Modified Cyclodextrins Solubilize Elemental Sulfur in Water and Enable Biological Sulfane Sulfur Delivery

Sarah G. Bolton and Michael D. Pluth*

Department of Chemistry and Biochemistry, Materials Science Institute, Knight Campus for Accelerating Scientific Impact, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA. E-mail: pluth@uoregon.edu

ABSTRACT: An important form of biological sulfur is sulfane sulfur, or S0, which is found in polysulfide and persulfide compounds as well as in elemental sulfur. Sulfane sulfur, often in the form of S8, functions as a key energy source in the metabolic processes of thermophilic Archaea and is found in sulfur-rich environments and can be metabolized both aerobically and anaerobically by different archaeans. Despite this importance, S8 has a low solubility in water (~19 nM), raising questions of how it can be solubilized and made chemically accessible in complex environments. Motivated by prior crystallographic data showing S8 binding to hydrophobic motifs in filamentous glycoproteins from the sulfur reducing Staphylothermus marinus an aerobe, we demonstrate that simple hydrophobic motifs, such as 2-hydroxypropyl β-cyclodextrin (2HPβ), are sufficient to solubilize S8 at concentrations up to 2.0 ± 0.2 mM in aqueous solution. We demonstrate that the solubilized S8 is chemically accessible, can be reduced with tris(2-carboxyethyl) phosphine (TCEP), and reacts with thiols to generate H2S. The thiol-mediated conversion of 2HPβ/S8 to H2S ranges from 80% to quantitative efficiency for Cys and glutathione (GSH). Moreover, we demonstrate that 2HPβ can catalyze the reduction of Cys-mediated reduction of S8 to H2S in water. Adding to the biological relevance of the developed systems, we demonstrate that treatment of Raw 264.7 macrophage cells with the 2HPβ/S8 complex prior to LPS stimulation reduces NO2− levels, which is consistent with known activities of bioavailable H2S and sulfane sulfur. Taken together, these investigations provide a new strategy for delivering H2S and sulfane sulfur in complex systems and more importantly provide new insights into the chemical accessibility and storage of S8 and S8 in biological environments.

Introduction

Sulfur is a long-standing critical component to life on Earth. Prior to the Great Oxidation Event approximately 2.4 billion years ago, during which time the Earth’s atmosphere became rich in O2, the atmosphere on Earth was weakly reducing.1 Volcanic activity was an abundant source of sulfur-containing species, and gases including sulfur dioxide (SO2) and hydrogen sulfide (H2S) were released and dissolved in pools or lakes.2 Spark discharge experiments using a simulated atmosphere containing H2S and a mixture of reducing gases thought to be present in the early Earth atmosphere have demonstrated abiotic synthesis of diverse organic compounds including amino acids.3 In conditions reflecting those near deep-sea vents rich in iron-sulfur compounds, both H2 and organosulfur compounds were generated from a mixture of H2S and FeS under a N2/CO2 atmosphere in acidic conditions.4 Sulfane sulfur (S8), which is most commonly found as elemental octasulfur (S8), is also found in these deep-sea environments and is another important source of biologically available sulfur.

Recently, interest in biological and synthetic S8 sources has increased significantly due to the connection between such species and the small biological signaling molecule H2S. H2S is produced endogenously from cysteine metabolism and serves signaling roles in diverse pathways. Along with carbon monoxide (CO) and nitric oxide (NO), H2S is now recognized as member of the family of small molecules often referred to as gasotransmitters, which are produced enzymatically and act upon specific molecular targets within cellular environments.5–7 One unique feature that distinguishes H2S from CO and NO is that sulfur has biologically-accessible oxidation states ranging from -2 to +6 and participates in a complex redox cellular landscape.8 In many eukaryotic organisms, H2S serves as a source of biologically available sulfur and is intrinsically tied to both organic and inorganic S8-containing species, including persulfides and related polysulfides/polysulfanes, in the S8 pool. This redox labile pool can generate H2S upon reduction or participate in transsulfidation reactions to transfer S8 moieties to cysteine residues.9 The biological activity of H2S is inextricably linked to the downstream production of S8-containing species, and understanding the generation, action, and translocation of S8 is critical to understanding the intertwined chemistry of reactive sulfur species in biology.

Despite this broad importance in both contemporary and evolutionary chemistry and biology, investigations into S8 activity in aqueous systems is challenging due to the complex reactivity of available S8 sources. Organic polysulfides, such as diallyl trisulfide (DATS) found in alliums including garlic, or other synthetic organic and inorganic...
polysulfides can act as sources of biologically available $S^0$. These systems present divergent reactivity based on the polysulfide chain length and pendant alkyl group. Inorganic polysulfides in particular are unstable in aqueous conditions and quickly equilibrate to different polysulfide mixtures. Despite these fundamental challenges, available $S^0$ sources demonstrate significant promise in different systems, ranging from anticancer properties in several human cell lines to enhanced antioxidative activity. In all of these cases, however, the production of byproducts obfuscates the role of $S^0$.

