Introduction

Hydrogen sulfide, one of the reactive sulfur species (RSS), has proved to be a key molecule in many diseases.1–3 Studies have shown that H₂S and H₂Sₙ are redox partners in terms of chemical properties and coexist in biological systems.3,4 Recently, accumulated data have indicated the important roles of H₂Sₙ in signal transduction in physiological processes, such as cardiovascular system, ion channels, oxidative post-translational modification, antioxidation and cytoprotection.5–11 Furthermore, aberrant production and physiological levels of H₂Sₙ are found to be correlated with physiological processes and diseases such as sulfur redox balance and liver disease.12,13 Nonalcoholic fatty liver disease (NAFLD), referring to a wide spectrum of liver damage that ranges from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis, represents the most common chronic liver disease in developed countries. Clarification of the functions of signaling molecules in NAFLD offers important insights into the pathogenesis of the disease, drug development and therapeutic evaluation.14,15 Even though some research studies have implied the existence of H₂Sₙ in liver disease,12,16,17 the role and signaling pathway of H₂Sₙ in the process of NAFLD have not been revealed due to the lack of suitable analytical methods for selectively monitoring H₂Sₙ in living biological systems.

Some in vitro analysis techniques including UV-spectroscopy, cold cyanolysis and mass spectrometry have been described to detect H₂Sₙ5,18 while fluorescence imaging has received widespread attention in H₂Sₙ bioanalysis due to its unique advantages in live cell imaging, such as excellent biocompatibility and high spatial and temporal resolution.19 Previous efforts in designing H₂Sₙ fluorescent probes were mainly based on conjugating functional groups, such as 2-fluoro-5-nitro-benzoic ester, phenyl-2-(benzoylthio)benzoate, and cinnamate ester, with hydroxyl-containing fluorophores.4,20,21 However, these functional groups may also react with abundant biothiols and active hydrolases inside cells, which could cause interference in H₂Sₙ detection. Only a few probes have been designed based on other electrophilic receptors, such as derivatives of xanthene or silicon-rhodamines.22–24 However, there are currently no methods suitable for selectively sensing H₂Sₙ from other persulfides (RSSH). At the same time, poor solubility, concentration fluctuation and unsatisfactory photo-stability make it more difficult to accurately monitor the changes of H₂Sₙ in live-cell imaging. To address these problems, we report a first-generation through-bond energy transfer (TBET) ratiometric fluorescent probe, PPG-Np-RhPhCO, with significantly improved photostability, selectivity and biocompatibility for monitoring intracellular H₂Sₙ via a simple and general strategy. The design concept consists of three steps (Fig. 1): (i) screening of new recognition sites based on the
stable electrophilic receptor, enabling an optimal response site for specific detection of H$_2$S that is independent of pH and hydrolyses; (ii) construction of ratiometric probes, achieving accurate detection of H$_2$S by single-wavelength excitation; (iii) self-assembly of nanoparticles, improving the selectivity, photostability and water solubility. The nano-probe PPG-Np-RhPhCO was successfully applied for monitoring the generation of H$_2$S$_n$ in NAFLD, which to our knowledge is reported for the first time. Importantly, we also show that 3-mercaptopyruvate sulfurtransferase (MPST) and cystathionine γ-lyase (CSE) are largely responsible for participating in the regulation of H$_2$S$_n$. Furthermore, we revealed that ROS/H$_2$S/H$_2$S$_n$ crosstalk signaling pathways that increased ROS in the drug-treated NAFLD model would stimulate MPST and CSE to produce more H$_2$S, which causes significant enhancement of H$_2$S$_n$ via reaction with ROS or catalysis by MPST and CSE.

