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Modified cyclodextrins solubilize elemental sulfur in water and enable biological sulfane sulfur delivery†

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An important form of biological sulfur is sulfane sulfur, or S^0, which is found in polysulfide and persulfide compounds as well as in elemental sulfur. Sulfane sulfur, often in the form of S_8, functions as a key energy source in the metabolic processes of thermophilic Archaean organisms found in sulfur-rich environments and can be metabolized both aerobically and anaerobically by different archaeons. Despite this importance, S_8 has a low solubility in water (~19 nM), raising questions of how it can be made chemically accessible in complex environments. Motivated by prior crystallographic data showing S_8 binding to hydrophobic motifs in filamentous glycoproteins from the sulfur reducing *Staphylothermus marinus* anaerobe, we demonstrate that simple macrocyclic hydrophobic motifs, such as 2-hydroxypropyl β-cyclodextrin (2HPβ), are sufficient to solubilize S_8 at concentrations up to 2.0 ± 0.2 mM in aqueous solution. We demonstrate that the solubilized S_8 can be reduced with the common reductant *tris*(2-carboxyethyl)phosphine (TCEP) and reacts with thiols to generate H_2S. The thiol-mediated conversion of 2HPβ/S_8 to H_2S ranges from 80% to quantitative efficiency for Cys and glutathione (GSH). Moreover, we demonstrate that 2HPβ can catalyze the Cys-mediated reduction of S_8 to H_2S in water. Adding to the biological relevance of the developed systems, we demonstrate that treatment of Raw 264.7 macrophage cells with the 2HPβ/S_8 complex prior to LPS stimulation decreases NO_2^- levels, which is consistent with known activities of bioavailable H_2S and sulfane sulfur. Taken together, these investigations provide a new strategy for delivering H_2S and sulfane sulfur in complex systems and more importantly provide new insights into the chemical accessibility and storage of S^0 and S_8 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 O_2, the atmosphere on Earth was weakly reducing.[1] Volcanic activity was an abundant source of sulfur-containing species, and gases including sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) were released and dissolved in pools or lakes.[2] Spark discharge experiments using a simulated atmosphere containing H_2S 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 H_2 and organosulfur compounds were generated from a mixture of H_2S and FeS under a N_2/CO_2 atmosphere in acidic conditions.[4] Sulfane sulfur (S^0), which is most commonly found as elemental octasulfur (S_8), is also found in these deep-sea environments and is another important source of biologically available sulfur.

Recently, interest in biological and synthetic S^0 sources has increased significantly due to the connection between such species and the small biological signaling molecule H_2S. H_2S is produced endogenously from cysteine metabolism and serves signaling roles in diverse pathways. Along with carbon monoxide (CO) and nitric oxide (NO), H_2S 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 H_2S 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, H_2S serves as a source of biologically available sulfur and is intrinsically tied to both organic and inorganic S^0-containing species, including persulfides and related polysulfides/polysulfanes, in the S^0 pool. This redox labile pool can generate H_2S upon reduction or

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participate in transpersulfidation reactions to transfer S^0 moieties to cysteine residues.[9]

Prior investigations into the reaction between elemental sulfur and H_2S have demonstrated the formation of inorganic polysulfide ions ($S^{2-}{}_{n>1}$), which are also important intermediates in sulfur cycling in sediments.[10] Interestingly, biologically produced sulfur particles from *Thiobacillus* sp. W5 react with HS^- to generate mixed polysulfides more efficiently than when compared S_8 (α-S_8) directly.[11] All of these examples highlight the important role and interconnected roles of the reactivity of S^0 and intermediate polysulfide formation upon reaction with sulfhydryl nucleophiles. Connecting with the biological activity of H_2S, the generation, action, and translocation of S^0-containing species 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 S^0 activity in aqueous systems are challenging due to the complex reactivity of available S^0 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.[12] Inorganic polysulfides in particular are unstable in aqueous conditions and quickly equilibrate to different polysulfide mixtures.[13] Despite these fundamental challenges, available S^0 sources demonstrate significant promise in different systems, ranging from anticancer properties in several human cell lines[14–17] to enhanced antioxidative activity.[18] 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 solubility of 6.4 μg L^{-1} (19(6) nM) at 25 °C in water[18] and ~4 μg L^{-1} in seawater.[19] Despite this low solubility, S^0 found in volcanic deep-sea environments can be readily metabolized by thermophilic Archaean organisms found in these sulfur-rich habitats.[5] As one example, species of the order *Sulfolobales* can derive energy from the metabolism of S^0 *via* both aerobic and anaerobic pathways.[20,21] Similarly, *Acidianus ambivalens* can utilize S^0 as both an electron donor and acceptor. Taken together, such organisms may provide clues into possible mechanisms of stabilizing and activating bioavailable S^0 in aqueous environments.

