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A two-photon “turn-on” fluorescent probe based on carbon nanodots for imaging and selective biosensing of hydrogen sulfide in live cells and tissues

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Determination of hydrogen sulfide (H$_2$S) in live cells and tissues is still a challenge for evaluating the key roles that H$_2$S plays in the physiological and pathological processes. In this work, a “turn-on” two-photon fluorescent (TPF) sensor for H$_2$S was developed, in which carbon nanodot (C-Dot) was employed as a two-photon fluorophore due to its large two-photon absorption cross-section ($\sigma$) and AE-TPEA-Cu$^{2+}$ complex (AE-TPEA = N-(2-aminoethyl)-N,N,N'-tris(pyridin-2-ylmethyl)ethane-1,2-diamine) was first designed as a specific receptor for H$_2$S. The fluorescence of C-Dot conjugated with AE-TPEA (C-Dot-TPEA) was quenched upon the addition of Cu$^{2+}$. Then, the fluorescence was restored after the addition of H$_2$S, because Cu$^{2+}$ could be released from TPEA binding site when H$_2$S interacted with the Cu$^{2+}$ ion. The designed C-Dot-TPEA-Cu$^{2+}$ fluorescent sensor exhibited high specificity for H$_2$S over biothiols, sulphur-containing compound, reactive oxygen species (ROS), and other biological interferences. Meanwhile, a broad linear range from 5 µM to 100 µM was obtained and the detection limit was achieved to 0.7 µM. In addition, the C-Dot-based TPF probe exhibited bright two-photon fluorescence, favourable photo stability against light illumination and pH change, and low cytotoxicity. Accordingly, the nanohybridized TPF sensor with high selectivity and sensitivity, as well as the fascinating properties of C-Dot themselves, successfully provided a new way to TPF imaging and biosensing of H$_2$S in live cells and tissues. We believe this is the first report of TPF imaging and biosensing of H$_2$S in live cells and tissues using specially engineered C-Dot-based nanosystem.

Introduction

Over the past decades, two-photon fluorescent (TPF) probes have attracted much attention for their applications in the field of biomedical imaging and biosensing, because of the low background signal, deep tissue penetration depth, reduced photobleaching, and low phototoxicity associated with the use of near infrared two-photon excitation for TPF probes.1-5 Recently, carbon nanodot (C-Dot) have emerged as promising TPF probes due to their unique properties such as high water solubility, favourable biocompatibility, and long-term photostability.6-12 However, exploration of C-Dot for two-photon imaging and sensing still remains at an early stage. In this work, we report the design and synthesis of a two-photon fluorescent probe using C-Dot-based inorganic-organic nanohybrids for imaging and biosensing of physiological hydrogen sulfide (H$_2$S) in live cells and tissues.

Although H$_2$S is well known for the rotten egg smell, recent studies have demonstrated that H$_2$S is the third gaseous transmitter, in addition to nitric oxide (NO) and carbon monoxide (CO).13-14 H$_2$S contributes to various physiological processes, including relaxation of vascular smooth muscles, mediation of neurotransmission, regulation of inflammation and O$_2$ sensing, and it can also protect against ischemia/reperfusion injury.15-19 However, disruption of the H$_2$S level is linked to various diseases such as Alzheimer’s disease, Down’s syndrome, diabetes, and liver cirrhosis.20-23

So far, several elegant techniques have been developed for the detection of H$_2$S, including colorimetric and electrochemical assays, gas chromatography, and metal-induced sulfide precipitation.24-29 Fluorescence imaging and biosensing with spatial or temporal resolution for typical cell-based or live tissue experiments have attracted great attention in recent years.30-38 However, most of fluorescent probes for H$_2$S have been evaluated using one-photon microscopy and require relatively short excitation wavelengths,31-35 limiting their use in deep-tissue imaging because of the shallow penetration depth (<80 µm). Besides, the design and synthesis of new TPF probes from small molecules with suitable water solubility, biocompatibility and photostability is still very challenging.