Results and discussion

Design and screening of recognition sites

Although several fluorescent probes for H$_2$S$_n$ were prepared based on the introduction of aromatic ester groups into fluorophores, their poor stability resulting from the susceptibility to pH and hydrolase could easily cause false signals, thereby limiting their application in live-cell imaging. Several persulfide probes based on xanthene derivatives that are susceptible to nucleophilic attack and regulate the fluorescence changes for hydropolysulfide detection have been reported, but they lack the specificity of H$_2$S$_n$. To address these concerns, unsaturated double bonds containing different electron-withdrawing groups were introduced into the benzene moiety of xanthene dyes (Fig. 1 and Scheme S1†). It was proposed that the reaction with H$_2$S$_n$ produces persulfide intermediates via nucleophilic addition and then rapidly undergo intramolecular spirocyclization because of the high nucleophilicity and bis-nucleophiles of persulfide intermediates. As a proof of concept, seven xanthene derivatives bearing different substituents containing unsaturated double bonds (RhCHO1, RhIndo2, RhThia3, RhCN4, RhCOOE5, RhPhCO6, and RhAton7 in Scheme S2†) were synthesized and tested as candidates for new recognition site screening. To examine the fluorescence response toward different RSS, S$_8$, S$_2$O$_3^{2-}$, SO$_3^{2-}$, HS$O_3^-$, SO$_4^{2-}$, Na$_2$S, GSH, Cys, Hey, Na$_2$S$_2$ and Na$_2$S$_4$ were incubated with the probe in phosphate buffer (25 mM PBS mixed with 1% MeCN, pH 7.4) separately (Fig. 2, S1 and S2†). RhPhCO6 was chosen as the target recognition site due to fast response to H$_2$S$_n$ (<1 min, Fig. S3a†) and resistance to pH (pH 4.0–11.0, Fig. S3b†). The higher reaction activity of RhPhCO6 toward H$_2$S$_n$ than of the other six compounds was further explained by density functional theory (DFT) calculations (Fig. 2 and S4†), and it was a result of proper electrostatic charges of the β-carbon of the C=–C bond and the medial carbon of rhodamine in RhPhCO6, as well as steric hindrance. The reaction between unsaturated double bonds and H$_2$S$_n$ was followed by rapid intramolecular spirocyclization (Fig. S5†), as evidenced in the high resolution mass spectrum (Fig. S6†) and $^1$H NMR spectrum (Fig. S7†). Furthermore, to examine the possible reasons for the specific response of RhPhCO6 towards H$_2$S$_n$ rather than H$_2$S, time-dependent $^1$H NMR spectra of the reaction between RhPhCO6 and H$_2$S$_n$ or H$_2$S were recorded (Fig. S7 and S8†). The excellent selectivity is due to the ultra-fast reaction between RhPhCO6 and H$_2$S$_n$, which is probably caused by the special alpha effect of H$_2$S$_n$. Even novel recognition sites in RhPhCO6 exhibit excellent response towards H$_2$S$_n$, but a reaction with a high concentration of GSH and Cys could cause some interference with intracellular H$_2$S$_n$ sensing. Therefore, the self-assembly strategy of amphiphilic polymers was further utilized to increase the selectivity, which will be detailed in the next paragraph.
Construction of the ratiometric fluorescent nano-probe

The ratiometric signal offers more accurate detection of intracellular analytes due to its resistance to the influence of probe concentration and various cellular environments. Several ratiometric probes for H_2S have been reported recently, whose ratiometric mechanisms were achieved by intramolecular charge transfer (ICT) and Förster resonance energy transfer (FRET). Ratiometric probes based on through-bond energy transfer (TBET) usually exhibit higher energy transfer efficiency than those based on FRET even without spectral overlap. Thus, a ratiometric fluorescent probe Np-RhPhCO for sensing H_2S was constructed by combining a naphthalene unit (donor) and RhPhCO (acceptor) via Through-Bond Energy Transfer (TBET) cassettes (Scheme 1). The synthesis of probe Np-RhPhCO started from commercially available 5-bromophthalide using bromination and condensation to yield Br-Rhod-COOH and then reduction, oxidation and alcohol oxidation to obtain Br-Rhod-CHO (Scheme 1 and S3†). And the receptor Br-RhPhCO was subsequently synthesized via the classic Wittig reaction. Lastly, Pd-catalyzed Negishi cross-coupling of Br-RhPhCO and the boronic acid pinacol ester of Np (Np-Borate) provided the TBET-based ratiometric probe Np-RhPhCO (synthesis and characterization details in the ESI†). As reported before, the naphthalene–rhodamine TBET platform showed two well-separated absorbance peaks at 355 nm and 565 nm with emission peaks at 486 nm and 591 nm, respectively (Fig. S9†).

