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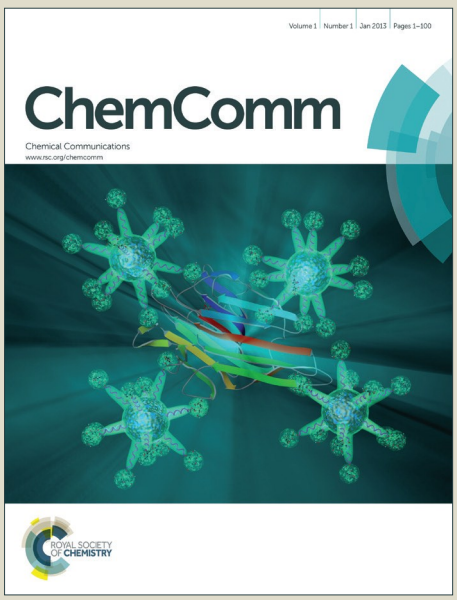

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NIR light controlled release of caged hydrogen sulfide based on upconversion nanoparticles

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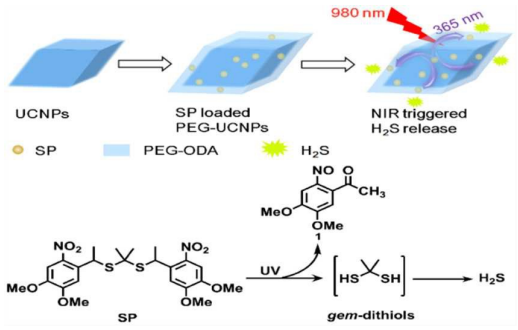
The NIR light induced H_2S release platform based on UCNPs was constructed. Under NIR light excitation, UCNPs can emit UV light which triggers H_2S release in a spatial and temporal pattern. The platform was also employed to real-time monitor the delivery process in vivo, which may provide a new way for the use of H_2S-based therapeutics for a variety of diseases.

In the past decade, hydrogen sulfide (H_2S), identified to be a novel gaseous transmitter, as carbon monoxide and nitric oxide, can be act as a cell-signalling mediator in physiology and pathology.[1] Recently, the application of H_2S in medical therapy has drawn much attention. Studies have shown that H_2S has great therapeutic potential in treatment of cancer,[2] inflammation[3] and neurodegenerative disease.[4] The rapid growth of the H_2S biomedical research has led to the concomitant need of the H_2S donor, since it has great therapeutic potentials of exogenous administration of H_2S.[5] Despite the great progress with respect to H_2S biomedical application, remote control the release of H_2S inside cells or tissue in a spatial and temporal precision manner remains a critical challenge.

So far, several intracellular controlled release strategies on the basis of photolysis, hydrolysis, thiol activation or bicarbonate activation,[5] have been developed to direct endogenous production of the H_2S in living cell. Among them, photo-induced release, which can achieve spatial- and temporal-specific control release of H_2S from donors, has been currently recognized as a new direction for their promising biomedical practices in H_2S release *in vivo*.[6] However, one major problem which exists in current photo-induced release relies on the short wavelength UV irradiation, which is suffered from low tissue penetration and biological damage. Thus, a new way to effectively trigger H_2S release with minimum side effects in living tissues is highly required.

Recently, upconversion nanoparticles (UCNPs) have attracted much attention in the fields of biological imaging and targeted delivery based on their novel optical properties.[7] UCNPs can absorb long wavelength NIR light and emit narrow and sharp emissions ranging from UV to visible,[8] which enable them to work as promising NIR-induced delivery or release cargos for biological studies.[9] It is well known that NIR light is safer and can penetrates several centimetres in tissue.[10] However, to the best of our knowledge, there is no report in literature of the use of NIR light excitation to emit UV light to spatially and temporally trigger H_2S release *in vitro* and *in vivo*.



Scheme 1. Construction of SP-loaded PEG-UCNPs platform for NIR-triggered H_2S release.

Herein, we present a simple NIR light reactive nanoparticle carrier system to exert target H_2S release in living cells on the basis of UCNPs (see Scheme 1). In this system, $LiYF_4$:Yb/Tm nanoparticles were chosen as light nanotransducers due to their strong emission at 365 nm with 980 nm irradiation. An amphiphilic compound polyethylene glycol-octadecylamine (PEG-ODA) was then coated on the surface of $LiYF_4$:Yb/Tm nanoparticles to convert hydrophobic nanoparticles into aqueous phase. At the same time, propane-2,2-diylbis((1-(4,5-dimethoxy-2-nitrophenyl)ethyl)sulfane) (SP), as a new H_2S donor (see Scheme S1 in ESI), was synthesized for the first time and loaded at the surface of UCNPs through hydrophobic interactions. Upon 980 nm laser irradiation, the upconverted UV light emitted from UCNPs is supposed to photo-cleavage SP to gem-dithiols through luminescence resonance energy transfer (LRET) effect. As we know, gem-dithiols are unstable and apt to hydrolysis

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to release H_2S[6] (showed in Scheme 1). Upon the nanocarriers arrive at the specific physiological targets, H_2S release can be trigged by 980 nm laser, thus giving a remotely, non-invasive and sustainable stimulus to trigger spatially and temporally controllable H_2S release.