An attractive approach to investigate the chemical biology of $S^0$ is to use the most common and simplest form of sulfane sulfur: $S_8$. However, use of $S_8$ directly is hindered by its low water solubility of 6.4 µg/L (19(6) nM) at 25 °C. Despite this low solubility, $S_8$ found in volcanic deep-sea environments can be readily metabolized by thermophilic Archaean organisms found in these sulfur-rich habitats. As one example, species of the order Sulfolobales can derive energy from the metabolism of $S_8$ via both aerobic and anaerobic pathways. Similarly, *Acidianus ambivalens* can utilize $S_8$ as both an electron donor and acceptor. Taken together, such organisms may provide clues into possible mechanisms of stabilizing bioavailable $S^0$ in an aqueous environment.

![Figure 1](image1.png)

**Figure 1.** Structure of the tetrameric right-handed coiled-coil component of the tetrabrachion of the archaean *S. marinus* (PDB: 5JR5) demonstrating two hydrophobic pockets in the core capable of encapsulating $S_8$. Highlighted residues are colored according to their hydrophobicity, where red indicates stronger hydrophobicity.

One potential strategy for $S_8$ solubilization and activation can be gleaned from the archaean *Staphylothermus marinus*, a strict sulfur reducing anaerobe that requires $S^0$ as its terminal electron acceptor. Found near hot deep-sea vents, *S. marinus* is coated in thermostable filamentosus glycoprotein structure called tetrabrackon that protrude from its surface. The tetrabrackon of *S. marinus* is composed of a four-stranded parallel coiled-coil structure with a hydrophobic core that is particularly stable. The 24 kDa right-handed coiled-coil structure of the tetrabrackon contains hydrophobic cavities that have been found to encapsulate two $S_8$ molecules (PDB: 5JR5). Closer inspection of these $S_8$-binding cavities revealed that the sulfur motifs were held in place by van der Waals forces with aliphatic amino acid side chains leucine and isoleucine (Figure 1). Further supporting this observation that hydrophobic motifs can increase $S_8$ solubility, Steudel and Holdt demonstrated that the solubility of $S_8$ in water can be increased using surfactants, with $S_8$ concentrations reaching up to 0.1 mM in saturated hexadecyl(trimethyl)ammonium bromide (CTAB) solutions, although reactivity studies were not reported. Taken together, these prior observations support that this approach to solubilize $S_8$ in aqueous environments may be more general and could also lead to new approaches to enable chemical accessibility of $S^0$-containing species in biological environments.

Understanding the intrinsic strategies for stabilizing simple $S^0$-containing sources in solution remains a key unmet need that could have significant impacts in broad fields ranging from contemporary chemical biology of reactive sulfur species to greener synthetic methods for sulfur-containing compounds. Here we report that hydrophobic interactions within cavity-containing molecules, such as cyclodextrins (CDs), can be used to significantly solubilize $S_8$ in aqueous solutions, and that this solubilized $S^0$ is both chemically and biologically accessible. Specifically, we use 2-hydroxypropyl β-cyclodextrin (2HPβ) to generate 2HPβ/$S_8$ solutions that are stable and quantifiable, react with thiols to generate $H_2S$, exert antioxidant activities in cell models of oxidative stress, and increase intracellular $S^0$ levels.

**Scheme 1. Solubilization and chemical accessibility of $S_8$ in 2-hydroxypropyl β-cyclodextrin (2HPβ).**

**Results and Discussion**

The simplest form of $S^0$, $S_8$, is readily available in high purity as a sublimed chalky yellow solid. Unfortunately, its use in biological applications is severely hampered by its hydrophobicity and low water solubility. The solubility of $S_8$ has been calculated to be only 1.9(6) x 10⁻⁸ mol/kg (or 1.5(2) x 10⁻⁷ M $S^0$), which is multiple orders of magnitude below biologically relevant $S^0$ concentrations. Despite this low solubility, solid $S_8$ has been demonstrated to be...
biologically accessible by erythrocytes to produce H$_2$S, which highlights the potential for increasing the bioavailability of S$_8$ in different systems. Moreover, these prior results suggest that biological pathways for sulfane sulfur activation from elemental sulfur may be accessible if S$_8$ could be solubilized in aqueous environments.

Motivated by the binding of S$_8$ to the hydrophobic pockets of the archaeon S. marinus and increased solubility in hydrophobic environments, we envisioned that watersoluble compounds with hydrophobic interiors could enable similar S$_8$ binding. Building from this hypothesis, we utilized CDs, which are cyclic oligosaccharides that contain a hydrophobic interior and that are widely utilized to bind and solubilize hydrophobic compounds. This solubilization is due, in part, to the hydrophobic interior of CDs, which promotes encapsulation and binding of a nonpolar guest, whereas the hydrophilic exterior enables water solubility. CDs are available in different sizes/volumes, and naturally produced CDs include α-CD, β-CD, and γ-CD, which contain 6, 7, and 8 glucose units, respectively. The choice of CD depends upon the size of the nonpolar compound to be solvated and the properties of the system being studied. Although natural CDs have limited water solubility, modification of the ring periphery with hydroxypropyl groups results in significant increases in solubility. In particular, 2-hydroxypropyl β-CD (2HPβ) and 2-hydroxypropyl γ-CD (2HPγ) have been used extensively, including in drug formulations to enable delivery of otherwise hydrophobic and insoluble compounds.

