**Mesoporous silica nanoparticles for glutathione-triggered long-range and stable release of hydrogen sulfide†**

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Mesoporous silica nanoparticles (MSNs) that can stably load therapeutic drugs and release them in response to a specific trigger are of great interest in disease diagnosis and treatment. However, the controlled-release of gaseous drug molecules such as hydrogen sulfide (H\(_2\)S) from a long-range and stable MSN-based system still presents a great challenge. Herein, a MSN-based glutathione (GSH)-triggered controlled-release H\(_2\)S system has been fabricated with high entrapment efficiency (99.0 ± 0.3\%) and loading content (44.2 ± 0.1\%) of diallyl trisulfide (DATS). After the addition of GSH (2 mM), DATS-MSN (100 \(\mu\)g mL\(^{-1}\)) steadily releases moderate amounts of H\(_2\)S (peaking at the 4th hour, ~60 \(\mu\)M) in phosphate buffer solution (PBS). The release of H\(_2\)S in plasma is similar to a physiological process (peaking at the 4th hour) and the DATS-MSN remains in the plasma of a rat’s system over 9 hours without significantly affecting the blood pressure, heart rate and cardiac function. Moderate quantities of nanoparticles can be taken up by cardiomyocytes in vitro, while in vivo study shows that nanoparticles mainly accumulate in the liver and spleen, affecting the H\(_2\)S level in these organs. Furthermore, DATS-MSN shows excellent biocompatibility, as well as superior cytoprotection and an isolated heart protection effect of H\(_2\)S under ischemic/reperfusion injury. This study provides a new insight into controlled-release applications of MSN-based H\(_2\)S releasing systems both in vitro and in vivo.

**Introduction**

Mesoporous silica nanoparticles are one of the most promising and versatile drug carrier candidates, which meet the needs of ideal nanovehicles.\(^1\)–\(^5\) Drugs delivered by MSNs show several advantages compared to free drugs such as improved solubility, stability, therapeutic efficacy, and biocompatibility.\(^6\)–\(^10\) Despite these promising benefits, the release of gas molecule drugs from MSN frameworks in a controlled fashion still presents a challenge. Stimuli-responsive MSN-drug formulations have a great potential to solve this problem due to their unique response to specific triggers. Examples of the most widely used intracellular or extracellular stimuli in the design of stimuli-responsive MSNs include pH, redox potential, light, temperature, and enzyme.\(^11\)–\(^14\) These smart drug delivery systems are designed to release their payloads at targeted organelles and tissues for *in vitro* and *in vivo* applications, respectively.\(^15\)–\(^17\)

In recent years, hydrogen sulfide (H\(_2\)S) has been recognized as the most common gaseous transmitter along with nitric oxide (NO) and carbon monoxide (CO). H\(_2\)S is endogenously synthesized from cysteine, which is activated by several enzymes such as cystathionine \(\beta\)-synthase (CBS), cystathionine \(\gamma\)-lyase (CSE), and 3-mercaptopyruvate sulfur-transferase (3-MST). The gaseous transmitter provides various physiological and pathophysiologic effects in different tissues, especially in the cardiovascular system. For example, H\(_2\)S can exert cytoprotection and anti-apoptotic effects by attenuating ischemia reperfusion injury in myocardium, preserving cardiac function.\(^18\)\(^,19\) Moreover, H\(_2\)S also provides vasodilation and anti-hypertensive effects by opening the
adenosine triphosphate sensitive potassium channels of vascular smooth muscles cells.20 Furthermore, H2S is also involved in many physiological processes, including anti-inflammation,21 antioxidant defense responses,22 neuromodulation,23 inhibiting insulin resistance,24 atherogenesis,25 anti-platelet activation,26 and metabolic suppression.27 The underlying mechanisms of these biological functions are currently being studied with increasing interest.

H2S studies have nowadays been largely promoted by realising ideal H2S donors. Previous studies employed many different H2S donors with different features. Sodium hydrosulfide (NaHS), the most commonly used H2S donor, can rapidly increase the H2S concentration. However, the instant release of H2S cause many adverse effects in the body such as rapid fall in blood pressure,28 and it cannot mimic the slow and continuous process of H2S generation in vivo. Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate (GY4137), a derivative of Lawesson’s reagent, reaction process.34 Among these decomposition products, donor, but converts into oxidized glutathione (GSSG) in the face functionality, 1,2,16 as well as its good dispersity in aqueous medium, which can reformulate poorly water-soluble drugs.7,8}


to elucidate whether the long-range and stable releasing donor causes the better biofunctionality of H2S. It is also necessary to identify the biocompatibility of DATS-MSN, as well as its ability of being captured by cells and different organs, which would affect H2S distribution in vivo. The study provides a unique MSN-based platform for controlled release of H2S in vitro and in vivo.