In recent years, nanoparticles used to improve the photo-stability and biocompatibility of small organic molecules have attracted increasing attention in the fields of biosensing, in vivo imaging and drug release. In addition, it has been reported that the selectivity can be improved by encapsulating an organic fluorescent probe in a self-assembled supramolecular system of an amphiphilic polymer. In order to achieve the application in a complex living system, we tried to encapsulate the small

Scheme 1 Synthesis of the ratiometric probe Np-RhPhCO. (i) NBS, AIBN, CHCl_3, reflux. (ii) H_2O, reflux. (iii) p-Toluenesulphonic acid, propionic acid, 3-diethylaminophenol, 90 °C. (iv) H_2SO_4, CH_3OH, 80 °C. (v) LiiH_4, dry THF, 0 °C. (vi) p-Chloranil, CH_2Cl_2/CH_3OH, rt. (vii) 1,1,1-Triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1H)-one, CH_2Cl_2, rt. (viii) (Benzoylmethylene)triphenylphosphorane, LiCl, CHCl_3, 70 °C. (ix) The boronic acid pinacol ester of Np (Np-Borate), K_3PO_4, Pd(dppf)Cl_2, 1,4-dioxane/H_2O, reflux.
molecule ratiometric probe \textit{Np-RhPhCO} into the self-assembled nanoparticles based on an amphiphilic copolymer to construct a more suitable organic nano-probe. 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) and poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (mPEG-PGG-PEG) were applied to prepare the nano-probes, respectively (Fig. S10\textendash13†). Among them, \textit{PPG-Np-RhPhCO} with a 33 nm average hydrodynamic size (Fig. 3b and S12b†) exhibited a 21-fold ratiometric signal (\(I_{486}/I_{591}\)) enhancement in the presence of \(H_2S_n\) (Fig. 3c and d), displaying a better response than DSPE-Np-RhPhCO. The detection limit was calculated to be as low as 9.4 nM, indicating the great potential to detect endogenous \(H_2S_n\) in live cells. Importantly, 7 \(\mu M\) \(Na_2S_2\) triggered a prominent enhancement of the fluorescence intensity ratio (\(I_{486}/I_{591}\)), while negligible signal fluctuation was obtained in the presence of other analytes (30 \(\mu M\) to 1 \(mM\)) (Fig. 3e). Notably, compared with \textit{Np-RhPhCO}, the nanoprobe \textit{PPG-Np-RhPhCO} showed less response towards bulky biothiols and their hydropolysulfides (Cys, GSH, CysSSH and GSSH) even with a longer incubation time (2.5 h) (Fig. 3f and g), which is probably due to physical blocking of the interaction. In addition, \textit{PPG-Np-RhPhCO} is capable of responding toward \(H_2S_n\) over a wide pH range (pH 4.0\textendash11.0) (Fig. S12a†).

Another key parameter of ideal fluorescent probes for living cell imaging is stability, primarily including long-term stability and photostability. The formed nano-probe showed good stability with only slight spectral changes for ten days at room temperature (Fig. S13†). Furthermore, illumination with continuous laser scanning showed that the fluorescence signal of \textit{PPG-Np-RhPhCO} remained stable over 3600 s, while the intensity of the parent probe \textit{Np-RhPhCO} decreased by 62 percent, demonstrating the significantly improved photostability of the nano-probe (Fig. 3h). This was ascribed to the fact that the outer polymer micelle provides an effective shelter protecting the inner organic small molecule probe \textit{Np-RhPhCO} from the surrounding environment. 80

