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Controlled release of hydrogen sulfide significantly reduces ROS stress and increases dopamine levels in transgenic *C. elegans*†

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Hydrogen sulfide, an endogenous signalling molecule, is central to several pathophysiological processes in mammalian systems. It scavenges reactive oxygen species and is known to ameliorate dopaminergic neuronal degeneration in neurotoxin-induced Parkinson's disease models. The rapid volatilization of H_2S from spontaneously releasing sulfide salts being a challenge, we describe peptide conjugates which exhibit tris(2-carboxyethyl)phosphine mediated "slow and sustained" H_2S release. These conjugates reduced hydrogen peroxide-induced oxidative stress and significantly increased dopamine levels in transgenic *C. elegans*.

Hydrogen sulfide, a foul smelling metabolic poison under normal circumstances, is also known to be an endogenous neuronal modulator and a critical gasotransmitter, along with other therapeutically relevant gaseous molecules such as nitric oxide and carbon monoxide.[1,2] Three enzymes namely cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfur-transferase (MPST) are involved in the endogenous synthesis of H_2S in mammalian systems by converting cysteine or cysteine derivatives to H_2S in different tissues and organs.[3]

It is involved in important physiological functions in cardiovascular systems,[4] cancer,[5] modulation of inflammatory processes[6] and neuroprotection,[7] to name a few. The latter property is important as oxidative stress caused by reactive oxygen species (ROS) and imperfect redox states exposes neural cells to damage thereby leading to significant sensory and functional losses in neurodegeneration.[8] H_2S can react with ROS such as hydrogen peroxide, hydroxyl radicals, and superoxide radical anions, to show antioxidant action in a cellular environment. Such studies have interesting implications toward the elucidation of bacterial resistance mechanisms.[9]

Recent studies revealed that impairment in the endogenous H_2S production also leads to several neurodegenerative diseases such as Parkinson's disease (PD).[10] Hydrogen sulfide scavenges ROS and consequently showed antioxidant action.[11] Oxidative stress causes increased ROS levels inside the cells and is considered as one of the main causes of neuronal cell death. In PD, dopaminergic neurons degenerate mainly due to increased ROS levels, resulting in decreased dopamine levels in the brain.[12,13] Loss of dopamine results in the appearance of motor and non-motor symptoms such as bradykinesia, postural instability, cognitive deficits *etc.* Notably, various *in vitro* and *in vivo* PD models suggest that H_2S not only reverses the progression of PD but also has a protective effect on PD.[10,14,15]

Owing to the difficulty in handling malodorous H_2S gas, most of the biological studies are performed with an aqueous solution of sodium sulfide (Na_2S) and sodium hydrogen sulfide (NaHS). However, these sulfide salts spontaneously release H_2S and its concentration decreases rapidly due to volatilization, making it difficult to control the deliverable concentrations of these gases for therapeutic benefits. To overcome these limitations, several synthetic donors capable of releasing H_2S under physiological conditions have been developed.[16–20] Among which GYY4137[21–23] and dithiolthione (DTT)[24] are better explored, but they also release H_2S in an uncontrolled manner.

Peptide-based self-assembled structures present exciting delivery prospects due to their simple structure, biocompatibility and chemical diversity.[25–28] Recently our group has successfully demonstrated the beneficial role of peptide based soft structures in intracellular nitric oxide delivery.[29,30] Peptide-based platforms such as amphiphilc gels,[31,32] nanoribbons or nanocoils,[33] have emerged as an interesting approach for H_2S delivery in biological systems. Herein, we describe the design and synthesis of novel H_2S releasing peptide constructs which self-assembled in aqueous medium into nanostructures and released H_2S in a controlled manner. Furthermore, these conjugates exhibited excellent potential for neutralizing hydrogen peroxide-induced oxidative stress and showed a significant enhancement of dopamine levels in transgenic *Caenorhabditis elegans* (*C. elegans*).

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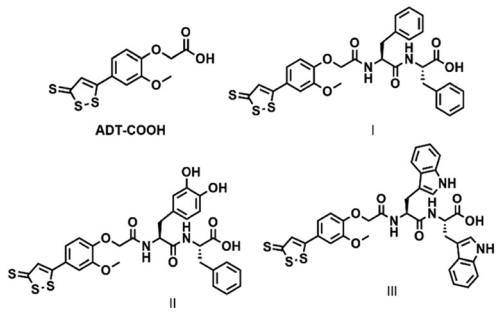


Fig. 1 Structures of H_2S releasing conjugates used in this study.