Herein, we develop a TPF “turn-on” fluorescent sensor for H$_2$S with high selectivity and sensitivity, in which C-Dot was
employed as a TPF fluorophore and AE-TPEA/Cu²⁺ complex (AE-TPEA= N-(2-aminoethyl)-N,N,N',N'-tris(pyridin-2-ylmethyl)ethane-1,2-diamine) was designed as a specific receptor for H₂S. In our previous work, AE-TPEA was used as a recognition element for Cu²⁺ and AE-TPEA/Cu²⁺ showed high stability in the presence of biological metal ions and amino acids.

As illustrated in Scheme 1, the fluorescence of C-Dot conjugated with AE-TPEA (C-Dot-TPEA) was quenched upon the addition of Cu²⁺. Then, as expected, the fluorescence was restored after the addition of H₂S, because Cu²⁺ could be released from the AE-TPEA/Cu²⁺ complex when H₂S bond to the Cu²⁺ center. The designed C-Dot-TPEA-Cu²⁺ fluorescent sensor demonstrated high selectivity for H₂S over other potential interferences. Furthermore, the present biosensor showed a broad linear range of 5–100 μM and a low detection limit of 0.7 μM. Finally, the significant analytical performance of the biosensor, as well as good photo stability and low cytotoxicity of C-Dot, established a reliable fluorescent probe for TPF imaging and biosensing of H₂S in live cells.

**Fig. 1** Working principle of the C-Dot-TPEA-Cu²⁺ fluorescent probe for H₂S detection.

**Experimental**

**Reagents and chemicals**

Graphite rods were purchased from Alfa Aesar. Methanol, ethanol, dichloromethane, petroleum ether, ethyl acetate, acetonitrile, diethyl ether, tetrahydrofuran, L-5Glutathione (GSH), L-cysteine (L-Cys), DL-Homocysteine (DL-Hcy), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), metal salts, and silica gel (200-300 meshes) were obtained from Sinopharm. Sodium sulfite (Na₂SO₃), sodium thiosulfate (Na₂S₂O₃), and Triton X-100 were obtained from Aladdin. Sodium sulfide nonahydrate (Na₂S·9H₂O), N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), methyl thiazolyl tetrazolium (MTT), ethylenediamine (99%), and N,N,N',N'-tetraakis(3-(dimethylaminopropyl)ethane-1,2-diamine) was designed as a specific receptor for Cu²⁺ and AE-TPEA/Cu²⁺ showed high stability in the presence of biological metal ions and amino acids.

**Preparation of C-Dot**

C-Dot was synthesized according to the previously reported electrochemical method. Briefly, 0.3 g NaOH was dissolved in 100 mL ethanol/H₂O (99.5:0.5, v:v) by using graphite rods (diameter of 0.5 cm) as both anode and cathode. C-Dot was obtained at a current intensity of 40 mA cm⁻² for 12 h. The raw C-Dot solution was treated with a suitable amount of MgSO₄ (5–7 wt%) and stirred for 20 min. Then, it was left to stand for 24 h to remove the salts and water. Afterwards, the ethanol in the purified C-Dot solution was removed by vacuum distillation. To introduce carboxyl groups on the surface of C-Dot, the aqueous C-Dot solution was mixed with concentrated nitric acid, and the mixture was heated at reflux for 24 h. The excessive acid was finally neutralized by NaOH and then removed by dialyzing against Milli-Q water.