Monitoring endogenous \(H_2S_n\) generation in live cells
Inspired by the excellent \textit{in vitro} response, we then investigated the performance of \textit{PPG-Np-RhPhCO} for detecting endogenous generation of \(H_2S_n\) in live cells. The cytotoxicity of \textit{PPG-Np-RhPhCO} in cells was first examined by standard MTT assays, indicating negligible cytotoxicity on the human liver cell line HL-7702 (L02) after 24 h of incubation (Fig. S14†). In good agreement with the \textit{in vitro} photostability test, the probe \textit{PPG-Np-RhPhCO} exhibited significantly higher photostability than the parent small-molecule probe \textit{Np-RhPhCO} in live cells under continuous excitation (Fig. S15†). To demonstrate the capability of \textit{PPG-Np-RhPhCO} for detecting endogenous \(H_2S_n\), we next performed live-cell imaging under different stimulation conditions. As shown in Fig. 4a and c, L02 cells incubated with \textit{PPG-Np-RhPhCO} showed weak fluorescence in the green channel (425\textendash475 nm) but strong fluorescence in the red channel (570\textendash620 nm) in the cytosol, indicating a low level of \(H_2S_n\) in the normal state. A lower \(F_{\text{Green}}/F_{\text{Red}}\) value was observed when 300 \(\mu M\) N-ethylmaleimide (NEM, a RSS blocking agent\textsuperscript{44}) was pre-treated for 30 min to cause the depletion of endogenous polysulfide and mercapto compounds. In contrast, addition of
and 1 mM GSH provide only slight fluorescence ratio ($F_{\text{Green}}/F_{\text{Red}}$) gradually increased as a function of incubation time, while introduction of 1 mM dl-propargylglycine (PAG, a commercial CSE irreversible inhibitor) yielded a clearly reduced fluorescence ratio signal. Moreover, the capability of the nano-probe PPG-Np-RhPhCO for imaging H$_2$S$_n$ was further confirmed via two-photon imaging in live cells, zebrafish, and fresh liver tissues (Fig. S16 and S17†).

**H$_2$S and MPST involved endogenous H$_2$S$_n$ generation in NAFLD**

Nonalcoholic fatty liver disease (NAFLD) is a common type of liver disease that evolves from simple steatosis to hepatitis and liver cancer. Determination of signaling molecules in the process of NAFLD offers insights into disease prevention, drug development and therapeutic evaluation. H$_2$S has been studied for decades to elucidate its functions in NAFLD; however, H$_2$S$_n$ as the partner of H$_2$S coexisting in the organism, has not been investigated in NAFLD due to the lack of suitable probes. Herein, we tried to examine the existence of H$_2$S$_n$ in NAFLD and further reveal the possible pathway of H$_2$S$_n$ generation. The NAFLD model was produced via incubating normal liver L02 cells with 0.5 mM FFA for 12 h, which was confirmed by the significantly increased lipid droplets and triglycerides based on Oil Red O staining experiments and triglyceride test kits (Fig. S18†). As shown in Fig. 5a and b, treatment with FFA leads to a huge increase in the ratiometric signal ($F_{\text{Green}}/F_{\text{Red}} = 3.9$), indicating the high level of H$_2$S$_n$ in NAFLD. Interestingly, an increase of the fluorescence ratio was observed in both normal L02 cells and FFA-stimulated L02 cells when a higher concentration of Na$_2$S (the donor of H$_2$S) was produced, indicating that the key role of H$_2$S is the regulation of endogenous H$_2$S$_n$ (Fig. 5d). Moreover, a higher level of endogenous H$_2$S in NALFD was observed after staining with the reported H$_2$S probe TPC-N$_3$ (Fig. S19†), which further suggests a positive correlation between H$_2$S and H$_2$S$_n$ production in NAFLD.

![Fig. 4 Imaging exogenous/endogenous H$_2$S$_n$ with the nano-probe PPG-Np-RhPhCO (40 μg mL$^{-1}$, 4.8 μM).](Image)

(a) Confocal microscopy images of H$_2$S$_n$ in live L02 cells with the nano-probe. The first column: incubation with the nano-probe for 2 h; the second column: pretreatment with 500 μM NEM for 0.5 h prior to incubation with the nano-probe for 2 h; the third column: incubation with 30 μM Na$_2$S$_2$ for another 20 min after treatment under the conditions in the second column; the fourth to seventh columns: incubation with Na$_2$S$_2$ (30 μM), Na$_2$S$_4$ (30 μM), Na$_2$S (50 μM), or GSH (1 mM) for another 20 min, respectively, after treatment with the nano-probe for 2 h. (b) Confocal microscopy images of H$_2$S$_n$ in live RAW 264.7 cells with the nano-probe PPG-Np-RhPhCO (4.8 μM) after stimulation with LPS. Live RAW 264.7 cells were incubated with the nano-probe for 2 h in the absence and presence of LPS (1 μg mL$^{-1}$) for 6 h and 12 h, respectively. The fourth column: live RAW 264.7 cells were pretreated with dl-propargylglycine (PAG, 1 mM) for 2 h before incubation with LPS (1 μg mL$^{-1}$) and probes. (c) Average fluorescence intensity ratios ($F_{\text{Green}}/F_{\text{Red}}$) of live L02 cells under the conditions in (a). (d) Average fluorescence intensity ratios ($F_{\text{Green}}/F_{\text{Red}}$) of live RAW 264.7 cells under the conditions in (b). Data are mean ± s.e.m. (n = 5 independent experiments; 80 cells). Statistical significance was calculated with unpaired two-tailed Student’s t-tests. *p < 0.05, **p < 0.01, ***p < 0.001. Green channel (425–475 nm), red channel (570–620 nm), pseudocolor ratio images (green channel/red channel). $\lambda_{ex} = 405$ nm. Scale bar: 20 μm.