One potential strategy for S_8 solubilization and activation can be gleaned from the archaeon *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 filamentous glycoprotein structure called tetrabrachion that protrude from its surface. The tetrabrachion of *S. marinus* is composed of a four-stranded parallel coiled-coil structure with a hydrophobic core that is particularly stable.[22] The 24 kDa right-handed coiled-coil structure of the tetrabrachion 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 (Fig. 1).[23] Additional computational investigations have investigated the transit pathways for exchange of water and S_8 within these structures.[24] 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.[25] In addition, the stability of sulfur nanoparticles and sulfur sols, which can be produced both industrially and from sulfur oxidizing bacteria,[26] can also be modulated by different types of surfactants.[27,28] 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.

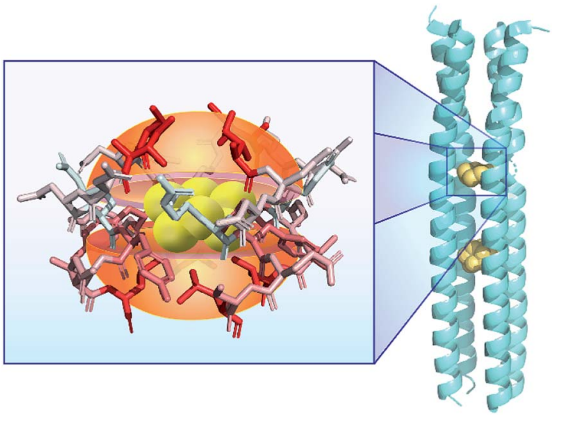
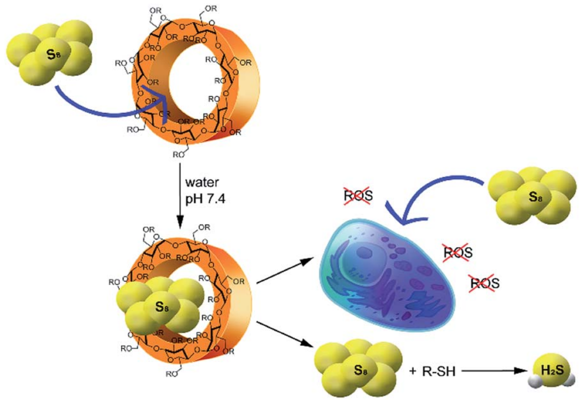


Fig. 1 Structure of the tetrameric right-handed coiled-coil component of the tetrabrachion of the archaeon *S. marinus* (PDB: 5JR5) demonstrating two hydrophobic pockets in the core capable of encapsulating S_8. Highlighted residues are colored according to their hydrophobicity, where red indicates stronger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

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).

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



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

biological applications is severely hampered by its hydrophobicity and low water solubility. The solubility of S_8 has been calculated to be only $1.9(6) \times 10^{-8}$ mol kg^{-1} (or $1.5(2) \times 10^{-7}$ M S^0), which is multiple orders of magnitude below biologically relevant S^0 concentrations.[18] These results suggest that even small modifications to increase S_8 solubility in water can result in increased reactivity toward polysulfide formation. Moreover, efficient reduction of S_8 to H_2S will also require stabilization and solubilization of intermediate polysulfides or persulfide intermediates. Despite this low solubility, solid S_8 has been demonstrated to be biologically accessible by erythrocytes to produce H_2S,[29] 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 water-soluble compounds with hydrophobic interiors could enable similar S_8 binding.[30] 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.[31] This solubilization is due, in part, to the hydrophobic interior of CDs, which promotes encapsulation and binding of a non-polar 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.[32]