The Yb^{3+}/Tm^{3+} co-doped UCNPs were prepared according to the protocol from the literature.[11] The morphologies of the UCNPs were characterized by transmission electron microscopy (TEM). As shown in Figure 1a, the UCNPs are monodisperse rhombus with the long axis around 100 nm. From the high resolution transmission electron microscopy (HRTEM) image (Figure S1), it also can be seen that the interplanar spacing for all lattice fringes is 0.41 nm, which corresponds to the (101) planes of tetragonal-phase of $LiYF_4$.[11] To improve the dispersion stability of nanoparticles in aqueous solutions, amphiphilic PEG-ODA synthesized from mPEG and ODA, was then bound with UCNPs through the lipophilic interaction between octadecyl chain and oleic acid at the surface of UCNPs. After coated with PEG-ODA the nanoparticles disperse well in water (see Figure 1c). In the TEM image of PEG-ODA modified UCNPs (PEG-UCNPs), a very thin layer was observed at the edge of UCNPs which mainly attributes to the high hydrophilic nature of PEG (see Figure 1b). The successful PEG-lyation was also confirmed by IR spectroscopy (Figure S2). The IR spectrum of PEG-UCNPs shows a band at 1,100 cm^{-1}, which is the characteristic stretching vibration of C-O-C bonds of PEG. In contrast, the PEG-modification did not affect the optical properties of UCNPs (see Figure S3). H_2S donor SP was loaded on PEG-UCNPs through the lipophilic interaction. The size distribution of PEG-UCNPs and SP loaded PEG-UCNPs were analysed by dynamic light scattering (DLS) measurement (Figure S2). The average particle size of PEG-UCNPs are around 156 nm in water, after SP loading the size of PEG-UCNPs has a little increase. The loading capacity for SP on the surface of PEG-UCNPs was determined by UV-Vis method (see Figure S4-S6), and the loading amount was estimated to be about 34,000 SP molecules loaded on one particle of UCNP. To investigate the H_2S release mechanism, reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) measurements were performed. As displayed in Figure S7, the retention time for SP is 13.8 min. By contrast, a main peak at 4.4 min was observed after UV irradiation for 10 min. The eluates at 4.4 min were collected and then analysed by HRMS. The molecular ion peak located at *m/z* 210.0759 (Figure S8), is assigned to $C_{10}H_{12}NO_4^+$, which further demonstrates the formation of compound **1**. Thus, the results confirmed the SP photo-cleavage mechanism shown in Scheme 1 and SP could be employed as a H_2S donor.

The LRET efficiency between the UCNPs and SP is of great importance for the NIR-induced H_2S release. Hence, the LRET effect between UCNPs and SP was assessed. Figure 1d shows the emission spectra of the UCNPs dispersed in aqueous solution before and after SP loading under excitation at 980 nm. SP shows an absorption band between 300–400 nm, and the emission intensity of UCNPs at 350–370 nm is significantly decreased after SP loading, which could be ascribed to the LRET effect or inner filter effect.[12] We also tested the fluorescence spectrum of the mixture of UCNPs and SP at the same concentration in the SP-loaded PEG-UCNPs, and little change in fluorescence was observed (Figure S9), suggesting that the fluorescence quenching around 365 nm is not caused by inner filter effect. These results confirmed that there is efficient LRET effect between PEG-UCNPs and SP, which is probably due to the short distance between UCNPs and SP and the perfect match between UCNPs emission and SP absorption.

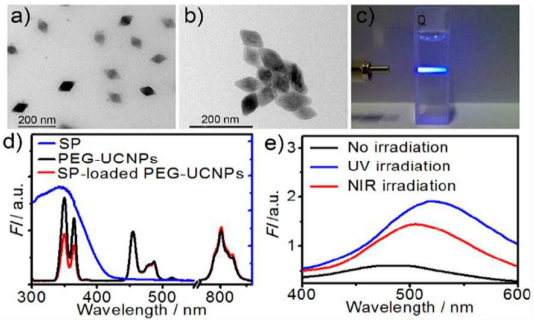

Figure 1. (a) TEM images of the as-prepared UCNPs and (b) PEG-UCNPs; (c) photos of the PEG-UCNPs in water (~3.5 mg mL^{-1}) under 980 nm laser irradiation (20 W cm^{-2}). (d) UV-vis absorption spectrum (blue line) of SP; emission spectra of UCNPs (black line) and SP-loaded PEG-UCNPs (red line). (e) Fluorescence spectra of DNS-Az in the presence of SP-loaded PEG-UCNPs with 365 nm UV light irradiation for 5 min or 980 nm laser irradiation for 2 h.

