed and a solution of NaOH (2.0 g, 50 mmol) in 30 mL H_2O was added followed by Bu_4NBr (161 mg, 0.5 mmol). Bromoacetophenone (4.0 g, 20 mmol) was then added and the mixture was stirred vigorously at 60 ℃ under nitrogen for 3 hrs. After cooling, the solid was collected by filtration and washed thoroughly with dichloromethane. The organic phase was filtered and the solvent was removed under vacuum. The residue was recrystallized from ethyl acetate to provide the product as a white solid (4.51 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 7.2 Hz, 2 H), 7.56 (t, *J* = 7.2 Hz, 1 H), 7.44 (t, *J* = 7.2 Hz, 2 H), 7.20 – 7.35 (m, 5 H), 3.75 (s, 2H), 3.66 (s, 2 H).

Compound 2. To a solution of thiourea (1.52 g, 20 mmol) in 95% ethanol (50 mL) was added *p*-xylene dibromide (2.64 g, 10 mmol) slowly. The mixture was heated to reflux for 5 hrs. After cooling to room temperature, the solvent was removed and a solution of NaOH (2.0 g, 50 mmol) in 30 mL H_2O was added followed by Bu_4NBr (161 mg, 0.5 mmol). Bromoacetophenone (4.0 g, 20 mmol) was then added and the mixture was stirred vigorously at 60 ℃ under nitrogen for 3 hrs. After cooling, the solid was collected by filtration and washed thoroughly with dichloromethane. The organic phase was filtered and removed solvent under vacuum. The residue was recrystallized from ethyl acetate to provide the product as a white solid (3.54 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 7.6 Hz, 4 H), 7.56 (t, *J* = 7.6 Hz, 2 H), 7.45 (t, *J* = 7.6 Hz, 4 H), 7.29 (s, 4 H), 3.73 (s, 4 H), 3.66 (s, 4 H).

Compound 3. To a solution of thiourea (1.52 g, 20 mmol) in 95% ethanol (50 mL) was added 4-vinylbenzyl chloride (3.05 g, 20 mmol) slowly. The mixture was heated to reflux for 5 hrs. After cooling to room temperature, the solvent was removed and a solution of NaOH (2.0 g, 50 mmol) in 30 mL H_2O was added followed by Bu_4NBr (161 mg, 0.5 mmol). Bromoacetophenone (4.0 g, 20 mmol) was then added slowly and the mixture was stirred vigorously at 60 ℃ under nitrogen for 3 hrs. After cooling, the solid was collected by filtration and washed thoroughly with dichloromethane. The organic phase was filtered and removed solvent under vacuum. The residue was recrystallized from dichloromethane/MeOH to provide the product as a white solid (4.79 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 7.2 Hz, 2 H), 7.57 (t, *J* = 7.2 Hz, 1 H), 7.45 (t, *J* = 7.2 Hz, 2 H), 7.35 (d, *J* = 7.2 Hz, 2 H), 7.31 (d, *J* = 7.2 Hz, 2H), 6.69 (dd, J_1 = 17.4 Hz, J_2= 10.8 Hz, 1 H), 5.73 (d, *J* = 17.4 Hz, 1 H), 5.23 (d, *J* = 10.8 Hz, 1 H), 3.74 (s, 2 H), 3.66 (s, 2 H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 194.4, 136.8, 136.6, 136.4, 135.4, 133.3, 129.4, 128.7, 128.6, 126.3, 113.8, 35.8, 35.7. HRMS calculated for $C_{17}H_{17}OS$ 269.1000, found 269.0994 $[M+H]^+$.

Polymer P1. AIBN (3 mg) was added to a 10 mL THF solution containing compound **3** (100 mg) and poly(ethylene glycol) methyl ether methacrylate (**PEGMA**, Mn = 500, 1.00 g). The solution was bubbled with N_2 for 10 minutes and heated at 60°C for 4 hours under dark. After cooling to room temperature, the solution was precipitated into diethyl ether and the polymer was collected as a viscous solid (0.43 g). Mn 23.3 kDa, PDI 2.06; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.93 (broad, 2 H), 7.56 (broad, 1 H), 7.46 (broad, 1H), 6.94-7.24 (broad, 4H), 4.07 (broad, 14 H), 3.50-3.95 (m, 260 H), 0.80-2.00 (m, 37 H).

Hydrogel G1. AIBN (3 mg) was added to a 10 mL THF solution containing compound **3** (100 mg) and poly(ethylene glycol) methyl ether methacrylate (**PEGMA**, Mn = 500, 1.00 g) and ethylene glycol dimethacrylate (100 mg) . The solution was bubbled with N_2 for 10 minutes and heated at 60°C for 24 hours under dark. The solution changed to a gel and solvent was removed under vacuum. After washing and swelling with water, the hydrogel was dried again providing a solid (1.20 g). The gel has a swelling ratio of 13 in PBS buffer which was defined as the ratio of the weight of the swollen hydrogel (W_s) to the weight of the dried hydrogel (W_d): $Q = W_s/W_d$.

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Polymer P2. AIBN (15 mg) was added to a 15 mL THF solution containing compound **3** (100 mg) and styrene (10.0 g). The solution was bubbled with N_2 for 10 minutes and heated at 60°C for 24 hours under dark. After cooling to room temperature, the solution was precipitated into methanol and the polymer was collected as a white solid (0.43 g). Mn 78.7 kDa, PDI 1.53; The ^{1}H NMR spectrum is shown in Figure S15.