**Synthesis of AE-TPEA**

N,N,N',N'-tris(pyridin-2-ylmethyl)ethane-1,2-diamine (TPEA) was first synthesized using a modified literature procedure. Then, TPEA (4.31 g, 12.9 mmol), N-(2-bromoethyl)phthalimide (3.28 g, 12.9 mmol), anhydrous K₂CO₃ (4 g, 28.9 mmol), and KI (0.215 g, 1.29 mmol) were mixed in 40 mL of CH₂CN, the reaction was stirred at reflux for 24 h under nitrogen atmosphere. Concentration and purification of the obtained brown oil by basic alumina column chromatography (a mixture of CH₂Cl₂–CH₃OH as eluent) produced a tan solid. Next, to its solution in anhydrous ethanol (35 mL) was added hydrazine hydrate (0.625 mL, 12.9 mmol). After the product was cooled to room temperature, HCl (10.8 mL, 129 mmol) was added and the mixture was stirred for another hour at room temperature. The white solids were filtered off and the ethanol was removed by vacuum distillation. To this aqueous mixture was added NaOH. The aqueous phase was extracted three times with CH₂Cl₂ and the combined organic layers were dried over Na₂SO₄, filtered, and the solvent was removed, resulting in the product (N-(2-aminoethyl)-N,N,N',N'-tris(pyridin-2-ylmethyl)ethane-1,2-diamine, AE-TPEA) as a brown oil. Yield=40%. H NMR (400 MHz, CDCl₃): 8.53–8.48 (m, 3H, C₂H₃N), 7.64–7.10 (m, 9H, C₂H₄N), 3.81 (m, 4H, CH₂N), 2.72–2.67 (m, 6H, CH₂CH₃), 2.51 (t, 2H, CH₂CH₂), 1.60 (br, 2H, NH₂), 1.3 C NMR (100 MHz, CDCl₃): δ 160.0, 159.6, 148.9, 148.8, 136.3, 136.2, 122.8, 122.7, 121.9, 121.8, 66.0, 60.8, 57.8, 52.5, 52.3, 39.7. TOF MS El+: calculated for [M+H⁺] 376.2375, found 376.2377.

**Preparation of C-Dot-TPEA**

EDC/NHS (20 mM) and AE-TPEA (2 mM) were added into the obtained C-Dot solution and stirred for ~2 h. Finally, the nanohybrids were separated from the free EDC/NHS and AE-TPEA molecules by three cycles of concentration/dilution (10:1), using a Nanosep centrifugal device (Pall Corporation, MW cutoff of 3 kDa), and re-dispersed in PBS buffer (pH=7.4).

**Instruments and methods**

Optical absorption spectra were recorded by an Agilent 8453 UV-vis-NIR spectrophotometer. Infrared spectroscopic data were collected by infrared spectrometer (AVATAR-370DTGS).
In order to observe the morphology of C-Dot, transmission electron microscopy (HR-TEM, JEOL 2100, Japan) was employed. X-ray photoelectron spectroscopy (XPS) was carried out by PHI-5000C ESCA system (Perkin Elmer) with Mg K\(\alpha\) radiation (h\(ν\)=1253.6 eV). All binding energies (BEs) were referred to as the C 1s peak (284.6 eV) arising from surface hydrocarbons (or adventitious hydrocarbon). AFM images were recorded by Picoscan 2100 MI, USA. One-photon fluorescence spectra were obtained on a VARIAN Cary-Eclipse 500 fluorescence spectrophotometer. Two-photon spectra were recorded on a spectrometer (HORIBA Model iiiR550) and the pump laser beam came from a mode-locked Ti: sapphire laser system (Coherent Mira900). The cell images were taken by a confocal laser scanning microscope (Leica TCS SP8) equipped with a mode-locked Ti: sapphire laser source (Mai Tai DeepSee, 80 MHz, ~90 fs).