To test our general hypothesis, we treated aqueous solutions of 2HPβ with a 10-fold excess of solid S$_8$. We chose to start our investigations with the β-CD structure because the cavity volume (262 Å$^3$) is an ideal match for S$_8$ (∼149 Å$^3$; 57% cavity occupancy), based general preference for encapsulated guests to occupy ∼55% of host volume. After stirring as solution of 2HPβ with S$_8$ in water for several days and subsequent filtration to remove residual insoluble S$_8$, we observed a strong absorbance at 263 nm in the UV-vis spectrum, which is a characteristic absorbance of S$_8$. By contrast, stirring S$_8$ in water under identical conditions but in the absence of 2HPβ failed to produce a significant S$_8$ absorbance (Figure 2). To test the stability of the solubilized 2HPβ/S$_8$, we next assessed whether the solution could be precipitated, filtered, and re-dissolved without loss of S$_8$. We precipitated the 2HPβ/S$_8$ complex with acetone, isolated the solid, and re-dissolved the resultant solid in buffer (Figure S1). In these experiments, we observed that the same absorbance from the original and re-dissolved solutions, confirming the stability of the solubilized system in both the liquid and solid state.

Figure 2. Comparison of the UV-vis spectra of S$_8$ in water with S$_8$ in aqueous solutions containing 2HPβ. Conditions: 4 mg of S$_8$ in either 5 mL of PBS 7.4 PBS or 5 mL of 25% w/w 2HPβ in pH 7.4 PBS. Solutions were stirred for one day and then filtered prior to absorbance measurement.

To determine which components of the 2HPβ complex were responsible for S$_8$ solubilization, we next evaluated S$_8$ solvation in the presence of glucose and hydroxypropyl cellulose (HPC) as models for the sugar units of the 2HPβ macrocycle and the hydroxypropyl motif, respectively. After stirring an excess of S$_8$ to solutions of each saccharide in pH 7.4 phosphate buffered saline (PBS) buffer, the solutions were filtered, and UV-vis spectra were recorded (Figure 3). As shown in Figure 2, the characteristic absorbance at 263 nm corresponding to S$_8$ was significantly larger for 2HPβ (orange, 687 μM in this solution) than for glucose (aqua) or HPC (yellow). These data suggest that the cyclic structure and cavity of the CD are key components required for S$_8$ solvation. We next investigated the importance of the 2HPβ hydroxypropyl groups by testing S$_8$ solubilization with β-CD (lacking the 2-HP groups). We treated β-CD in pH 7.4 PBS buffer with excess S$_8$ and stirred for one month (Figure S2). After filtering the solution, we failed to observe any solubilized S$_8$ by UV-vis spectroscopy, which suggests that the hydroxypropyl groups are required for S$_8$ solubilization.

Figure 3. UV-vis spectra of 640 mg S$_8$ in 10 mL pH 7.4 aqueous solutions containing 365 mg of 2HPβ, HPC, or glucose. 2HPβ demonstrates superior S$_8$ solvating ability to both non-cyclic saccharides.
To further investigate the requirement of the cyclic structure and presence of a cavity for S₈ solubilization, we also investigated whether 2HPβ (cavity volume: 427 Å³) could solubilize S₈. We compared the S₈ solubilization in 25% w/w solutions of 2HPβ and 2HPγ in both pH 7.4 PBS buffer and in water and observed significantly less S₈ solubilization from 2HPγ than from 2HPβ (Figure S3). Taken together, these experiments strongly support that both the cyclic / cavity-containing structure and the presence of the hydrophobic core and hydrophilic exterior are critical for the observed S₈ solution by 2HPβ.

Building from these investigations, our next goal was to determine how much S₈ was solubilized in 2HPβ solutions. However, due to the low solubility of S₈ in water only the extinction coefficient for S₈ in methanol, 6730 M⁻¹ cm⁻¹ at 263 nm, has been reported. We sought to confirm that the extinction coefficient for S₈ in methanol could still be used to quantify aqueous S₈ in the 2HPβ/S₈ complex. To do this, we prepared a stock solution of S₈ in methanol that yielded an S₈ concentration of 278 μM. Using this as a standard of comparison, we then used the extinction coefficient value of S₈ in methanol to measure the concentration of S₈ in an existing 2HPβ/S₈ solution. We diluted this solution to match the calculated value of [S₈] in the methanol stock solution and compared the resulting curves. If the extinction coefficient does not change between 2HPβ/S₈ and S₈ in methanol, the calculated [S₈] for the 2HPβ/S₈ stock solution should be correct, and dilution from this value to the S₈ concentration in the methanol stock solution should yield an identical concentration and thus an identical curve. The resultant curves (Figure S4) are similar with the 2HPβ/S₈ solution containing an S₈ concentration of 247 μM, the percentage difference equation demonstrates a difference of 12% between the methanol/S₈ and 2HPβ/S₈ solutions. This confirms that the extinction coefficient does not change appreciably between these two systems and confirms that the S₈ extinction coefficient in MeOH can be used to approximate solubilized [S₈] in the 2HPβ system. Using this extinction coefficient at 50% w/w 2HPβ, we measured the S₈ concentrations of up to 2.0 ± 0.2 mM (16 mM S₈) in water. When compared to the background solubility of S₈ in water, this constitutes ~10³-fold enhancement in S₈ solubility.