2-(2-Methoxy-4-(5-thioxo-5*H*-1,2-dithiol-3-yl)phenoxy)acetic acid (**ADT-COOH**), which contains the H_2S releasing dithiolethione (DTT) moiety, was selected for peptide conjugation due to its chemopreventive[34] and neuroprotective[35] properties (Fig. 1). Aromatic dipeptides such as Phe–Phe, Dopa–Phe and Trp–Trp were selected for conjugation with **ADT-COOH** to give conjugates **I, II** and **III**, respectively. These peptides are known to form well-defined self-assembled solution-phase morphologies in the shape of fibers and spherical structures.[36–38]

ADT-COOH was synthesized partially following a known protocol as outlined in Scheme 1a.[39] Peptide conjugates were synthesized by employing both solid-phase and solution-phase methods (Scheme 1b). The desired dipeptide was assembled on the resin and coupled with **ADT-COOH** followed by cleavage from the resin to afford target conjugates, which revealed the formation of soft structures in solution as characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM) (Fig. 2). The AFM micrographs of conjugate **I** reveal fiber-like structures of nanoscale dimensions, whereas conjugates **II** and **III** afforded spherical soft structures in aqueous medium. Dynamic light scattering measurements of these conjugates showed good agreement with the microscopy results (Fig. 3).

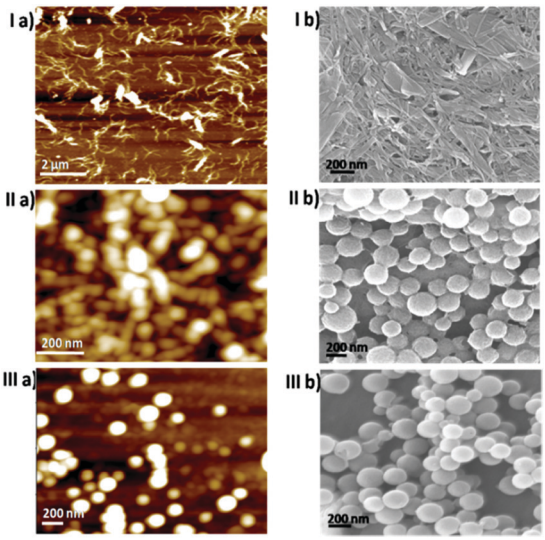
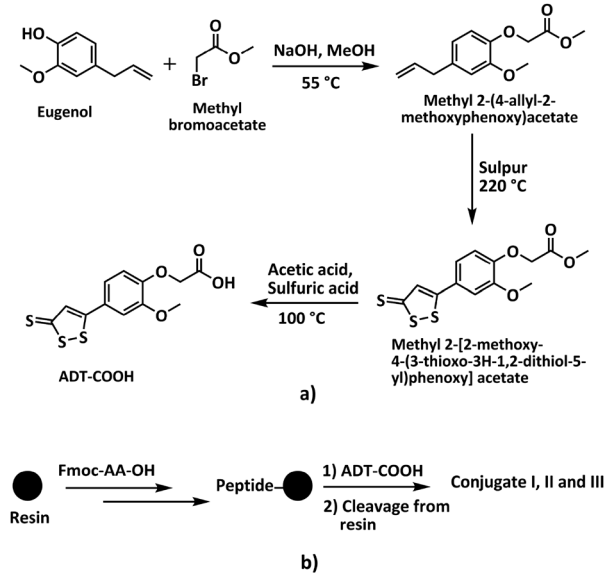


Fig. 2 AFM micrographs (**Ia–IIIa**) and SEM micrographs (**Ib–IIIb**) of conjugates **I**, **II** and **III** at 1 mM concentration on the copper surface.

The H_2S-releasing ability of these three donor conjugates was evaluated by incubating them with tris(2-carboxyethyl)phosphine (TCEP, used as a reducing agent) in phosphate buffer solution at 37 °C. H_2S release was detected and quantified using the methylene blue method.[40] H_2S release was quantified by measuring the absorbance at 670 nm on a UV-Vis spectrophotometer. Although a precise mechanism of TCEP-induced H_2S release is not known, it is believed that TCEP first reduces the disulfide bond to finally release H_2S.[41] In brief, a solution of conjugates (100 μM) in phosphate buffer (pH 7.4) was incubated with TCEP (1 mM) at 37 °C. Aliquots of this mixture were taken at specified time points and treated with solutions of MB cocktail, followed by incubation for 15 minutes at 37 °C. The absorption of the mixture was recorded at 670 nm. The concentrations of H_2S released were calculated using standard Na_2S curves (Fig. 4).