**Cell culture and cytotoxicity assay**

Cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing high glucose supplemented with fetal bovine serum (10 %, v/v), penicillin (100 units mL\(^{-1}\)), and streptomycin (100 µg mL\(^{-1}\)). The cellular cytotoxicity of C-Dot-TPEA-Cu\(^{2+}\) was tested by means of a standard MTT (methyl thiazolyl tetrazolium) assay. Firstly, the cells (~3×10\(^4\) cells mL\(^{-1}\)) were seeded onto a 96-well microtiter plate in an atmosphere of 5 % CO\(_2\) and 95 % air at 310 K humidified incubator for 48 h. Then, the cells were incubated in the fresh culture medium containing the C-Dot-TPEA-Cu\(^{2+}\) with different concentrations for 48 h. Thereafter, 100 µL of the new culture medium containing MTT (10 µL, 5 mg mL\(^{-1}\)) was added to each well followed by incubation for 4 h to allow the formation of formazan dye. Finally, the supernatant was removed before 150 µL of DMSO was added to each well and the plate was shaken for 10 min. By measuring the absorbance at 490 nm in a Multiskan MK3 microplate photometer (Thermo Scientific), the cell viability values were determined (at least 3 times) according to the following formula: cell viability (%) = the absorbance of experimental group/the absorbance of blank control group × 100%.

**Apoptosis assay**

HeLa cells were incubated with C-Dot-TPEA-Cu\(^{2+}\) at concentrations of 0.02, 0.06, 0.10 mg mL\(^{-1}\) for 24 h. Cells floating in the cell medium were collected by centrifuge while adherent cells were collected by treating with trypsin-EDTA. After washing with PBS, cells were then stained with FITC- annexin V (Molecular Probe) and Propidium Iodide (PI, Aldrich) following the standard protocol. A Becton-Dickinson flow cytometer was used for the flow cytometry measurements.

**In vitro fluorescence imaging**

Firstly, one day before imaging studies, the cultured cells were passaged and plated on a Petri dish. The culture media was replaced with 1 × PBS (pH=7.4) containing the C-Dot-TPEA probes, and the cells were incubated for ~1 h at 310 K. The cells were washed with 1 × PBS (pH=7.4) three times. The fluorescence imaging of HeLa cells were obtained with a spectral confocal and multiphoton microscopes (Leica TCS SP8). One-photon fluorescence images were obtained with an excitation wavelength at 488 nm and a collection window in the 520-700 nm range, and two-photon fluorescence images were excited at 800 nm and collected in the 440-650 nm range. Then, 100 µM CuCl\(_2\) were added and further incubated for ~1 h before imaging. Finally, 50 and 100 µM Na\(_2\)S were added and incubated with cells for ~30 min, and then fluorescence images were acquired.

**Preparation and staining of lung cancer tissue slices**

Tissue slices were prepared from the human lung cancer A549 cells. A total of 2×10\(^6\) A549 cells diluted in 200 µL of serum-free DMEM medium were injected subcutaneously into the right flank of 6 to 8-week-old BALB/c nude mice to inoculate tumors. On day 15 after A549 inoculation, mice were sacrificed. Tumors were removed and embedded with O.C.T (Sakura Finetek, USA, Torrance, CA) for frozen sections. The tissues were cut into 250 µm-thick slices using a vibrating-blade microtome. Slices were treated with C-Dot-TPEA nanoprobe (0.2 mg mL\(^{-1}\)) for 6 h at 310 K, then with CuCl\(_2\) (500 µM) for 1 h, and Na\(_2\)S (500 µM) for ~30 min. The slices were washed three times with 1×PBS and transferred to slice boxes for observation. The two-photon microscopy images of lung cancer tissue slices labeled with the nanoprobe were obtained at depths of 90–180 µm.

**Results and discussion**

Characterization of C-Dot-TPEA probe

The C-Dot was synthesized according to the previously reported electrochemical method. A typical transmission electron microscopy (TEM) image (Fig. 2A) shows that the as-synthesized C-Dot is ~5 nm in size. The corresponding atomic force microscopy (AFM) image (Fig. 2B, top) revealed a typical topographic height of 4.7–5.2 nm (Fig. 2B, bottom), suggesting that the average thickness of C-Dot is ~5 nm. The infrared (IR) spectrum of the C-Dot is given in Fig. 2C. The peaks located at ~2973 and 1563 cm\(^{-1}\) correspond to the C–C stretch of polycyclic aromatic hydrocarbons. The peak observed at ~1666 cm\(^{-1}\) indicates the existence of carbonyl (C=O) groups, while the band at ~3405 cm\(^{-1}\) is ascribed to the OH stretching.
mode. These data demonstrate that the as-synthesized C-Dot was surrounded by –COOH and/or –OH groups.