We next studied the light-induced release of H_2S from the platform, where dansylazide (DNS-Az) was used as a fluorescence probe to monitor the generation of H_2S (Scheme S2 and Figure S10). Firstly, the controlled release properties of the developed platform under UV light irradiation was carried out. As shown in Figure 1e, upon irradiation with UV light for 5 min, the fluorescence emission peak of DNS-Az shifts from 480 nm to 520 nm and the fluorescence intensity is significantly increased, indicating H_2S release caused by UV irradiation achieved.[13] Then, SP-loaded PEG-UCNPs were exposed to NIR light, the intensity around 520 nm is also increased and significantly much higher than that without light irradiation, which indicates the successful H_2S release triggered by NIR light from the platform. NIR-controlled H_2S release was further measured *in vitro* with varying NIR light intensity and irradiation time. As shown in Figure 2, the concentration of H_2S is increased when prolonging the irradiation time or increasing the irradiation intensity. In contrast, there's no significant H_2S release from SP solution in the absence of UCNPs at the same experimental condition (Figure S11). It indicates that the H_2S release is mainly triggered by NIR light in the presence of UCNPs, and can be controlled by either NIR irradiation time or NIR irradiation intensity. As a proof of concept, in our experiment the highest H_2S concentration released from SP-loaded PEG-UCNPs is 31 μM with the 1 mg mL^{-1} concentration of SP-loaded PEG-UCNPs under 20 W cm^{-2} of irradiation intensity for 2 h. In the further biological application, the H_2S concentration can be tuned by varying the concentration of the SP-loaded PEG-UCNPs, NIR irradiation time or irradiation intensity. It should be noted that H_2S could affect the biological process even when the concentration is in the range of 1–10 pmol per second per milligram protein.[14] Thus, our H_2S delivery platform will be able to satisfy the widely requirement of further application.

The cytotoxicity of SP-loaded PEG-UCNPs to both normal cells and cancer cells were also measured with MTT method.[15] For either L929 fibrosarcoma cells or MCF-7 breast cancer cells, the cell

survival rates are above 80% (see Figure S12) when incubated with the SP-loaded PEG-UCNPs. In addition, SP and PEG-UCNPs as well as NIR light irradiation also show low cytotoxicity (see Figure S12). These results suggest that the SP-loaded PEG-UCNPs are low cytotoxicity and have potential to be further used in biological system.

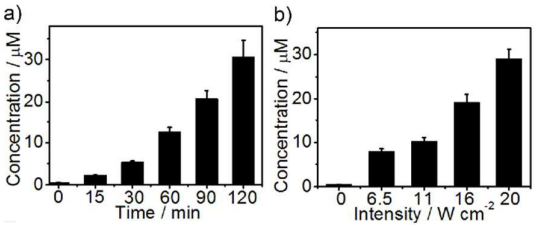

Figure 2. (a) H_2S release as a function of irradiation time from SP-loaded PEG-UCNPs (1 mg mL^{-1}) under 980 nm laser (20 W cm^{-2}). (b) H_2S release as a function of irradiation intensity from the SP-loaded PEG-UCNPs (1 mg mL^{-1}) under 980 nm laser for 2 h. Error bars mean S.D. (n =3).

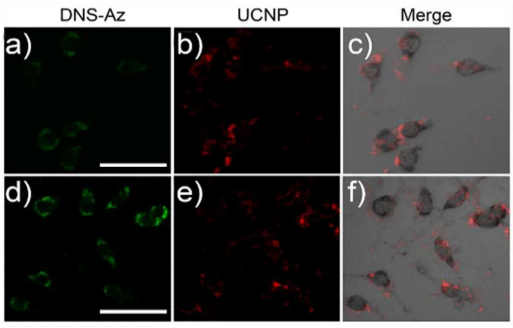

Figure 3. CLSM images of L929 cells (a-c) before and (d-f) after 980 nm laser irradiation. The cells were treated with SP-loaded PEG-UCNPs (2 mg mL^{-1}) and DNS-Az (50 μM) in CTAB (100μM) solution. The merged image represents the overlay of UCNP channel and bright field channel. The scale bars are 20 μm.