Results

Characterization of DATS-MSN

Transmission electron microscopy (TEM) images indicate that the mesoporous silica nanoparticles are monodisperse with uniform size and regular mesopores (Fig. 1a, b). The diameter of MSN is ~225 ± 35 nm (n = 3). The Brunauer–Emmett–Teller (BET) surface area of the MSNs is measured to be ~813 m² g⁻¹ (Fig. 1c). The pore size distribution curve (Fig. 1d) indicates that the obtained MSN has uniform pore size of ~2 nm. As shown in Fig. S1 (ESI†), the hydrodynamic diameter for DLS test is ~289 nm (PDI = 0.08). Due to the high superficial area and interaction between DATS and Si–OH, the entrapment efficiency of DATS-MSN is ~99.0 ± 0.3% (n = 3), suggesting that the overwhelming majority of drugs is combined to MSN. The calculated drug loading contents of the samples are ~44.2 ± 0.1% (n = 3). As shown in Fig. S2 (ESI†), the content of DATS released in 24 h is relatively low at ~8.4 ± 1.1% (n = 3).

![Fig. 1 Characterization of DATS-MSN. (a, b) TEM (a) and enlarged TEM (b) images. (c, d) Surface area measurement (c) and pore distribution (d) by multipoint BET analysis.](Image)
**In vitro H₂S release of DATS-MSN**

DATS-MSN released moderate amounts of H₂S in PBS steadily, which could be regulated by GSH concentration and pH condition. DATS and DATS-MSN are both stable in PBS (100 mM, pH 7.4), as shown by a H₂S-selective microelectrode. After adding 2 mM GSH, different H₂S release curves are observed for the two systems. The release of H₂S from DATS-MSN is much slower and continuous. The curve still continues to rise after 80 min. In contrast, DATS releases H₂S relatively rapidly, peaking at about 35 min before starting to fall. Compared to the peak, the real time picoamps current shows a significant decline after more than 1 h. As a control, H₂S generation from NaHS is also quite rapid. The concentration instantly peaks in seconds and rapidly decreases nearly to the baseline after half an hour without reaching a plateau (Fig. 2a).

For high-performance liquid chromatography (HPLC) analysis, H₂S generation of DATS-MSN in PBS (100 mM, pH 7.4) with GSH is shown in Fig. 2b. The maximum value is reached at the 4th hour (peaking time), while the plateau time is between 2–6 h, after which the H₂S concentration decreases gradually. In the presence of different concentrations of GSH, DATS-MSN significantly releases different amounts of H₂S. H₂S concentrations are apparently reduced by the decrease of GSH concentration from 2 mM and 200 μM to 20 μM (Fig. 2b). The release of H₂S from DATS-MSN is pH dependent (Fig. 2c) as it is significantly inhibited in an acidic environment (pH 6.5) compared with neutral (pH 7.4) environment, while alkaline PBS (pH 8.0) did not apparently affect H₂S release. Temperatures (37, 20 and 4 °C) have negligible impacts on the generation and release of H₂S (Fig. 2d).

**Fig. 2** Release of H₂S from DATS-MSN in PBS solution. (a) DATS (10 μg mL⁻¹), DATS-MSN (10 μg mL⁻¹) and NaHS (10 μM) with GSH (2 mM) (pH 7.4, 37 °C). Drugs are added at the 5th min, and GSH is added at the 10th min. (b) DATS-MSN (100 μg mL⁻¹) with different concentrations of GSH (2 mM, 200 μM and 20 μM) at pH 7.4 and 37 °C. (c) DATS-MSN (100 μg mL⁻¹) in different pH (8.0, 7.4 and 6.5) with GSH (2 mM) at 37 °C. (d) DATS-MSN (100 μg mL⁻¹) with different temperatures (37 °C, 20 °C and 4 °C) at time points 2, 4 and 6 h with GSH (2 mM) at pH 7.4. (a) Measurements are assessed by a H₂S-selective microelectrode, and representative tracings are obtained by at least 6 similar measurements. (b–d) Measurements are performed by HPLC (mean ± SEM, n = 6).