Scheme 1 (a) Synthesis of **ADT-COOH** and (b) general scheme for the synthesis of conjugates **I**, **II** and **III**.

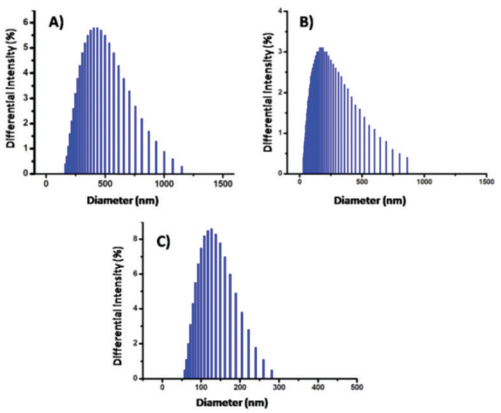


Fig. 3 Size distribution of conjugates **I** (A), **II** (B) and **III** (C) by dynamic light scattering measurements.

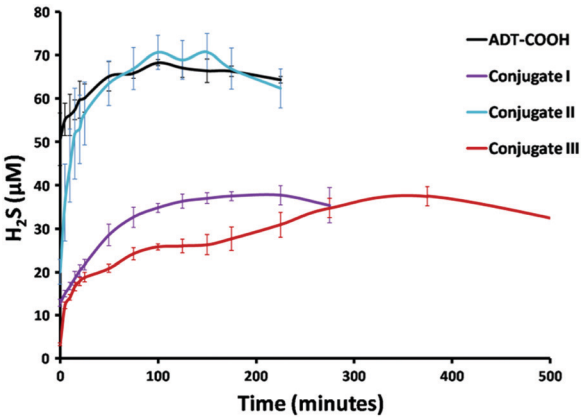


Fig. 4 *In vitro* H_2S release from **ADT-COOH**, conjugates **I**, **II** and **III** (100 μM) on incubation with tris(2-carboxyethyl)phosphine (TCEP, 1 mM) at 37 °C. Each bar represents the mean ± SD of three independent experiments.

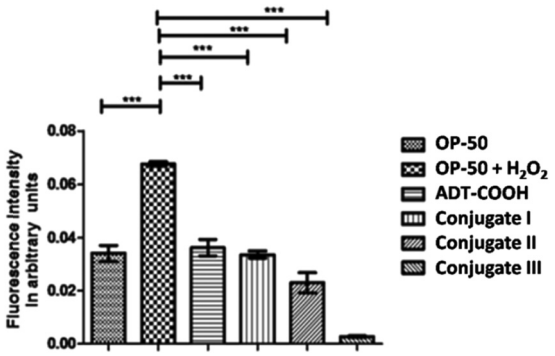


Fig. 5 ROS estimation using DCFDA in wild type N2 strain of *C. elegans* on treatment with 1 mM of compound **ADT-COOH**, **I**, **II** and **III** in the presence of 20 mM concentration of H_2O_2 (as positive control). Number of worms in each set is 100 and observations were measured in triplicate (***$P < 0.05$).

ADT-COOH released H_2S instantly on incubation with TCEP and reached the maximum H_2S concentration in solution in 50 min. Conjugate **I** slowly released H_2S in a controlled manner to reach the maximum concentration around 225 min, whereas conjugate **II** released H_2S faster than conjugate **I**, to reach the maximum concentration at 175 min. However, conjugate **III** released H_2S in a slow controlled fashion to reach the maximum concentration around 350 min. It appears that the difference in release kinetics could be ascribed to the morphology of these conjugates.