On the other hand, AE-TPEA was designed and synthesized by substituting TPEA with N-(2-bromoethyl)phthalimide in acetonitrile followed by deprotection of the product with hydrazine hydrate. Then, AE-TPEA molecules were conjugated with C-Dot by using EDC and NHS as catalysts. The modification process of AE-TPEA on the C-Dot was tracked by IR spectroscopy and X-ray photoelectron spectroscopy (XPS, Supporting Information (SI), Fig. S5). As shown in Fig. 2C, the peak obtained at ~1660 cm\(^{-1}\) indicates the existence of carbonyl (C=O) groups, while the band located at ~1477 cm\(^{-1}\) is ascribed to the bending vibration of the -NH group. The peak observed at ~3358 cm\(^{-1}\) corresponds to the –NH stretching. The result demonstrated the formation of new amide group on the C-Dot surface. In addition, two peaks ascribed to N 1s were observed in XPS after the modification of AE-TPEA on the C-Dot surface (SI, curve b in Fig. S5). The first peak located at ~399.1 eV is attributed to the pyridinic nitrogen type. The second peak observed at ~401.5 eV is interpreted as nitrogen from amide group. The XPS results confirm the successful modification of AE-TPEA on the C-Dot surface.

As shown in Fig. 3 (curve a), the UV-Vis absorption peak of the C-Dot at 250–300 nm represents a typical absorption of an aromatic π system.\(^\text{14}\) The fluorescence intensity of the C-Dot is strong enough to be easily seen with the naked eye. Upon excitation at 400 nm, the bare C-Dot show a strong emission at 495 nm as demonstrated in Fig. 3 (curve b). The two-photon fluorescence spectrum was a little red shift, compared with one-photon fluorescence spectrum (curve c and b). Using rhodamine B as a standard, the fluorescence quantum yield (Φ) of C-Dot was calculated to be about 10%. In addition, the two-photon absorption cross-section (σ) of C-Dot was estimated by determining the two-photon luminescence intensities of the specimen and a reference under the same experimental conditions. By using rhodamine B as the reference, the σ value for the C-Dot at 800 nm was estimated to be 32000 ± 4000 GM (Goeppert-Mayer unit, with 1 GM = 10\(^{30}\) cm\(^{-1}\) s/photon). The result is comparable with that for the two-photon C-Dot prepared by a different method and those for other two-photon luminescent nanomaterials including CdSe and CdSe/ZnS.\(^\text{15-18}\) The high σ value for the C-Dot should be very benefit for two-photon imaging and biosensing in live cells, deep tissues, and animals.

![Fig. 3](image-url)  
**Fig. 3** (a) UV-vis absorption spectrum, (b) one-photon (400 nm excitation) and (c) two-photon (800 nm excitation) fluorescence spectra of the as-prepared C-Dot.

One-photon and two-photon detection of H\(_2\)S

The fluorescent Cu\(^{2+}\) titration in the C-Dot-TPEA solution (0.04 mg mL\(^{-1}\)) was first performed upon one-photon excitation (taking account of the further cell experiments, 488 nm was selected as the excitation wavelength). As shown in Fig. 4A, the fluorescence in the 500–700 nm range shows continuous quenching with the addition of Cu\(^{2+}\). The fluorescent peak intensity (λ\(_{em}\) = 560 nm) has a good linearity with the concentration of Cu\(^{2+}\) in the range of 5–100 μM (Fig. 4B). As expected, upon the addition of Na\(_2\)S (a commonly employed H\(_2\)S donor), a strong emission turn-on with a little red shift is clearly observed (Fig. 4C). Moreover, the fluorescent peak intensity is in a good linearity with the concentration of Na\(_2\)S in the range of 5–100 μM (Y=6.5789X+185.2631, R\(^2\)=0.9928, Fig. 4D).