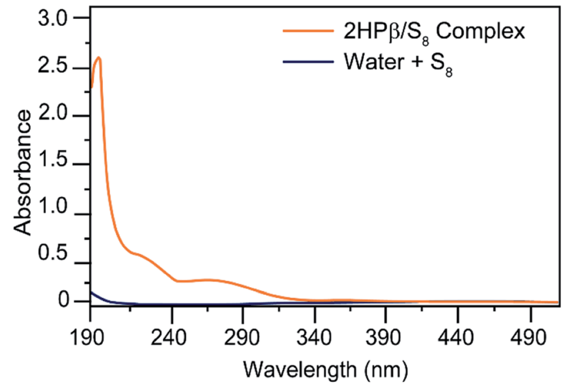


Fig. 2 Comparison of the UV-vis spectra of S_8 in water with S_8 in aqueous solutions containing 2HPβ. Conditions: 4 mg of S_8 in either 5 mL of PBS 7.4 PBS or 5 mL of 25% w/w 2HPβ in pH 7.4 PBS. Solutions were stirred for one day and then filtered prior to absorbance measurement.

To 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.[33] 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.[34] By contrast, stirring S_8 in water under identical conditions but in the absence of 2HPβ failed to produce a significant S_8 absorbance (Fig. 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 (Fig. 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.

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 (Fig. 3). As shown in Fig. 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 (Fig. S2†). After filtering the solution, we failed to observe any solubilized

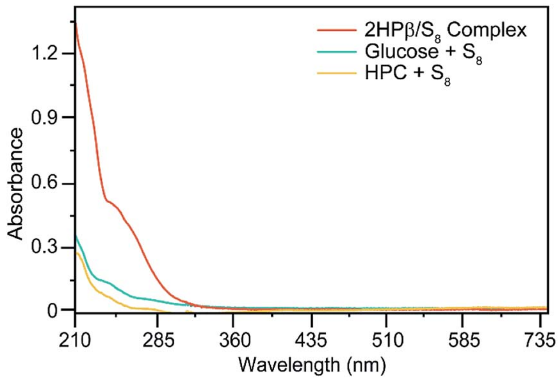


Fig. 3 UV-vis spectra of 640 mg S_8 in 10 mL pH 7.4 aqueous solutions containing 365 mg of 2HPβ, HPC, or glucose. 2HPβ solvates significantly more S_8 than either HPC or glucose.

S_8 by UV-vis spectroscopy, which suggests that the hydroxypropyl groups are required for S_8 solubilization.

To further investigate the requirement of the cyclic structure and presence of a cavity for S_8 solubilization, we also investigated whether 2-hydroxypropyl γ-CD (2HPγ; cavity volume: 427 $Å^3$) could solubilize S_8. We compared the S_8 solubilization in 25% w/w solutions of 2HPβ and 2HPγ in both pH. 7.4 PBS buffer and in water and observed significantly less S_8 solubilization from 2HPγ than from 2HPβ (Fig. 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 S_8 solvation by 2HPβ.

Building from these investigations, our next goal was to determine how much S_8 was solubilized in 2HPβ solutions. However, due to the low solubility of S_8 in water we used the extinction coefficient for S_8 in methanol, 6730 M^{-1} cm^{-1} at 263 nm, which has been reported previously.[34] To confirm that the extinction coefficient for S_8 in methanol could be used to measure S_8 concentrations in the 2HPβ/S_8 complex in water, we first prepared a solution of S_8 in MeOH at a known concentration (278 μM). We then used the known S_8/methanol extinction coefficient to measure the concentration of S_8 in an existing 2HPβ/S_8 solution. We then diluted this solution to match the calculated value of [S_8] in the methanol stock solution and compared the resulting absorbance traces. If the extinction coefficient remains constant between 2HPβ/S_8 and S_8 in methanol, then the calculated [S_8] for the 2HPβ/S_8 stock solution should be correct, and dilution from this value to the S_8 concentration in the methanol stock solution should yield an identical concentration and thus an identical absorbances.

The resultant curves (Fig. S4†) are similar to the 2HPβ/S_8 solution containing an S_8 concentration of 250 μM, which is ∼12% different between the methanol/S_8 and 2HPβ/S_8 solutions. This observation confirms that the extinction coefficient does not change appreciably between these two systems. On the basis of these comparisons, we have used the molar extinction coefficient of S_8 in MeOH to quantify S_8 in the 2HPβ. These quantified concentrations are further supported by the quantitative S_8 conversion to H_2S by thiols (*vide infra*), which provides additional support for the value of the reported S_8 concentrations in the 2HPβ system. Applying this extinction coefficient to the 50% w/w 2HPβ solution, provides an S_8 concentration of 2.0 ± 0.2 mM (16 mM S^0) in water. When compared to the background solubility of S_8 in water, this constitutes $\sim 10^5$-fold enhancement in S_8 solubility.