Having established that S₈ is readily solubilized in aqueous solution of 2HPβ we next sought to investigate the stoichiometry and magnitude of the interaction between S₈ and 2HPβ. To probe these interactions, we monitored the observed [S₈] in solution as a function of increasing [2HPβ] (from 0-45% w/w) that were prepared with a 10-fold molar excess of solid S₈ in solution. Under these conditions, the activity of S₈ in solution remains constant, and increasing the [2HPβ] should result in a concomitant increase in [S₈] in solution. After stirring each solution for several days to ensure equilibrium, the solutions were filtered, and the S₈ concentration were measured by UV-vis spectrophotometry (Figure 4a). As expected, the measured [S₈] increased linearly with increasing [2HPβ], which further supports a direct interaction between S₈ and the 2HPβ complex. The above constant activity data can be used to determine the binding stoichiometry and affinity between 2HPβ and S₈ through equation (i). Under these conditions, the total S₈ content is defined as S₈ in equation (i), and the concentration of unbound S₈, held constant throughout experiments to ensure constant activity, is defined as s₈ (1.9(6) x 10⁻⁷ mol/kg). The constant n represents the binding stoichiometry.

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\log \frac{S₈}{s₈} = \log K + n \cdot \log[2HPβ]
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Generating a log-log plot with the parameters of equation (i) demonstrates that the relationship between 2HPβ and S₈ is consistent with 1:1 binding (Figure 4b). Upon performing linear regression analysis, we obtained a Kₘ value of 3.4 ± 0.05 x 10⁻⁵ M⁻¹ for the 2HPβ/S₈ complex, which is much stronger than the typical 10⁻³-10⁻⁴ M⁻¹ binding affinities observed in βCD systems. Although the above analyses are supportive of a 1:1 binding stoichiometry, we cannot definitively exclude equimolar higher order interactions from our data.

We repeated these binding stoichiometry experiments with 2HPγ and 2-hydroxypropyl α-CD (2HPα) (Figure S5). Our expectation was that these differently-sized CD hosts would not solubilize or bind S₈ as efficiently as 2HPβ. For example, if S₈ were bound within 2HPα or 2HPγ, cavity occupancies of 86% and 32% would be observed, respectively, which is outside of the range of most host-guest interactions. Consistent with these expectations, 2HPγ solvated less significantly less S₈ and exhibited less linear binding behavior when compared to 2HPβ (Figure S5(a)). Similarly, 2HPα solubilized very little S₈ with significant deviations from a well-defined binding relationship (Figure S5(c,d)). These experiments further support that the size complementarity between S₈ and 2HPβ is an important factor in solubilization.

Building from our data supporting S₈ solubilization in the 2HPβ, we next sought to determine whether the solubilized S₈ is chemically accessible. To investigate this question, we first determined whether tris-(2-carboxyethyl)phosphine (TCEP), which reacts with S' to generate an oxidized phosphate-sulfide product, could access the solubilized S₈ and generate the characteristic P=S and P=O products upon phosphate-mediated reduction and subsequent hydrolysis. Conveniently, this conversion can readily be monitored qualitatively by ³¹P NMR spectroscopy with

![Figure 4](image-url)
TCEP ($\delta = 15.2$-$15.8$ ppm), the resultant TCEP sulfide ($\delta = 51.5$ ppm), and the associated TCEP oxide ($\delta = 53.0$ ppm), all having characteristic NMR resonances. Prior to TCEP addition, the $^{31}$P NMR spectrum of S$_8$ in a 25% w/w 2HP$\beta$ solution in pH 7.4 buffer only shows a peak at $\delta = 0$ ppm from the PBS (Figure 5a). After TCEP addition and incubation overnight at room temperature, however, the $^{31}$P NMR spectrum showed new peaks corresponding to unreacted TCEP, as well as the phosphine sulfide and oxide peaks at $\delta = 53.0$ and 51.5 ppm, respectively. To confirm that the peaks were from oxidized TCEP products, we repeated the TCEP incubation with TCEP and S$_8$ in MeOD (Figure S6a) and K$_2$S$_5$ in D$_2$O (Figure S6b). In both cases, we observed the same peaks in the $^{31}$P NMR spectrum at $\delta = 53.0$ and 51.5 ppm, confirming product formation. In the absence of the 2HP/$\beta$/S$_8$, only the TCEP peak is observed, which confirms that TCEP oxide is not formed from adventitious oxidation. Similarly, addition of TCEP to a solution of 2HP$\beta$ did not generate TCEP oxide (Figure 5b). Taken together, these results demonstrate that the solubilized S$_8$ in the 2HP$\beta$/S$_8$ solution is chemically accessible and can react directly with reductants.