**Cytotoxicity assays and in vitro cellular uptake**

DATS-MSN shows no significant toxicity (cell viability >80%) even at concentrations up to 100 μg mL⁻¹ (Fig. 3a), suggesting that it can be safely used as a H₂S donor *in vitro*. Orange fluorescence of Cyanine 3 labelled MSN (Cy3-MSN) is observed in the cytoplasm, proving that the nanoparticles are phagocytozed by cardiomyocytes successfully (Fig. 3b, c). The fluorescent intensity of the nanoparticles is proportional to their number concentration, and a moderate concentration in cardiomyocytes was observed.

**Protection effect of DATS-MSN from hypoxia/reoxygenation induced damage**

Cell counting kit-8 (CCK-8) assay indicates that cell viability is protected by all NaHS, DATS and DATS-MSN pretreatment compared with the control after hypoxia/reoxygenation procedure, however, this protection effect in the DATS-MSN group is significantly better than in either the NaHS or DATS group (Fig. 3d). The lactate dehydrogenase (LDH) activity measurement shows that LDH activity is the lowest in the DATS-MSN group after the hypoxia/reoxygenation procedure (Fig. 3e), although a decrease of LDH activity is observed in all pretreating groups.

**In vivo H₂S release of DATS-MSN in plasma**

H₂S release from DATS-MSN in plasma *in vivo* is slow and mimics the physiological process. After vein injection of DATS-MSN, H₂S concentration in rat plasma increases from the first time point (30 min), peaks at the 4th hour, and continues to increase over the 9 h experiment. In contrast, although NaHS apparently increases the plasma H₂S concentration soon after administration (peaking at 1 h), H₂S levels reduces to baseline after 3 h. Direct administration of the same amount of DATS
shows a smaller increase of \( \text{H}_2\text{S} \) concentration and shorter affecting time than that of DATS-MSN (Fig. 3f).

**Biodistribution of DATS-MSN and \( \text{H}_2\text{S} \) in organs**

3D reconstruction of fluorescence light imaging tomography (FLIT) shows that nanoparticles in the mice body are mainly detected in the region of the liver, spleen and kidney, indicating the accumulation of nanoparticles in these organs (Fig. 4a). The fluorescence signal intensity peaks at 2 h, followed by a gradual decrease at 24 h and 7 d. At 7 d, the mice body only contains slight fluorescence signals. Rat \( \text{ex vivo} \) fluorescence imaging shows similar results. After euthanasia at 2 h, 24 h and 7 d after DATS-MSN administration, the fluorescence signals of the liver, kidney, spleen, lung, heart and brain collected are shown in Fig. 4b. The strongest fluorescence signal intensity is observed at 2 h, mainly detected in the liver, spleen and kidney, maintaining high levels for at least 24 h. At 7 d, the fluorescence signals of these organs almost decrease to the same level as the whole body except for a slight enhancement in the liver (Fig. 4c). Compared to the control, \( \text{H}_2\text{S} \) contents in the liver, kidney, spleen, heart, lung and brain are all clearly elevated at 2 h, especially in the liver (Fig. 4d). The \( \text{H}_2\text{S} \) content in organs decreases in a time-dependent way. At 24 h after injection, the \( \text{H}_2\text{S} \) level in just the liver and spleen is slightly higher than that in control. At 7 d, \( \text{H}_2\text{S} \) content in all organs falls back to the control level.

**Effect of DATS-MSN on blood pressure, heart rates and cardiac function**

DATS-MSN makes little difference to either mean artery pressure or heart rates within 1 h (Fig. S3, ESI†). In contrast, NaHS causes immediate and time-dependent fall in the rat’s mean artery pressure in 1 h (Fig. S3a, ESI†). Furthermore, heart rates are inhibited by NaHS administration, but not by DATS-MSN (Fig. S3b, ESI†). Neither NaHS nor DATS-MSN shows obvious heart inhibition, with no significant difference in both cardiac structure (left ventricular end-diastolic volume and left ventricular end-systolic volume) and function (ejection fraction and fractional shortening) data compared to that in control (Table S1, ESI†).

**Hematological, serological and histological examinations**

After injection with DATS-MSN for 2 h, 24 h and 7 d, all treated rats survived with normal activity. No abnormal symptoms or behavior is observed. There is no visible erythema or necrosis at the injection regions. As shown in Table S2 (ESI†), at 2 h, 24 h and 7 d, there is no significant difference on the red blood cells (RBC), white blood cells (WBC), platelets (PLT), hemoglobin (HGB) and hematocrit (HCT) when comparing the DATS-MSN treated rats to those in the control group. Similarly, no difference in WBC distribution (neutrophils and lymphocytes) is observed between the control and treated animals. Moreover, the levels of indicators of hepatic and renal function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum creatinine (CRE) and urea nitrogen in the serum (BUN), are all similar in the DATS-MSN treated group and control group at each time point. As shown in Fig. S4 (ESI†), compared with the control, all tissues (liver, kidney spleen, heart, lung, and brain) show no obvious pathological change in hematoxylin and eosin (H&E) examination at 2 h, 24 h and 7 d after DATS-MSN injection, indicating that DATS-MSN causes no significant tissue toxicity.