Typical procedure for monitoring H_2S release from thiobenzaldehydes. Water soluble polymer **P1** (20 mg) was dissolved in 1 mL PBS buffer. 20 µL *tert*-butyl amine was added and the mixture was irradiated under a long wave (355 nm) UV lamp. Aliquots (20 µL) was taken from the reaction mixture at different time intervals to the freshly prepared methylene blue cocktail (1% w/v of $Zn(OAc)_2$ (100 µL) in H_2O, 30 mM $FeCl_3$ (200 µL) in 1.2 M HCl, 20 mM of N,N-dimethyl-1,4-phenylenediamine sulfate (200 µL) in 7.2 mM HCl). The assay was kept at room temperature for 30 min and diluted with PBS buffer to a final volume of 1.5 mL and measured absorbance at 670 nm. The solution and PBS buffer were degassed and sealed prior to initiation of the assay.

Cell culture. To observe H_2S uptake by live cells, 3T3 fibroblast cells were seeded at $2.5x10^4$ cells/cm^2 densities in Dulbecco's Modified Eagle Medium (DMEM)-high glucose media supplemented with 10% fetal calf serum. Polystyrene films were sterilized by washing with 80% ethanol twice, 5 minutes each. Then, the films were washed with PBS 3 times. After 24 hours, the media was removed and replaced by PBS and UV was irradiated for 5 minutes. Then, equal volume of **WSP-1** solution (10 µM) were added to each well. After 30-60 minutes incubation, PBS and probe were removed and the wells were washed 3 times with PBS.
For cytotoxicity assay, hydrogels were soaked in 80% ethanol for 5-10 minutes and washed 3 times with PBS and immersed in culture media for 5 minutes before adding to tissue culture plated. Cells were seeded at $1.5x10^4$ cells/cm^2 density in 96-well plate (day 0) for 1 day before adding the hydrogels. The hydrogels were added to culture plated at day 1 and UV irradiated 5 min at day 1, 2 and 3 once per day. At day 3, hydrogels were taken out and CCK-8 cytotoxicity assay were performed according to manufacturer protocol. 100 µL of fresh media and 10 µL of CCK-8 solution were added to each well. Cells were then incubated for 4 hours and the absorbance at 450 nm was measured by microplate reader. One-way ANOVA with Tukey's multiple comparison tests were used to compare different groups.

Human blood collection, platelet-rich plasma and platelet-poor plasma preparation. The recruitment of participants and collection of blood specimens was approved by the Monash University Human Research Ethics Committee (Project CF07/0141-2007/0025). Human blood was collected from healthy volunteers who had not taken anti-platelet medications in the past 10 days via venepuncture into tri-sodium citrate (3.2% w/v final). Signed informed consent was obtained prior to participation. The blood was centrifuged (200 g, 15 min) to yield platelet-rich plasma (PRP), supplemented with 0.84 mg/mL glycine and further centrifuged (1200 g, 5 min) to yield platelet-poor plasma (PPP).

Light transmission aggregometry. Aggregation of PRP samples (PPP used as blank) was determined via light transmission (AggRAM Helena Laboratories) after the addition of adenosine diphosphate (ADP, 10 µM) at 37℃ with stirring at 600 rpm. PRP samples were supplemented with 3 mg/mL polymer-H_2S donor treatment (versus phosphate buffered saline (PBS) and exposed to UV light for 1 minute (versus no UV exposure). UV exposure was obtained from a Philips 8W long wave (355 nm) UV lamp placed onto the PRP samples. A representative aggregation trace was presented and the maximum aggregation obtained in those condition from 8 blood donors was presented as mean value ± SEM (n=8).

P-Selectin expression. Flow cytometry was used to measure the expression of P-Selectin by activated platelets after H_2S donor treatment with UV exposure. PRP prepared as outline above was diluted in PBS (x5) deposited in a 96 well plate with 3 mg/mL polymer-H_2S donor (versus PBS) and exposed to UV light for 1 minute (versus no UV exposure). UV exposure was obtained from a Philips 8W long wave (355 nm) UV lamp placed onto the 96 well plate (one plate for UV exposed samples and one plate for non UV exposed samples). Following the 1 minute UV exposure, PRP sample were then activated by 10 µL ADP 60 µM and labelled with a P-Selectin monoclonal Phycoerythrin (PE) coupled antibody conjugated to (ThermoFisher Scientific). A PE-coupled IgG1 isotype-matched control antibody was used to exclude nonspecific binding. After 20 min incubation at 37℃, the PRP samples were analysed by flow cytometry (Fortessa, HTS module, BD Biosciences). FlowJo software (Tree Star Inc., USA) was used for data analysis. Platelet populations were gated according to their typical granularity and the level of P-Selectin expression was measured from the PE fluorescence signal associated to the platelets. The emission in the Yellow green (562 nm laser) 582/15 channel ([YG]582_15-A) was observed and plotted on 2D histograms. The thresholds for P-Selectin positive platelets were adjusted for optimal signal-to-noise ratio and the percentage of P-Selectin positive platelet was measured for each PRP groups. Results were presented as mean values ± SEM (n=5).

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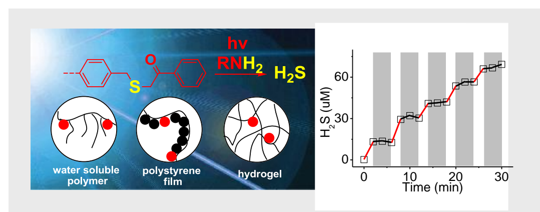

Unexpected H_2S donors: one pot reaction to synthesis, two control factors to release. A facile and versatile approach to H_2S release has been developed by trapping the photo generated thiobenzaldehydes with amines. The H_2S release process can be controlled with light and an amine. In addition to the crystalline small molecule H_2S donor, water soluble polymer, polystyrene film and hydrogel based H_2S donor materials were easily achieved. The new materials showed excellent biocompatibility and have been used for controlled delivery of H_2S to cells and anti-platelet application.

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