![Figure 5](image)

Figure 5. $^{31}$P spectra in 2HP$\beta$/S$_8$ or 2HP$\beta$ solutions incubated with TCEP. (a) 25% w/w solution of 2HP$\beta$ with 500 mg S$_8$ before (top) and after (bottom) addition of 10 mg TCEP with overnight incubation. (b) 25% w/w 2HP$\beta$ without sulfur in PBS before (top) and after (bottom) TCEP addition and incubation; no oxidized product peaks are observed.

To further the potential biological relevance of the solubilized S$_8$ in the 2HP$\beta$/S$_8$ complex, we next determined whether the solubilized S$_8$ could be reduced by thiols to release H$_2$S. One important feature of S$^0$ that contributes to its role in biology is its ability to release H$_2$S upon reduction by thiols. Polysulfides, such as DATS, are well established to release H$_2$S after reaction with thiols and are used broadly as exogenous sources of S$^0$. Although prior studies have investigated how different functional groups on polysulfide motifs impacts H$_2$S release, one limitation of these systems is that all of these compounds also generate organic byproducts upon H$_2$S release. As expected, a higher S$^0$ content in polysulfides leads to greater H$_2$S release, although tetrasulfides appear to be the largest synthetically-accessible and consistently-stable polysulfides. Using a similar logic of trying to maximize the S$^0$ content per donor motif, we envisioned that the solubilized 2HP$\beta$/S$_8$ complex could also function as an entirely new approach to deliver S$^0$ and/or H$_2$S. Importantly, since S$_8$ is comprised entirely of S$^0$ it should be an effective donor, with the only byproduct being 2HP$\beta$.

To determine whether the S$_8$ solubilized in the 2HP$\beta$/S$_8$ system is accessible to thiols, and to quantify resulting H$_2$S release, we treated a 2HP$\beta$/S$_8$ solution (25 µM S$_8$, 200 µM S$^0$ in 50% w/w 2HP$\beta$) with 1 mM (5 equiv. with respect to S$^0$ atoms) of cysteine or reduced glutathione (GSH) under air-free conditions in pH 7.4 PBS. We measured H$_2$S release at different time points during the reaction by using the methylene blue assay, a colorimetric assay that measures H$_2$S production through the formation of the methylene blue dye (Figure 6). Calculated efficiency values assume all S$^0$ atoms can react to form H$_2$S. After 45 minutes, we observed 160 ± 5 µM H$_2$S release (80% efficiency) from the 2HP$\beta$/S$_8$ in the presence of cysteine (green). Under identical conditions, treatment of the complex with GSH (red), the most abundant biological thiol, yielded 220 ± 7 µM H$_2$S release after 45 minutes, corresponding to stoichiometric reduction of each S$^0$ atom. In the absence of S$_8$ with only 2HP$\beta$ and 500 µM cysteine, no H$_2$S was observed from the methylene blue assay (yellow), which confirms that 2HP$\beta$ and thiols alone do not spontaneously generate H$_2$S or result in methylene blue formation. Similarly, in the absence of 2HP$\beta$ we did not observe any H$_2$S release from S$_8$ (50 µM if fully soluble) and cysteine (500 µM, blue), confirming the importance of 2HP$\beta$ to the accessibility of S$_8$. As a whole, these results show that S$_8$ is made chemically accessible in water by solubilization with 2HP$\beta$, and that this sulfur can be reduced to H$_2$S with biologically relevant thiols.

In addition to the above experiments, we also determined whether S$_8$ needed to be pre-solubilized with 2HP$\beta$ prior to reaction with thiols, or whether 2HP$\beta$ could act as a catalyst for S$_8$ conversion to H$_2$S by thiols in water. To answer this question, we added 224 µg S$_8$ solid and 500 µM cysteine to 180 µg 2HP$\beta$ in 40 mL pH 7.4 PBS buffer and monitored H$_2$S generation using the methylene blue method. Under these conditions, we observed a faster peaking time of 15 minutes, but also a slightly lower overall efficiency of 147 µM H$_2$S release (74% efficient) (Figure S6). These data indicate that S$_8$ does not need to be pre-solvated to the 2HP$\beta$ complex prior to thiol addition in order to facilitate reaction with thiols and subsequent H$_2$S release. These data may also support the role of 2HP$\beta$ as a phase transfer catalyst in these environments and that the rate of thiol-mediated reduction is faster than the rate of S$_8$ encapsulation. Expanding from the present system, these results suggest that hydrophobic motifs in more complex systems may enable chemical accessibility of transiently formed S$_8$ from different redox processes.
inorganic

Figure 6. Release of H₂S from S₈ solvated in 2HPβ in the presence of cysteine (green) or GSH (red). 2HPβ alone (yellow) does not release H₂S in the presence of cysteine, and the amount of S₈ in solution at 50 μM without 2HPβ (blue) is not high enough for appreciable release. All data points collected in triplicate, and the error determined via standard deviation.