**Protection effect of DATS-MSN from ischemic/reperfusion (I/R) injury**

The pretreatment of NaHS, DATS and DATS-MSN shows heart protection effect after I/R injury compared with the control, however, the best functional recovery is observed in the DATS-MSN group, with significantly greater left ventricle developed pressure (LVDP, systolic minus diastolic pressure), maximum rate of left ventricular pressure development (\(+\text{dp/dt max, } -\text{dp/dt max}\) ) and coronary flow than those from either NaHS or DATS group (Table S3, ESI†). Moreover, although less apoptotic cells proportion is observed, decreased LDH and creatine kinase (CK) levels in collected perfused buffers are demonstrated in all 3 pretreatment groups compared with that in the control. The effect is most significantly observed in the DATS-MSN group (Fig. S5, ESI†). These observations indicate that DATS-MSN provides the heart protection effect of \( \text{H}_2\text{S} \) in I/R injury, which is more effective than either NaHS or DATS.

**Discussion**

MSN is utilized to reformulate a natural \( \text{H}_2\text{S} \) donor—DATS, and to successfully synthesize a novel \( \text{H}_2\text{S} \)-releasing system activated by GSH. Unlike NaHS and free DATS, DATS-MSN can release moderate amounts of \( \text{H}_2\text{S} \) in a very slow and controllable way \( \text{in vitro} \). It can also continually elevate \( \text{H}_2\text{S} \) levels in plasma and
concentration. Free DATS decomposes and generates H$_2$S more slowly than NaHS, but still relatively faster than DATS-MSN. DATS-MSN slowly releases H$_2$S, which can be regulated by GSH concentration.

For the release mechanism of DATS-MSN in vitro, DATS molecules may gradually be released into the solution and interact with GSH molecules. Our study shows that the content of DATS released from DATS-MSN is limited. Therefore, a possible mechanism could be that GSH molecules move into the mesopores of MSN to react with DATS for H$_2$S generation, which may slow down the release profile (Fig. 5). H$_2$S generation from DATS-MSN is a GSH-dependent pathway, which is limited by a lower GSH concentration. It suggests that H$_2$S release from the new system can be easily controlled by regulating GSH concentration, which is suitable for in vitro experiments requiring consistent but adjustable H$_2$S concentrations. The original intention of the study is to gain a slow-releasing H$_2$S donor that can be applied in organ preservation solutions. Therefore, besides the physiological temperature (37°C) and room temperature (20°C), the temperature for organ preservation (4°C) has also been studied. Data show that H$_2$S release is not inhibited at a low temperature. In addition, H$_2$S production is apparently limited in an acidic environment.

For in vivo study and circulation of DATS-MSN, the DATS-MSN system can also be used as a H$_2$S donor in vivo. Compared with NaHS, DATS-MSN increases H$_2$S concentration in the bloodstream slowly. Although the GSH concentration is relatively low in plasma (under 2 μM), the reduction of NADP$^+$ to NADPH via the pentose phosphate pathway (PPP), supported by glucose as the main energy source, maintains the GSH pool and ensures a sufficient GSH supply. In addition, other biological thiols in plasma such as cysteine may compete with GSH for the DATS. The H$_2$S release profile in such complex environment could be quite different from that in an aqueous solution. In addition, H$_2$S release in vivo also involves the distribution and metabolism of DATS-MSN. It has been reported that the blood circulation lifetime of MSN with similar size (200 nm) was nearly 4 h. In this study, the plasma H$_2$S concentration started to fall at 4 h after DATS-MSN injection, which is slower than clearance of MSN nanoparticles. The relatively large DATS-MSN particles (~225 nm) possess suitable blood-circulation lifetime and well controlled releasing rate of H$_2$S, which also effectively demonstrate the exerting protective effect of H$_2$S. Without good dispersity and circulation effect of MSN, the administration of free DATS temporarily elevates plasma H$_2$S level, which is insufficient. Therefore, combining with MSN makes DATS a more suitable H$_2$S donor for in vivo studies, and size modification and surface functionality of MSN in the future may bring more benefits.