![Fig. 4](image-url)  
**Fig. 4** (A) One-photon fluorescence spectra of C-Dot-TPEA (0.04 mg mL\(^{-1}\)) upon addition of different concentrations of Cu\(^{2+}\) ions (0–150 μM) in phosphate buffer solution (PBS, pH 7.4). Excitation wavelength: 488 nm; (B) Plot of fluorescent peak intensity at 560 nm of C-Dot-TPEA with the addition of Cu\(^{2+}\); (C) One-photon fluorescence spectra of the mixture of C-Dot-TPEA (0.04 mg mL\(^{-1}\)) and Cu\(^{2+}\) (100 μM) upon addition of different concentrations of Na\(_2\)S (0–150 μM) in PBS (pH 7.4). Excitation wavelength: 488 nm; (D) Plot of fluorescent peak intensity of the C-Dot-TPEA-Cu\(^{2+}\) in the presence of increasing Na\(_2\)S concentrations. These experiments were repeated three times.

On the other hand, under two-photon excitation at 800 nm, the fluorescent intensity in the 440–650 nm range gradually decreases with increasing the concentration of Cu\(^{2+}\) as shown in Fig. 5A. The fluorescent peak at 495 nm gives a good linearity with the concentration of Cu\(^{2+}\) in the range of 5–100 μM as plotted in Fig. 5B. Similar to that upon one-photon excitation, the fluorescence can be obviously restored upon the addition of Na\(_2\)S (Fig. 5C). The CuS precipitate with large reflection and scattering which could be generated in the reaction between H\(_2\)S and C-Dot-TPEA-Cu\(^{2+}\) may contribute to the red shift of the fluorescent peak. The two-photon fluorescent peak intensity at 500 nm varied linearly with the concentration of Na\(_2\)S in the range of 5–100 μM (Y=12.2500X+657.5000, R\(^2\)=0.9897, Fig. 5D), which meets the requirement for the detection of H\(_2\)S in the biological system.\(^\text{52,53}\) The detection limit was estimated to be 0.7 μM (based on a signal-to-noise ratio of S/N=3). Meanwhile, the reaction between C-Dot-TPEA-Cu\(^{2+}\) and H\(_2\)S was observed to be complete in ~200 s (SI, Fig. S6).

Selectivity, stability, and cytotoxicity

The complexity of bioimaging and biosensing in live cells presents a great challenge to the analytical methods not only in sensitivity, but more importantly in selectivity. The selectivity...
for H$_2$S was demonstrated by the fluorescence changes (ΔF1) induced by potential interferences over the fluorescence changes by H$_2$S (ΔF). Firstly, various thiols (5 mM GSH or 0.5 mM L-Cys, DL-Hcy, 2-ME, and DTT), as well as sulphur-containing compounds (1 mM Na$_2$SO$_3$, Na$_2$S$_2$O$_3$, and KSCN), were examined. Remarkably, as shown in Fig. 6A (white bars), negligible fluorescence changes (<5%) are observed for both thiols and sulphur-containing compounds at higher concentrations, compared with that for 100 µM H$_2$S. In addition, the fluorescent probe C-Dot-TPEA-Cu$_{2+}$ did not show obvious fluorescence enhancement in response to reactive oxygen species (1 mM O$_2^-$, H$_2$O$_2$, OCl$^-$, HO$^-$, ROO$^-$ and O$_2$), nitrogen oxides (0.1 mM for NO and HNO) or ascorbic acid (AA, 5 mM). Moreover, these potential interferences including thiols, sulphur-containing compounds, ROS, NO, HNO and AA showed negligible effects on the fluorescent signal for H$_2$S sensing (Fig. 6A, black bars). The high selectivity of the present biosensor for H$_2$S should be ascribed to the specific affinity of the designed C-Dot-TPEA-Cu$_{2+}$ toward H$_2$S.