The accessibility of the solvated S₈ to biological thiols prompted us to investigate whether the solvated S₈ could be taken up into cells. Increasing intracellular levels of S₈ induces a cytoprotective effect by reducing oxidative stress, making direct S₈ donation desirable.³³ To this end, we treated HeLa cells with either 10 μM S₈ or 2HPβ/S₈ or an equivalent amount of 2HPβ alone for 24 hours. We then treated cells with the sulfane-sulfur selective fluorescent probe SSP₄.³³ We observed that cells treated with 2HPβ/S₈ showed a significant increase in fluorescence when compared with those treated with 2HPβ alone, which is consistent with the bioavailability of the solvibilized S₈ (Figure 7).³³ As a positive control, we repeated these experiments with HeLa cells that were treated with the inorganic polysulfide K₂S₈ as a source of S₈, which also showed a significant SSP₄ fluorescence response. These results support that the 2HPβ/S₈ system can cause significant increases in intracellular S₈ levels, though the efficiency of this uptake is not yet known.

Finally, we sought to determine whether the bioavailable solubilized S₈ could be used to access protective effects associated with S₈/H₂S. Both H₂S and S₈ species play important antioxidant and anti-inflammatory roles throughout biology and are effective reducing agents able to neutralize damaging oxidants and free radical species.³⁴ ³⁵ Polysulfides and persulfides containing S₈ have demonstrated potent antioxidative properties greater than those attributed to H₂S or thiols alone.³⁹ The well-studied antioxidant N-acetyl cysteine has also been shown to enhance the production of S₈.³³ As a whole, a common theme is that polysulfides, and their role as both H₂S and persulfide donor motifs, facilitates their protection against oxidative stress. Building from this observation, we reasoned that the high S₈ content of the 2HPβ/S₈ system should therefore make it an effective antioxidant in a cellular environment. With this in mind, we sought to determine whether the 2HPβ/S₈ system provided antioxidant potential in cellular environments.

We used the colorimetric Griess reagent to track relative levels of NO₃ metabolites in RAW 264.7 macrophage cells pretreated with either 2HPβ/S₈ or 2HPβ, and then lipopolysaccharide (LPS). In the presence of proinflammatory cytokines such as LPS, RAW 264.7 cells produce NO from inducible nitric oxide synthase (iNOS).³⁷ ³⁸ When formed, NO is rapidly oxidized to downstream NO₃ species, and NO₃⁻ can be quantified directly using the Griess assay, creating a colored product when cells are experiencing inflammation. Importantly, H₂S-releasing compounds have been previously demonstrated to significantly reduce NO₃⁻ formation in RAW 264.7 cells.³⁹ ⁴⁰ To investigate the activity of the 2HPβ/S₈ in this system, we plated RAW 264.7 macrophage cells on 24 well plates and the following day treated with the delivery vehicle for 2HPβ/S₈ (PBS), different concentrations of 2HPβ/S₈, or equivalent concentrations of 2HPβ alone for 24 hours. The cells were then washed and treated with 1 μg/mL LPS for another 24 hours. After incubation, the media was collected from each well and the amount of NO₃⁻ was quantified using the Griess assay. The absorbance values of each treatment condition were normalized to the vehicle, which received no anti-inflammatory treatment. As shown in Figure 8, addition of the 2HPβ/S₈ complex results in a significant and decrease in NO₃⁻ formation across a range of concentrations, as evidenced by the decrease of absorbance of the Griess product. These data are consistent with H₂S and S₈ release. The 2HPβ complex alone also results in a reduction in NO₃⁻ levels, but to a much lower extent than the 2HPβ/S₈ system. It is possible that both H₂S and sulfane sulfur species generated from the solubilized S₈ both play roles in the reduction of NO₃⁻ levels in this assay, which were taken together supports prior work in the field demonstrating that both H₂S and sulfane sulfur provide protective effects toward models of oxidative stress.

Figure 7. Fluorescent images of HeLa cells receiving 10 μM S₈ (top) the equivalent amount of 2HPβ alone (middle) or the inorganic polysulfide K₂S₈ (bottom) and imaged with the SSP₄ probe for S₈. Scale bar = 50 μm.
Conclusion

We have demonstrated that hydrophobic cyclodextrins can facilitate solubilization and chemical activity of S₈ in water. In addition to providing a new and significant approach to delivering S⁰/sulfane sulfur to aqueous and biological environments, these results provide fundamentally new insights that impact S₈ bioavailability. Building from the solubilization of S₈ by the 2HPβ system, these results may suggest that pools of oxidized sulfur can stably exist in biological hydrophobic structures such as proteins. Moreover, the demonstration that the 2HPβ system can effectively catalyze S₈ reduction to H₂S by thiols in water also highlights this approach as a method to limit S₈ accumulation. We anticipate that this and related systems currently under investigation in our lab will not only find utility as H₂S and sulfane sulfur delivery systems, but also in expanding investigations into how S⁰ is managed in more complex biological systems.