Oxidative stress is considered as one of the critical parameters responsible for neuronal degeneration. Therefore, elimination of reactive oxygen species (ROS) has long been recognized as a promising strategy for the treatment of neurodegenerative diseases such as Parkinson's disease. An increase in ROS, formed under different physiological stress conditions, worsens the outcome by serving as an early signal to mediate neuronal apoptosis or necrosis. The biology of *C. elegans* is highly useful in dissecting *in vivo* ROS functions, both at embryonic and postembryonic stages, thereby offering significant advantages in studying redox biology within a whole organismal environment.[42] Moreover, it is known that humans and *C. elegans* have similar core metabolic processes, which is a reason for using this worm as an important research tool to understand lipid and energy metabolism and metabolic fluxes in ageing, among others. In our study, the presence of cytochrome P450 genes and reductive metabolic enzymes in *C. elegans* provides an environment needed for H_2S release in constructs **I–III**.[43]

At first, we employed wild-type N2 strain of *C. elegans* as it represents an ideal model organism to study basic genetic and molecular mechanisms of human development and diseases. *C. elegans* were treated for 48 h with these conjugates, followed by 20 mM H_2O_2 (as positive control) for 1 h. Estimation of ROS was accomplished by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay using a reported protocol.[44] Of all conjugates, **III** exhibited excellent potential to reduce ROS load at 1 mM concentration, in wild-type N2 strain (Fig. 5).

Impaired body balance and control in PD originate from the progressive loss and degeneration of dopaminergic neurons in substantia nigra and resulting deprivation of dopamine in the striatum.[45] To further assess the role of these conjugates in PD-like symptoms, we employed transgenic NL5901 strain of *C. elegans* as a PD model. The strain expresses the human α-synuclein (α-Syn) protein fused to the yellow fluorescent protein, under control of a promoter, transgene *pkIs*2386, where the protein is expressed in the body wall muscle. Upon α-Syn expression the worms display a clear expression pattern which is quantifiable using fluorescence microscopy, thus giving a measure of effects associated with PD.[46] In a typical experiment, NL5901 worms were treated with **ADT-COOH** and conjugates **I**, **II** and **III**. After 48 h treatment, the worms from each group, approximated by random sample counts, to 5000 worms per group, were collected and sonicated in Milli Q water followed by quantification of dopamine content using LC-MS/MS. Our results showed that, with respect to the control, conjugate **III** significantly increased the levels of dopamine (Fig. 6).

It is known that H_2S is a reducing agent that increases the levels of intracellular glutathione, which in turn decreases the oxidative stress. Such an effect, specifically in dopaminergic neurons, would prevent damage to the dopaminergic system

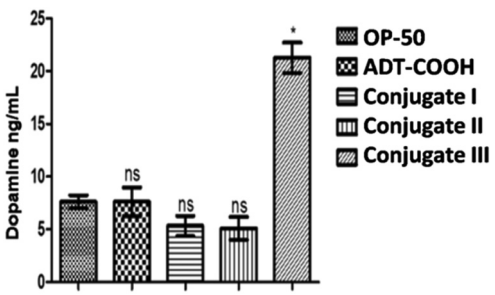


Fig. 6 HPLC/MS quantification of dopamine levels on treatment with compound **ADT-COOH**, **I**, **II** and **III** in NL5901 transgenic *C. elegans* (expressing human alpha synuclein YFP). Conjugate **III** significantly increases dopamine content. Data were analysed by using a simple Student t^2 test with means significantly different ($P < 0.05$). The number of worms is 5000.

thus augmenting the levels of dopamine. An important hallmark of PD is the inflammatory response; studies employing *in vitro* systems have reported that monoamine oxidase B (MAO-B), an enzyme primarily responsible for the oxidative degradation of neurotransmitter dopamine, is inhibited by H_2S releasing moieties.[47] Hence, in the present study, the observed dopamine enhancing effects could be attributed to the antioxidant and anti-inflammatory effects of H_2S. The properties of conjugate **III** in leading to slow and sustained release of H_2S probably result in its superior efficacy *vis-à-vis* countering dopamine decline.

In conclusion, we have developed a peptide-based system which releases hydrogen sulfide in a controlled manner. These conjugates self-assembled into fibres or spherical structures as a result of self-assembly, and they release hydrogen sulfide at a slower rate compared to **ADT-COOH**. These conjugates also significantly reduce ROS generated by hydrogen peroxide suggesting their promising antioxidant properties. Interestingly, conjugate **III** significantly increased the dopamine content, an important neurotransmitter crucial for transporting neuronal signals, which is pivotal for reward-motivated behavior and motor control through dopaminergic signaling.

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Conflicts of interest

There are no conflicts to declare.

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