Na$_2$S$_2$ (30 μM) or Na$_2$S$_4$ (30 μM), donors of H$_2$S$_n$, causes a significant ratiometric signal increase. Notably, 50 μM Na$_2$S and 1 mM GSH provide only slight $F_{\text{Green}}/F_{\text{Red}}$ changes, demonstrating the high selectivity of PPG-Np-RhPhCO towards H$_2$S$_n$ in living cells. Next, we further examined the capability of the nano-probe for detecting endogenous H$_2$S$_n$ generation in RAW 264.7 macrophages. It has been reported that bacterial endotoxin lipopolysaccharide (LPS) can induce the over-expression of cystathionine γ-lyase (CSE), which mediated cysteine metabolism for H$_2$S$_n$ formation in RAW 264.7 macrophages. Thus, we treated RAW 264.7 macrophages with the probe PPG-Np-RhPhCO in the presence of LPS with different incubation times (Fig. 4b and d). It was shown that the

$\lambda_{ex}$, and fresh liver tissues (Fig. S16 and S17†).
ROS/H$_2$S/H$_2$S$_n$ crosstalk signaling pathways in the NAFLD model

Previous literature showed that the reactive oxygen species (ROS) level in NAFLD increases greatly due to the progressive increase of oxidative stress levels caused by mitochondrial abnormalities in hepatic cells.$^{58}$ In addition, it is reported that ROS and RNS (including HClO, H$_2$O$_2$, O$_2^{=}$, NO, ONOO$^–$, etc.) react with H$_2$S to form H$_2$S$_n$, RSS$^–$, SO$_4^{2–}$, SO$_3^{2–}$, and S$_2$O$_3^{2–}$,$^{59–61}$ thus we assumed that higher ROS levels in the NAFLD model would also contribute to production and regulation of H$_2$S$_n$ (Fig. 5d). Moreover, there is growing evidence that long-term drug use for NAFLD patients could aggravate NAFLD and further increase the level of intracellular ROS,$^{53–56}$ Thus, a commonly used drug, acetaminophen (APAP, also known as paracetamol), was chosen to further elucidate the potential pathways of H$_2$S$_n$ production in a drug-treated NAFLD model.$^{56,57}$ As shown in Fig. S21,$^{†}$ both FFA and APAP can lead to higher levels of ONOO$^–$, one of the representative ROS, which was indicated by the reported probe MITO-CC.$^{58}$ It was shown that co-treatment with APAP and FFA causes higher ROS production than individual treatment separately (Fig. S20$^{†}$), probably due to the aggravation of oxidative stress caused by the synergistic effect of APAP and FFA.$^{56}$ Significantly, consistent enhancement of H$_2$S$_n$ and ROS under the above conditions was clearly observed (Fig. 6 and S21$^{†}$), strongly supporting our assumption that ROS plays an important role in the regulation of H$_2$S$_n$ production. We also investigated the change of the H$_2$S$_n$ level via two-photon tissue imaging of APAP-treated NAFLD mice, which was consistent with the results of cells imaging (Fig. S22$^{†}$).