Having established that S_8 is readily solubilized in aqueous solution of 2HPβ we next sought to investigate the stoichiometry and magnitude of the interaction between S_8 and 2HPβ. To probe these interactions, we monitored the observed [S_8] in solution as a function of increasing [2HPβ] (from 0–45% w/w) that were prepared with a 10-fold molar excess of solid S_8 in solution. Under these conditions, the activity of S_8 in solution remains constant, and increasing the [2HPβ] should result in a concomitant increase in [S_8] in solution. After stirring each solution for several days to ensure equilibrium, the solutions were filtered, and the S_8 concentration were measured by UV-vis spectrophotometry (Fig. 4a). As expected, the measured [S_8] increased linearly with increasing [2HPβ], which further supports a direct interaction between S_8 and 2HPβ. The above constant activity data can be used to determine the binding stoichiometry and affinity between 2HPβ and S_8 through eqn (1).[35] Under these conditions, the total S_8 content is defined as S_t in eqn (1), and the concentration of unbound S_8, held constant throughout experiments to ensure constant activity, is defined as s_0 (1.9(6) × 10^{-8} mol kg^{-1}).[18] The constant n represents the binding stoichiometry.

$$\log \frac{S_t - s_0}{s_0} = \log K_a + n \times \log[2\text{HP}\beta] \quad (1)$$

Generating a log–log plot with the parameters of eqn (1) demonstrates that the relationship between 2HPβ and S_8 is consistent with 1 : 1 binding (Fig. 4b). Upon performing linear regression analysis, we obtained a K_a value of 3.4 ± 0.05 × 10^5 M^{-1} for the 2HPβ/S_8 complex, which is higher than the typical 10^2–10^3 M^{-1} binding affinities observed in βCD systems.[36] 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) (Fig. S5†). Our

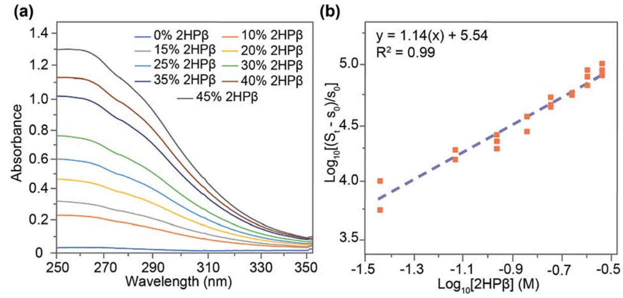


Fig. 4 (a) UV-vis spectra of increasing concentrations of 2HPβ with a 10-fold excess of S_8. (b) Log–log plot of quantified [S_8] as a function of increasing [2HPβ]. Trials were performed in triplicate.

expectation was that these differently-sized CD hosts would not solubilize or bind S_8 as efficiently as 2HPβ For example, if S_8 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.[30] Consistent with these expectations, 2HPγ solvated less significantly less S_8 and exhibited less linear binding behavior when compared to 2HPβ (Fig. S5a†). Similarly, 2HPα solubilized very little S_8 with significant deviations from a well-defined binding relationship (Fig. S5c and d†). These experiments further support that the size complementarity between S_8 and 2HPβ is an important factor in solubilization.

Building from our data supporting S_8 solubilization with 2HPβ we next sought to determine whether the solubilized S_8 is chemically accessible. To investigate this question, we first determined whether the reductant *tris*(2-carboxyethyl)phosphine (TCEP), which is a commonly-used and biologically compatible reductant used to reduce disulfides and other sulfane sulfur species, could access the solubilized S_8 and generate the characteristic P═S and P═O products upon phosphine-mediated reduction and subsequent hydrolysis. Conveniently, this conversion can readily be monitored qualitatively by ^{31}P NMR spectroscopy with 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 2HPβ solution in pH 7.4 buffer only shows a peak at $\delta = 0$ ppm from the PBS (Fig. 5a). After TCEP addition and incubation overnight at room temperature, however, the ^{31}P NMR spectrum showed peaks corresponding to unreacted TCEP, as well as the