Fig. 5 (A) Two-photon fluorescence spectra of C-Dot-TPEA (0.04 mg mL$^{-1}$) upon addition of different concentrations of Cu$_{2+}$ (0–150 µM) in PBS (pH 7.4). Excitation wavelength: 800 nm; (B) Plot of fluorescence intensity at 495 nm of C-Dot-TPEA with the addition of Cu$_{2+}$; (C) Two-photon fluorescence spectra of the mixture of C-Dot-TPEA (0.04 mg mL$^{-1}$) and Cu$_{2+}$ (100 µM) upon addition of different concentrations of Na$_2$S (0–150 µM) in PBS (pH 7.4). Excitation wavelength: 800 nm; (D) Plot of fluorescence intensity at 500 nm of the C-Dot-TPEA-Cu$_{2+}$ in the presence of increasing Na$_2$S concentrations. These experiments were repeated three times.

Significantly, the as-prepared C-Dot-based fluorescent probe which can freely disperse in water with transparent appearance exhibited good photostability. As shown in Fig. 6B, negligible changes (<5%) of the fluorescent intensity can be obtained up to 2 h under continuous excitation at 800 nm. Moreover, no significant fluorescence changes for the C-Dot-TPEA-Cu$_{2+}$ probe are observed over the biologically relevant pH range from 5.0 to 9.0 (Fig. 6C). These results indicate that superior to organic dyes, the C-Dot-based probe demonstrated excellent stability against light illumination, pH variation, and air condition, which would be very benefit for imaging and biosensing in live cells and other biological environments.

For further biological application point of view, MTT assays were carried out to evaluate the cytotoxicity of the C-Dot-based probe to HeLa, RAW264.7 and HEK 293T cells. As expected, the viability of both normal cells and cancer cells declined only by < 9% after their incubation with 0.10 mg mL$^{-1}$ fluorescent probes for 48 h at 37 °C (Fig. 6D). Thus, the as-prepared C-Dot-TPEA-Cu$_{2+}$ probe can be considered to be low toxic for the H$_2$S detection in live cells.

Next, apoptosis assay were carried out to confirm the biocompatibility of C-Dot-TPEA-Cu$_{2+}$ by flow cytometry (FACS) measurements. Taking HeLa cells as an example, they were first incubated with C-Dot-TPEA-Cu$_{2+}$ at concentrations of 0.02, 0.06, and 0.10 mg mL$^{-1}$ for 24 h. Then, the cells were stained by FITC-annexin V and Propidium iodide (PI) to label the apoptosis cells and necrotic cells respectively for FACS measurement (SI, Fig. S7). It is demonstrated that the C-Dot-TPEA-Cu$_{2+}$ probes have good biocompatibility to HeLa cells.

Moreover, to investigate the photo-toxicity associated with laser irradiation, HeLa cells were first irradiated at 800 nm for 1 h by a femtosecond laser. From the bright-field images of HeLa cells (SI, Fig. S8), no obvious changes were observed before and after the irradiation. Then, to determine the health of HeLa cells after irradiation, a standard live/dead staining procedure using commercially available propidium iodide (PI) was further performed. As shown in the inset of Fig. S8B (SI), when the irradiation-prefreated HeLa cells were incubated with PI, to which they are normally impermeable, no intracellular fluorescence signal at 620 ± 20 nm was observed in the confocal fluorescence imaging. This confirmed that the PI hardly crossed the cell membrane, and that the irradiation-prefreated HeLa cells were normal healthy cells.
One- and two-photon imaging of H\textsubscript{2}S in live cells and tissues