Experimental Section

Materials and Methods

Reagents were purchased from Fisher, Oakwood, and Tokyo Chemical Industry (TCI) and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories. ³¹P NMR spectra were recorded on a Bruker 500 MHz instrument. UV-vis spectra were recorded on an Agilent Cary 100 UV-vis spectrophotometer under standard conditions. Air-free techniques were performed under an inert N₂ atmosphere using Schlenk technique or in an Innovative Atmospheres glove box.

Procedures

CD/S₈ Complex Formation. Different weight percentages (0-45%) of 2-hydroxypropyl β-cyclodextrin (2HPβ) were added to pH 7.4 PBS buffer prepared from Millipore tablets in nanopure water in a stirred scintillation vial. A 10x molar equivalent of solid elemental sulfur (S₈) (Fisher) was added to this solution and shaken by hand until mixed. The vial was then stirred for several days at room temperature. After stirring, the solutions were taken up in plastic syringes and filtered through syringe filters (0.45 or 0.2 μm) to yield a clear solution that is colorless at low sulfur/cyclodextrin (CD) concentrations and yellow at higher concentrations. The S₈ concentration of each solution was quantified by UV-vis spectrophotometry in quartz cuvettes using the extinction coefficient of S₈ in methanol (εₘ₈ = 6730 M⁻¹ cm⁻¹).

S₈ Absorbance in MeOH versus buffer. To measure the absorbance of S₈ in methanol, 1.0 mg of S₈ was added to 7.0 mL of MeOH and shaken vigorously. The solution was then filtered into a quartz cuvette and the S₈ concentration was measured using the UV-vis absorbance at 263 nm. A stock solution of 2HPβ/S₈ in water was diluted to the same S₈ concentration and also measured by UV-vis. Both spectra were then overlaid to determine the percent difference of the S₈ concentration value.

Comparison of S₈ Solvation Between 2HPβ and Other Saccharides. Scintillations vial were charged with 640 mg S₈ followed by 10 mL of pH 7.4 PBS buffer or 10 mL of pH 7.4 PBS buffer containing 365 mg of either 2HPβ, hydroxypropyl cellulose (HPC), or glucose. The vials were sealed and stirred for three days, after which they were filtered and analyzed by UV-vis. Similar experiments were also performed with a 1.56% w/w solution of the parent non-functional β-CD in 8 mL pH 7.4 PBS buffer and 100 mg S₈. The resultant heterogenous solution was stirred for one month, filtered, and measured as described above. To evaluate S₈ solubilization in 2-hydroxypropyl γ-CD (2HPγ), a 25% w/w solution of 2HPγ or 2HPβ was prepared in 7.5 mL water or pH 7.4 PBS buffer. To each solution, 200 mg S₈ was added and solutions were stirred for two weeks, after which they were filtered and analyzed as described above.

Measurement of Binding Affinity of Elemental Sulfur to 2HPβ. Solutions of 2HPβ or other CDs of increasing weight percentage from 0 to 45% in pH 7.4 PBS were prepared from a 50% w/w stock solution of 2HPβ. To each of these vials were added 10-fold molar equivalents of S₈, and the resultant solutions were stirred for several days. After stirring, the solutions were filtered and the S₈ content quantified as described above. The measured S₈ concentrations from each sample were plotted against the 2HPβ concentration in a log-log plot described by equation (i). The apparent Kₛ value was obtained by linear regression analysis using Microsoft Excel’s LINEST function. These experiments were performed in triplicate.

³¹P NMR Spectroscopy of Trapped Sulfur. NMR tubes were charged with 25% w/w 2HPβ/S₈ solutions in pH 7.4 PBS buffer and ³¹P NMR spectra were recorded, after which a 30-fold molar excess of TCEP was added. The NMR tubes were incubated overnight at room temperature, after which additional ³¹P NMR spectra were recorded. The presence of reductant-labile sulfur was characterized by the formation of TCEP oxidation products, including TCEP sulfide or TCEP oxide formed by hydrolysis. The parent TCEP peak is at δ = 15.2-15.8 ppm, and the oxidized oxygen-containing and sulfur-containing product peaks are at δ =
53.0 (P=O) and 51.5 (P=S) ppm, respectively. To ensure that 2HPβ was not causing TCEP oxidation directly, the above experiments were performed with a solution containing only 25% w/w 2HPβ in pH 7.4 PBS and no oxidized TCEP products were observed.