For the uptake of MSN into cells, the clearance of MSN in blood circulation is mainly due to capture by various tissues, and intracellular uptake is one of the most important characters of nanoparticles. MSN can efficiently “enter” a variety of mammalian cells, and it is observed in this study that moderate quantities of nanoparticles were taken up by cardiomyocytes in vitro. Endocytosing is an important way by which drugs are being delivered into targeted cells, especially for biological molecules that lack specific membrane-bound receptors. It has been reported that H$_2$S production from DATS molecule is largely mediated by exofacial membrane protein thiols but not by intracellular thiols, moreover, the intracellular GSH concentration (2 mM) is much higher than that in the cellular exterior (2 μM). Thus, the endocytosis process of MSN may trigger larger amounts of H$_2$S production from DATS and change the intracellular H$_2$S levels. For nanoparticles with size larger than 100 nm, smaller nanoparticles passes through the cytomembrane more easily in considerable quantity. However, DATS-MSN is designed to be applied in a simple release environment, so the size of nanoparticles is relatively large and the intracellular process is controlled.

In accordance with previous studies that show nanoparticles taken up mainly by the reticuloendothelial system (RES) organs in vivo, the distribution of DATS-MSN affects H$_2$S level in different organs. At 2 h, almost all major organs collected show elevated H$_2$S content, which is due to the high H$_2$S level in blood circulation. However, only organs accumulating nanoparticles (liver and spleen) contain more H$_2$S than the control at 24 h. Along with the elimination of majority DATS-MSN, the H$_2$S level in all organs fell to the control level at 7 d. Two measures were performed to reflect the relative real condition of tissue H$_2$S. First, we designed a control group as the baseline. H$_2$S contents in the liver, kidney, spleen, heart, lung and brain were all clearly elevated compared to the control group. Second, to avoid H$_2$S loss from tissue samples, we also added ice-cold 5-sulfosalicylic acid into the samples to transit H$_2$S to HS – before

Fig. 5 A schematic of the mechanism of H$_2$S slow-release from DATS-MSN.
measurement, which further enhanced the stability of H2S during the measurement. Combined with MSN biodistribution, DATS-MSN is a unique H2S donor for a slow delivery of H2S to targeted organs after its clearance from blood circulation (such as at 24 h).

For the safety evaluation and toxicity, DATS-MSN shows a negligible cytotoxicity at the concentration required as H2S donors, proving the biocompatibility and safety of MSN in rat cardiomyocytes. For in vivo safety evaluation, unlike NaHS, obvious acute adverse effects like falling blood pressure and heart rates, as well as heart inhibition were not observed in DATS-MSN. The balance of toxicity and physiological functions of H2S depends on its concentration in the physiological environment. Our results indicated that the slow-releasing of H2S can avoid toxicity caused by rapid increase of H2S concentration. Although considerable liver and kidney accumulation of nanoparticles is observed, serological examination showed that liver and kidney function are all within the normal range. In addition, hematological data show no evidence of hemolysis or inflammation. H&E staining showed no significant tissue toxicity or irreparable impairment up to 7 d, indicating that rats suffer no obvious organ toxicity. Our results preliminarily prove that the working concentration of DATS-MSN is safe both in in vitro and in vivo studies.

The short effective residence time somewhat limits the biofunction effect of H2S. On the contrary, donors offering an accumulating H2S circumstance over a long time period are more potent in providing therapeutic functions. DATS-MSN also manifests better protection effect than free DATS, partially because of the poor solubility of DATS and its relatively fast H2S releasing process. Therefore, DATS-MSN is exactly an ideal H2S donor with a steady supply, to induce biofunctionality of H2S more effectively.

Conclusions

In summary, a novel GSH-triggered, water-dispersible H2S donor has been successfully synthesized, to slowly and controllably release moderate amounts of H2S. DATS-MSN elevates plasma H2S levels more effectively and sustainedly compared with either NaHS or DATS. The H2S content is elevated especially in the liver and spleen, which can be associated with the accumulation of nanoparticles in these organs. DATS-MSN can be safely used both in in vitro and in vivo, and can develop a superior myocardium protection effect of H2S from I/R injury in the cell and organ level. This study provides a consistent but tunable H2S condition, which can be applied to in vitro experiments such as cytoprotection and organ preservation. This ideal MSN-based platform allows for long-range and stable release of plasma H2S and its distribution to targeted organs. It also provides a new insight into control-releasing various gas molecules drugs based on MSNs and MSN-based nanoparticles.

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