Since we have revealed the function of H$_2$S in regulation of H$_2$S$_n$ generation in NAFLD, we next examined the H$_2$S level changes in the presence of a high concentration of ROS stimulated by APAP and FFA. Interestingly, consistent with ROS level changes, the H$_2$S level also increased more with co-treatment with FFA and APAP than when treated with FFA or APAP alone (Fig. 6d, S19 and S20$^{†}$). The consistent variation between ROS and H$_2$S is probably because of the redox homeostasis in living cells.$^{59–61}$ Moreover, treatment with the antioxidant ascorbic acid (VC, a natural, effective antioxidant$^{62}$) in NAFLD greatly reduced H$_2$S$_n$, H$_2$S and ROS levels simultaneously. Taken together, we assumed that direct reaction between H$_2$S and ROS is probably one of main pathways for producing H$_2$S$_n$, forming a ROS/H$_2$S/H$_2$S$_n$ crosstalk signaling pathway.

To further explore the functions of enzymes CSE, MPST and CBS in regulating the ROS/H$_2$S/H$_2$S$_n$ pathway, PAG (inhibitor of CSE), PDTC (inhibitor of MPST), and AOAA (inhibitor of CBS) were treated, respectively, prior to imaging H$_2$S$_n$ in APAP and FFA-treated L02 cells (Fig. 6a and b). Both H$_2$S$_n$ and H$_2$S displayed a significant decrease in the presence of PAG or PDTC, which points to the capability of CSE and MPST in regulating H$_2$S$_n$ and H$_2$S in the NAFLD model. However, the inhibitor AOAA had no obvious inhibitory effects maybe because the abundance of CBS in the liver is much lower than that of CSE.$^{63}$ These results were also consistent with the CSE and MPST western blot analysis results (Fig. 6c). Thus, the consistent changes of H$_2$S and H$_2$S$_n$ by regulation of CSE and MPST allow us to confirm the roles of H$_2$S, CSE and MPST in the H$_2$S$_n$ generation pathways (Fig. 6e). In combination with the participation of ROS in regulation of H$_2$S$_n$ generation, we proposed the ROS/H$_2$S/H$_2$S$_n$ crosstalk signaling pathways in Fig. 6e. Increased ROS in FFA-induced NAFLD stimulates MPST and CSE to produce more H$_2$S, which causes enhancement of H$_2$S$_n$ via direct reaction with ROS or catalysis by MPST and CSE.

In this work, to accurately and selectively image the intracellular H$_2$S$_n$, we introduced a new ratiometric fluorescent
Fig. 6 H$_2$S$_n$ imaging in drug-treated NAFLD. (a) Confocal fluorescence images of the nano-probe PPG-Np-RhPhCO (4.8 μM) in L02 cells under different conditions. The first column: incubation with the nano-probe for 2 h prior to imaging; the second and third columns: incubation with the nano-probe for 2 h after treatment with APAP (3 mM) or FFA (0.5 mM) for 12 h, respectively. The fourth to eighth columns: treatment with FFA (0.5 mM) for 12 h and VC (1 mM), PAG (1 mM), AOAA (300 μM) or PDTC (200 μM) for 2 h, respectively, and then with APAP (3 mM) for 12 h and with the nano-probe for 2 h. Green channel (425–475 nm), red channel (570–620 nm), pseudocolor ratio images (green channel/red channel). λex = 405 nm. Scale bar: 20 μm. (b) Average fluorescence intensity ratios ($F_{\text{Green}}/F_{\text{Red}}$) of live L02 cells under the conditions in (a). Data represent mean standard error (n = 5 independent experiments; 80 cells). Statistical significance in (a) was calculated with unpaired two-tailed Student’s t-tests. ***p < 0.001. Significance is expressed as # compare to the co-treatment with FFA and APAP, ##p < 0.05, ###p < 0.001. (c) Western blot analysis showing MPST and CSE expression in L02 cells upon treatment with FFA, APAP and inhibitor (PDTC or PAG), respectively. The MPST and CSE relative abundance was normalized with β-actin relative abundance. n = 3 independent experiments. (d) The relative levels of ONOO$^-$, H$_2$S and H$_2$S$_n$ in the NAFLD model. The data were based on fluorescence imaging of L02 cells stained with ONOO$^-$, H$_2$S and H$_2$S$_n$ probes (MITO-CC in Fig. S21† TPC-N3 in Fig. S20† and PPG-Np-RhPhCO in Fig. 6a), respectively, and were normalized with their respective control group. (e) Schematic of ROS/H$_2$S/H$_2$S$_n$ crosstalk signaling pathways that regulate H$_2$S$_n$ generation in the NAFLD model. Increased ROS in FFA-induced NAFLD stimulate MPST and CSE to produce more H$_2$S, which causes enhancement of H$_2$S$_n$ via direct reaction with ROS or catalysis by MPST and CSE. Black arrows denote the H$_2$S$_n$ generation pathway in FFA-induced NAFLD; red solid arrows and dashed arrows indicate ROS roles in the regulation of H$_2$S$_n$ in drug-treated NAFLD, respectively. VC represents antioxidants while PDTC and PAG indicate MPST and CSE inhibitors, respectively.
probe PPG-Np-RhPhCO, characterized by three main features that distinguish it from previous H$_2$S$_n$ sensors. First, rational design and fast screening offer new recognition sites for sensing H$_2$S$_n$, which avoid interference of pH and hydrolases in living cells. Second, introduction of nanoparticles formed by polymers enables the further improvement of selectivity of the probe, which is a prerequisite for applications in complex biological environments and a bottleneck for most small-molecule fluorescent probes. The encapsulation of fluorophores into nanoparticles works as a general method to improve the selectivity because it avoids the interaction with bulky potential competitors (Cys, GSH, CysSSH and GSSH), which were main interferences for previous H$_2$S$_n$ probes. Third, the nano-probe can offer more accurate detection of H$_2$S$_n$ through ratiometric imaging in living cells. The ratiometric quantification calculated from two signal channels can exclude the interference of probe concentration, which particularly benefits cell imaging under different stimulation conditions.