As demonstrated above, the advantages of high selectivity and sensitivity, together with small size, remarkable stability against illumination and pH, and especially good biocompatibility of C-Dot-based fluorescent probe substantially provide a reliable platform for TPF bioimaging and biosensing application. Herein, to demonstrate its competence in monitoring the change of H\textsubscript{2}S in HeLa cells, C-Dot-TPEA probes were first incubated with HeLa cells for ~1 h and then washed with 1×PBS (pH 7.4). Strong fluorescence images were observed upon 488 nm (one-photon, Fig. 7A) and 800 nm (two-photon, Fig. 7G) excitation. The overlay of fluorescence and bright-field channel reveals that the fluorescent signals were localized in the perinuclear region of the cytosol (Fig. 7B and 7H), indicating the excellent cell permeability of the C-Dot-TPEA probe. As expected, the fluorescence intensities obviously decreased after the incubation of cells with CuCl\textsubscript{2} (100 µM) for ~1 h at 310 K upon one- (Fig. 7C) or two-photon (Fig. 7J) excitation. Next, Na\textsubscript{2}S (50 µM and 100 µM) was added to the medium, a large intracellular fluorescence enhancement was observed under either one-photon (Fig. 7D and 7E) or two-photon condition (Fig. 7J and 7K). The fluorescence emission changes after the addition of different molecules were quantified and summarized in Fig. 7F (one-photon) and Fig. 7L (two-photon). Analogue studies in RAW264.7 and HEK 293T cells show similar results (SI, Fig. S9).

Then, we further investigated the utility of this two-photon fluorescence probe in tissues imaging. The two-photon fluorescence and bright-field images (Fig. 8A and 8B) show that the C-Dot-TPEA probe was evenly distributed in the lung cancer tissue slice. Furthermore, the two-photon excited fluorescence in lung cancer tissue slice was quenched upon addition of Cu\textsuperscript{2+} (Fig. 8C), suggesting that the C-Dot-TPEA probes response to Cu\textsuperscript{2+} sensitively. Then, after the addition of Na\textsubscript{2}S, the fluorescence was restored as shown in Fig. 8D. In addition, the two-photon fluorescence imaging along the z-direction (SI, Fig. S10) demonstrated that the C-Dot-TPEA-Cu\textsuperscript{2+} probe was capable of monitoring H\textsubscript{2}S changes at depth of 90–150 µm in living tissues using two-photon microscopy.

Conclusions

In summary, a “turn-on” TPF sensor for H\textsubscript{2}S has first been developed by using C-Dot as a two-photon fluorophore and designing TPEA-Cu\textsuperscript{2+} complex as a specific receptor for H\textsubscript{2}S. The present two-photon C-Dot-TPEA-Cu\textsuperscript{2+} fluorescent sensor exhibits high selectivity and sensitivity, broad linear range, and low detection limit. Meanwhile, the C-Dot-based TPF probe shows long-wavelength excitation, excellent stability against light illumination and pH, good permeability, and low cytotoxicity. Accordingly, the nanohybridized TPF sensor with high analytical performance, as well as the remarkable properties of C-Dot, has successfully been applied for TPF imaging and biosensing of H\textsubscript{2}S in live cells and tissues at the depth of 90–150 µm. This work has not only established a new approach to determination of H\textsubscript{2}S in living system, but also provided a methodology to designing TPF sensors for thiols and other species, which may play critical roles in the biological and pathological events.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (NSFC, 21175098), the National Nature Science Fund for distinguished young scholars (21325521), the Fundamental Research Funds for the Central Universities, and the Program of Shanghai Normal University (DZL123).

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Electronic Supplementary Information (ESI) available: NMR and MS spectra of AE-TPEA, apoptosis assay, and two-photon fluorescence microscopy images of RAW264.7 cells, HEK 293T cells and lung cancer tissue slice. See DOI: 10.1039/b000000x/

A “turn-on” two-photon fluorescent sensor for H₂S is developed, in which C-Dot is employed as a two-photon fluorophore and AE-TPEA-Cu²⁺ complex is first designed as a specific receptor for H₂S.