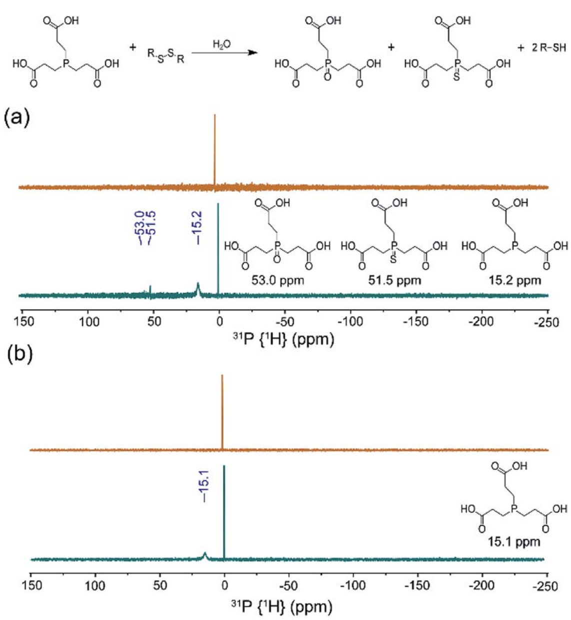


Fig. 5 ^{31}P {^{1}H} NMR spectra in 2HPβ/S_8 or 2HPβ solutions incubated with TCEP. (a) 25% w/w solution of 2HPβ with 500 mg S_8 before (top) and after (bottom) addition of 10 mg TCEP with overnight incubation. (b) 25% w/w 2HPβ without sulfur in PBS before (top) and after (bottom) TCEP addition and incubation; no oxidized product peaks are observed.

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 (Fig. S6a†) and K_2S_5 in D_2O (Fig. 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 2HPβ/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 2HPβ did not generate TCEP oxide (Fig. 5b). Taken together, these results demonstrate that the solubilized S_8 in the 2HPβ/S_8 solution is chemically accessible and can react directly with reductants.

To further the potential biological relevance of the solubilized S_8 in the 2HPβ/S_8 complex, we next determined whether the solubilized S_8 could be reduced by thiols to release H_2S. One important feature of S^0 that contributes to its role in biology is its ability to release H_2S upon reduction by thiols. Polysulfides, such as DATS, are well established to release H_2S 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_2S release, one limitation of these systems is that all of these compounds also generate organic byproducts upon H_2S release.[12,37] As expected, a higher S^0 content in organic polysulfides leads to greater H_2S 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 2HPβ/S_8 complex could also function as an entirely new approach to deliver S^0 and/or H_2S. Importantly, since S_8 is comprised entirely of S^0 it should be an effective donor, with the only byproduct being 2HPβ.

To determine whether the S_8 solubilized in the 2HPβ/S_8 system is accessible to thiols, and to quantify resulting H_2S release, we treated a 2HPβ/S_8 solution (25 μM S_8, 200 μM S^0 in 50% w/w 2HPβ) 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_2S release at different time points during the reaction by using the colorimetric methylene blue assay, which measures H_2S production by the formation of the methylene blue dye (Fig. 6). Calculated efficiency values assume all S^0 atoms can react to form H_2S. After 45 minutes, we observed 160 ± 5 μM H_2S release (80% efficiency) from the 2HPβ/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_2S release after 45 minutes, corresponding to stoichiometric reduction of each S^0 atom. In the absence of S_8 with only 2HPβ and 500 μM cysteine, no H_2S was observed from the methylene blue assay (yellow), which confirms that 2HPβ and thiols alone do not spontaneously generate H_2S or result in methylene blue formation. Similarly, in the absence of 2HPβ we did not observe any H_2S release from S_8 (50 μM if fully soluble) and cysteine (500 μM, blue), confirming the importance of 2HPβ to the accessibility of S_8. As a whole, these results show that S_8 is made chemically accessible in water by solubilization with 2HPβ, and that this sulfur can be reduced to H_2S with biologically relevant