Encouraged by the promising results of H_2S release with NIR light irradiation *in vitro*, we further examined the possibility of NIR light controlled H_2S release from SP-loaded PEG-UCNPs in living cells. DNS-Az was used as the fluorescence probe to determine the intracellular H_2S generation. As shown in Figure 3b and 3e the fluorescence of UCNPs distributing around both cell surface and cytosol indicates that PEG-UCNPs were uptaken by cells. The fluorescence emission of DNS-Az were shown in Figure 3a and 3d, from which we can see that both of the cell samples showed green fluorescence signals. Obviously, the cells which were irradiated with 980 nm light exhibit much stronger fluorescence intensity than the one without light exposure (Figure 3a and 3d). The fluorescence intensity within cells after NIR irradiation is about 1.7 times as high as that before NIR irradiation (analysed by ImageJ software). In contrast, in the absence of SP-loaded PEG-UCNPs, there is little difference in the intracellular fluorescence intensity before and after NIR irradiation (data not shown). Thus, the increase of intracellular H_2S concentration can be ascribed to H_2S release from SP-loaded PEG-UCNPs under NIR irradiation.

To further explore the potential of H_2S release from SP-loaded PEG-UCNPs in deep tissue triggered by NIR light, we performed ex-vivo experiment. Pork skin was used as the tissue model plated on the top of SP-loaded PEG-UCNPs solution because pork skin is thought to be very similar to human's (see Figure 4a).[16] As shown in Figure 4b after NIR irradiation for 0.5 h and 1 h the H_2S concentration was up to 3.7 μM and 5.6 μM, respectively. In contrast, when irradiated with UV light only a little H_2S was released under the same condition. It should be noted that without pork skin H_2S release triggered by UV irradiation was much higher than that by NIR light (see Figure 1e). Therefore, compared with UV light NIR light significantly improves tissue penetration and is preferred for the controlled release of H_2S in deep tissue.

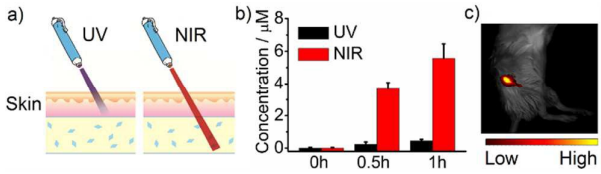

Figure 4. (a) Illustration the pork skin model used for in-deep tissue irradiation. (b) The ex-vivo H_2S release from SP-loaded PEG-UCNPs with UV (365 nm) or 980 nm laser irradiation for different time; (c) In vivo imaging of SP-loaded PEG-UCNPs.

UCNPs are supposed to be trackable *in vivo* because of their NIR fluorescence emission. To demonstrate the feasibility of *in vivo* imaging for SP-loaded PEG-UCNPs, we subcutaneously injected SP-loaded PEG-UCNP solutions into the back of Kunming mice, and then imaged it using the IVIS imaging system with the 980 nm laser as the excitation light. As shown in Figure 4c, SP-loaded PEG-UCNPs revealed strong NIR fluorescence emission under 980 nm irradiation, which can be used to track the SP-loaded PEG-UCNPs *in vivo* and facilitate to tune the H_2S release at the specific physiological targets.

In summary, we have developed an NIR light induced H_2S release platform based on UCNPs. Being exposed to 980 nm NIR laser, the functionalized SP-loaded PEG-UCNPs can emit UV light which is able to trigger H_2S release in both spatially and temporally controlled manner. Compared to the existing protocols for UV-controlled H_2S release, the use of unique NIR-to-UV properties of UCNPs would result in negligible photo-toxicity and guarantee high tissue penetration by NIR irradiation. Furthermore, H_2S delivery process *in vivo* can also be tracked in real-time with the NIR fluorescence emission of UCNPs, which is highly beneficial to the practical application of the H_2S-based therapeutics in a variety of diseases, such as cancer, inflammation as well as neurodegenerative diseases. The multiple advantages of PEG-UCNPs in H_2S release, NIR imaging and potential therapeutic applications could make it serve as an outstanding template in future biomedical research and therapeutic applications.

Improvements to this platform are still possible. The produced H_2S or the gem-dithiols as nucleophile can react with compound **1** before H_2S diffused into solution, thus affecting H_2S release efficiency, more efficient photoinduced H_2S donors should be developed. Because the excitation of UCNPs at 980 nm overlaps with water absorption peak at 970 nm, long time irradiation can lead

to overheating issue. However, this problem could be ameliorated by short interval irradiation in our experiment. In addition, novel UCNPs with other excitation wavelength such as 800 nm or 1490 nm are in development in our lab, which will avoid the overheating issue in another way.

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