**Evaluating Sulfur Content of Liquid and Precipitated 2HPβ/Sa Complex.** Two scintillation vials were charged with 25% w/w 2HPβ and a 10-fold excess of Sa in 10 mL pH 7.4 PBS buffer and stirred overnight. One solution was filtered and evaluated as described above. The other solution was filtered into a recrystallization dish filled with an excess of acetone, which results in formation of a white precipitate. The solvent was evaporated, and the resultant solid was collected and used to make a new 25% w/w solution, which was analyzed by UV-vis spectroscopy as described above.

**Measurement of H$_2$S Release from 2HPβ/Sa Complex in the Presence of Biological Thiols.** A round bottom flask was charged with 40 mL of degassed pH 7.4 PBS buffer, a stir bar, and either 1 mM L-cysteine or reduced GSH in an N$_2$-filled glove box. The solutions were removed from the glovebox and 500 µL of 2.0 mM Sa in 50% w/w 2HPβ (for a final Sa concentration of 25 µM) was added by syringe. For each time point, a 500 µL aliquot was removed by syringe and added to 500 µL of the methylene blue cocktail in a 1.5 mL plastic cuvette. (Methylene blue cocktail contains: 200 µL 30 mM FeCl$_3$ in 1.2 M HCl, 200 µL of 20 mM N,N-dimethyl-p-phenylene diamine in 7.2 M HCl, and 100 µL of 1% (w/v) Zn(OAc)$_2$.) The methylene blue reactions were incubated at room temperature for 1 hour, after which the absorbance at 670 nm was measured. For control experiments, the above procedure was repeated but with either 0.5 mg Sa (50 µM if fully soluble) or 0.9% w/w 2HPβ. All time points were collected in triplicate.

**Calibration Curve for Sulfide Measurement.** Solutions containing 500 µL of the methylene blue cocktail and 0.5 mL PBS containing 0.9% w/w 2HPβ were prepared in plastic cuvettes. A 100 mM stock solution of NaSH was prepared in 1.0 mL PBS under inert atmosphere. Solution was then diluted to 1 mM and added to the 1.0 mL solutions for final concentrations of 10, 20, 30, 40, 50, and 70 µM. The cuvettes were incubated at room temperature for 1 hour and absorbance at 670 nm was measured. All data points were measured in triplicate.

**Fluorescent S$^\circledast$ Imaging.** HeLa human cervical cancer cells (ATCC CCL-2) were cultured in DMEM containing phenol red, 10% premium grade fetal bovine serum (FBS), and 1% penicillin-streptomycin (PS) (10,000 units/mL penicillin and 10,000 µg/mL streptomycin). Cells were maintained at 37 °C under 5% CO$_2$. Cells were seeded in glass-bottomed 2 mL imaging dishes (MatTek) in DMEM containing 10% FBS, 1% PS and incubated overnight. The next day dishes were rinsed twice with FBS-free DMEM and incubated with test substances (solvated in PBS) in FBS-free DMEM for 24 hours. 2HPβ and 2HPβ/Sa stock solutions both contained 50% w/w 2HPβ, with equal volumes added to control and test dishes respectively. KS$_a$ was solvated in a ~1 mg/mL stock solution in water, and 10 µL were added to positive control dish. After treatment, dishes were again rinsed twice with FBS-free DMEM and incubated with NuRed and 10 µM SSP4 (Dojindo) separately in FBS-free DMEM according to manufacturer instructions for 15 minutes each. The NuRed (Invitrogen) concentration was two drops per 10 mL FBS-free DMEM. Dishes were rinsed twice with FluoroBrite DMEM (Thermo Fisher), and then imaged in this media. Image workup done with Fiji. 2HPβ/Sa SSP4 image normalized to 2HPβ only image, where this image’s intensity was turned down to background, and these identical settings applied to the 2HPβ/Sa image.

**Griess Assay.** RAW 264.7 murine macrophage cells (ATCC TIB-71) were cultured in DMEM containing phenol red, 10% premium grade fetal bovine serum (FBS), and 1% penicillin-streptomycin (PS) (10,000 units/mL penicillin and 10,000 µg/mL streptomycin). Cells were maintained at 37 °C under 5% CO$_2$. For the Griess assay, RAW 264.7 cells were seeded in sterile 12 well culture plates in DMEM containing 10% FBS, 1% PS and incubated overnight. The next day, the media was removed, and the cells were washed with PBS. The media was replaced with media containing either the 2HPβ/Sa complex or an equivalent w/w percentage of 2HPβ in FBS-free, phenol red-free DMEM. After a 24-hour incubation, cells are again rinsed and media is replaced with FBS-free, phenol red-free DMEM containing 1 µg/mL lipopolysaccharide (LPS). After another 24-hour incubation, 150 µL of media from each well was pipetted into a 96 well plate containing 20 µL mixed Griess reagent (Invitrogen) and then with mixed with 150 µL DI water. After incubation at room temperature for 30 minutes, plates were analyzed on a plate reader (BioTek Synergy 2).

**ASSOCIATED CONTENT**

**Supporting Information.** UV-vis spectra, NMR spectra, H$_2$S measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

* Corresponding Author
  * plut@uoregon.edu

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