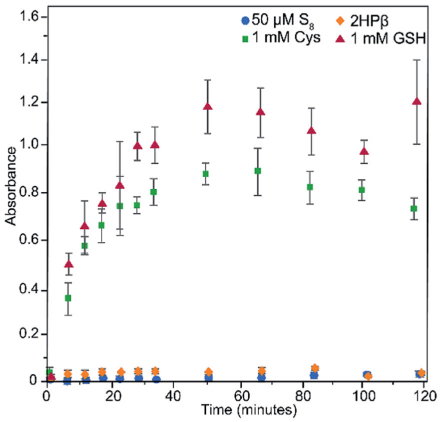


Fig. 6 Release of H_2S from S_8 solvated in 2HPβ in the presence of cysteine (green) or GSH (red). 2HPβ alone (yellow) does not release H_2S in the presence of cysteine, and the amount of S_8 in solution at 50 μM without 2HPβ (blue) is not high enough for appreciable release. All data points were collected in triplicate, and the error determined *via* standard deviation.

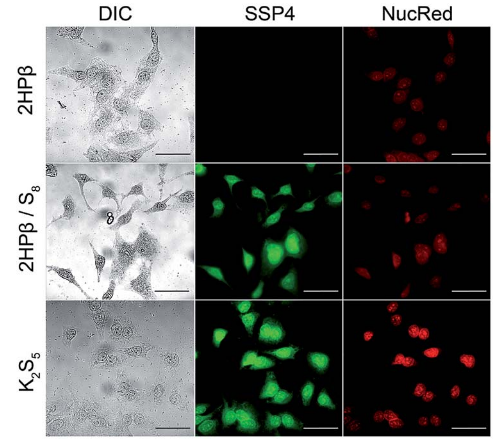


Fig. 7 Fluorescent images of HeLa cells treated with 2HPβ alone (top), 10 μM S_8 from a 2HPβ/S_8 solution (middle), or the inorganic polysulfide K_2S_5 (bottom) and imaged with the SSP4 probe for S^0. Scale bar = 50 μm.

thiols. Because this conversion requires the intermediate generation of persulfides and/or polysulfides en-route to H_2S release, these results may also suggest that the 2HPβ facilitates the solubilization of these reactive intermediates. Alternatively, this solubilizing environment may also help stabilize other forms of elemental sulfur, such as biosulfur or sulfur sols, which could facilitate further reactivity with sulfhydryl-containing nucleophiles.

In addition to the above experiments, we also determined whether S_8 needed to be pre-solubilized with 2HPβ prior to reaction with thiols, or whether 2HPβ could act as a catalyst for S_8 conversion to H_2S by thiols in water. To answer this question, we added 224 mg S_8 solid and 500 μM cysteine to 180 mg 2HPβ in 40 mL pH 7.4 PBS buffer and monitored H_2S 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_2S release (74% efficient) (Fig. S6†). These data indicate that S_8 does not need to be pre-solvated to the 2HPβ complex prior to thiol addition in order to facilitate reaction with thiols and subsequent H_2S release. These data may also support the role of 2HPβ 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.

The accessibility of the solvated S_8 to biological thiols prompted us to investigate whether the solvated S_8 could be taken up into cells. Increasing intracellular levels of S^0 induces a cytoprotective effect by reducing oxidative stress, making direct S^0 donation desirable.[38] Furthermore, the thiol-mediated reduction of S_8 to H_2S should proceed through persulfide and polysulfide formation, both of which should increase the levels of reactive sulfur species in cells. To evaluate cellular S_8 uptake, we treated HeLa cells with either 10 μM S_8 as 2HPβ/S_8 or an equivalent amount of 2HPβ alone for 24 hours. We then treated cells with the sulfane-sulfur selective fluorescent probe SSP4.[39] We observed that cells treated with 2HPβ/S_8 showed a significant increase in fluorescence when compared with those treated with 2HPβ alone, which is consistent with the bioavailability of the solubilized S_8 (Fig. 7).[40] As a positive control, we repeated these experiments with HeLa cells that were treated with the inorganic polysulfide K_2S_5 as a source of S^0, which also showed a significant SSP4 fluorescence response. These results support that the 2HPβ/S_8 system can cause significant increases in intracellular S^0 levels, though the efficiency of this uptake is not yet known.