To examine the application of the new H$_2$S$_n$ probe in live-cell imaging, we incubated PPG-Np-RhPhCO with the liver cell line, HL-7702 cells, and murine macrophage cell line, RAW 264.7 cells. Live-cell imaging demonstrated the capability of the probe in detecting endogenous H$_2$S$_n$ with good sensitivity and signal to background ratio (Fig. 4). The excellent performance of this probe in imaging and monitoring H$_2$S$_n$ inspired us to address the challenge of H$_2$S$_n$ production pathways. In the NAFLD model, we found that FFA could stimulate MPST expression, which promotes the generation of H$_2$S$_n$. Moreover, as the precursor, H$_2$S at higher concentration can promote the further increase of H$_2$S$_n$ in the NAFLD model. To further explore the roles of ROS in the production of H$_2$S$_n$, we introduced a widely used drug, APAP, to boost ROS production in the NAFLD model. Simultaneous treatment with APAP and FFA causes a dramatic increase of ROS, which stimulates MPST and CSE to increase the generation of H$_2$S to maintain redox homeostasis. It is also worth noting that higher expression of CSE in LO2 cells was observed under the treatment with FFA and APAP simultaneously, but not under treatment with FFA alone. This is probably because ROS at very high levels can stimulate CSE up-regulation to elevate the level of antioxidant H$_2$S to maintain the redox balance. A higher level of ROS and H$_2$S can generate more H$_2$S$_n$ via chemical reaction, forming one of main pathways for H$_2$S$_n$ production in NAFLD. Meanwhile, MPST and CSE which are highly expressed under FFA and APAP-stimulation can also directly induce H$_2$S to generate H$_2$S$_n$ forming another main pathway for H$_2$S$_n$ production in NAFLD. The ROS/H$_2$S/H$_2$S$_n$ pathways in the NAFLD model (Fig. 6e) reported in this work will provide new insights into future drug development and therapeutic evaluation for NAFLD patients.

**Conclusions**

In summary, we designed and developed a ratiometric fluorescent probe, PPG-Np-RhPhCO, for monitoring the generation of endogenous H$_2$S$_n$ in NAFLD. The new probe, PPG-Np-RhPhCO, was successfully applied to reveal the possible ROS/H$_2$S/H$_2$S$_n$ pathways in the NAFLD model. The excellent selectivity, photostability and biocompatibility of the probe PPG-Np-RhPhCO enable it to be a practical and direct tool for detecting and studying intracellular H$_2$S$_n$ in complex biological samples.

**Ethical statement**

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hunan University, and all animal experiments were approved by the Animal Ethics Committee of the College of Biology (Hunan University).

**Conflicts of interest**

There are no conflicts to declare.

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