Finally, we sought to determine whether the bioavailable solubilized S_8 could be used to access protective effects associated with S^0/H_2S. Both H_2S and S^0 species play important antioxidant and anti-inflammatory roles throughout biology and are effective reducing agents able to neutralize damaging oxidants and free radical species.[41,42] Polysulfides and persulfides containing S^0 have demonstrated antioxidative properties greater than those attributed to H_2S or thiols alone.[43] The well-studied antioxidant *N*-acetyl cysteine has also been shown to enhance the production of S^0.[38] As a whole, a common theme is that polysulfides, and their role as both H_2S and persulfide donor motifs, facilitates their protection against oxidative stress. The increased intracellular S_8 should further produce a diverse range of polysulfides in the cells. Building from this observation, we reasoned that the high S^0 content of the 2HPβ/S_8 system should therefore make it an effective antioxidant in a cellular environment. With this in mind, we sought to determine whether the 2HPβ/S_8 system provided antioxidant potential in cellular environments.

We used the colorimetric Griess reagent to track relative levels of NO_x metabolites in RAW 264.7 macrophage cells pretreated with either 2HPβ/S_8 or 2HPβ, and then lipopolysaccharide (LPS). In the presence of proinflammatory cytokines such

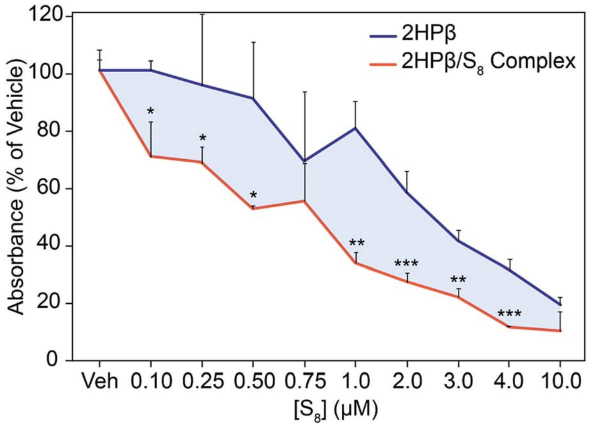


Fig. 8 Relative levels of NO_2^- in RAW 264.7 macrophage cells treated with different concentrations of 2HPβ/S_8 (orange) or equivalent concentrations of 2HPβ alone (blue). Data is normalized to the vehicle (PBS) treatment. Trials were performed in quadruplicate, and error determined by standard deviation.

as LPS, RAW 264.7 cells produce NO from inducible nitric oxide synthase (iNOS).[44,45] When formed, NO is rapidly oxidized to downstream NO_x species, and NO_2^- can be quantified directly using the colorimetric Griess assay. Importantly, H_2S-releasing compounds have been previously demonstrated to significantly decrease NO_2^- formation in RAW 264.7 cells.[46,47] To investigate the activity of 2HPβ/S_8 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/S_8 (PBS), different concentrations of 2HPβ/S_8, or equivalent concentrations of 2HPβ alone for 24 hours. The cells were then washed and treated with 1 μg mL^{-1} LPS for another 24 hours. After incubation, the media was collected from each well and the amount of NO_2^- was quantified using the Griess assay. The absorbance values of each treatment condition were normalized to the vehicle. As shown in Fig. 8, addition of the 2HPβ/S_8 complex results in a significant and decrease in NO_2^- formation across a range of concentrations, as evidenced by the decrease of absorbance of the Griess product. These data are consistent with H_2S and S^0 release. The 2HPβ alone also results in a reduction in NO_2^- levels, but to a much lower extent than the 2HPβ/S_8 system. It is possible that H_2S, polysulfides, and sulfane sulfur species generated from the solubilized S_8 all play roles in the reduction of NO_2^- levels in this assay, which when taken together supports prior work in the field demonstrating that both H_2S and sulfane sulfur provide protective effects toward models of oxidative stress.

Conclusions

We have demonstrated that hydrophobic cyclodextrins can facilitate solubilization and chemical activity of S_8 in water. In addition to providing a new and significant approach to delivering S^0/sulfane sulfur to aqueous and biological environments, these results provide fundamentally new insights that impact S_8 bioavailability. Building from the solubilization of S_8 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 S_8 reduction to H_2S by thiols in water also highlights this approach as a method to limit S_8 accumulation. We anticipate that this and related systems currently under investigation in our lab will not only find utility as H_2S and sulfane sulfur delivery systems, but also in expanding investigations into how S^0 is managed in more complex biological systems.

Conflicts of interest

There are no conflicts to declare.

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