CHINESE JOURNAL OF ANALYTICAL CHEMISTRY
Volume 46, Issue 1, January 2018
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chinese J. Anal. Chem., 2018, 46(1): 39–47

RESEARCH PAPER

A Förster Resonance Energy Transfer Ratiometric Probe Based on Quantum Dot-Cresyl Violet for Imaging Hydrogen Sulfide in Living Cells

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Abstract: Hydrogen sulfide (H_2S) has been confirmed as a significant endogenous gaseous signaling molecule involved in various physiological processes. In order to monitor H_2S in living cells, a Forster resonance energy transfer (FRET) ratiometric probe based on quantum dot-cresyl violet was developed. In this work, the quantum dot nanospheres via a facile ultrasonication emulsion strategy, and the mixture chloroform solution containing hydrophobic quantum dots and COOH-functionalized amphiphilic polymer were successfully transferred into the oil-in-water micelle. The negatively charged quantum dot nanospheres with quantum dots embedded in the polymer matrixes were successfully fabricated after the evaporation of chloroform. And then, these quantum dot nanospheres were condensed with positively charged cresyl violet-azide (CV-N_3) via electrostatic interaction to obtain the complexes (QDS-N_3). The as-prepared QDS-N_3 complexes were monodispersed nanospheres with an average diameter of about 120 nm. These complexes were taken up by the cell through endocytosis, and they were still stable even in wide pH range. In addition, the QDS-N_3 complexes exhibited no cellular toxicity which was verified by MTT assay. In this ratiometric probe, CV-N_3 as a FRET acceptor was conjugated to quantum dot nanospheres. The quantum dots emitted at 591 nm and served as the FRET donor; once the aryl azide on the CV-N_3 was reduced by H_2S to aniline, the probe emitted at 620 nm. The ratiometric probe allowed the elimination of interference of excitation intensity, intracellular environment and other factors. Furthermore, this method also offered a general protocol for preparing nanosensors for monitoring various small molecular in living cells.

Key Words: Quantum dot; Cresyl violet; Ratiometric fluorescence; Cell imaging; Hydrogen sulfide

1 Introduction

Hydrogen sulfide (H_2S) is the third endogenous signaling gasotransmitter following nitric oxide and carbon monoxide[1–4]. Abnormal level of endogenous H_2S has been found to be closely associated with a number of pathophysiological processes[5–12]. Consequently, monitoring of H_2S is of significance for the profound understanding of vital process and pathogenesis. Several methods have previously been explored for detection of H_2S, such as gas chromatography[13], electrochemical analysis[14], colorimetry[15], and UV absorbance[16], etc. However, these methods usually require destruction of the sample. In contrast, fluorescence detection has attracted much attention because of its great temporal and spatial sampling capability as well as high sensitivity for *in situ* and noninvasive analysis[17,18]. Although several fluorescent off-on H_2S probes have been developed[19–22], they are difficult to give precisely quantitative information on the H_2S concentration, because molecular emission intensity can be distinctly affected by excitation intensity, photobleaching, microenvironments, and local probe concentration[23]. By comparison, the ratiometric probe allows

Received 10 August 2017; accepted 7 September 2017
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This work was supported by the the National Natural Science Foundation of China (Nos. 21475102, 31671013), and the Young Talent Support Plan of Xi'an Jiaotong University, China.

DOI: 10.1016/S1872-2040(17)61062-4

the elimination of these interferences based on Förster resonance energy transfer (FRET).

In recent years, semiconductor quantum dots (QDs) have attracted much attention in the fields of biological imaging, targeted delivery and cancer diagnosis based on their unique optical properties of broad excitation and narrow, symmetric emission spectra with high photostability and long lifetime[24–30]. In this work, a FRET ratiometric probe based on quantum dot-cresyl violet was developed. Quantum dot nanospheres via a facile ultrasonication emulsion strategy was first synthesized, and the mixture chloroform solution containing hydrophobic quantum dots and COOH-functionalized amphiphilic polymer were successfully transferred into the oil-in-water micelle. The negatively charged quantum dot nanospheres with quantum dots embedded in the polymer matrixes were successfully fabricated after evaporation of chloroform. And then, these quantum dot nanospheres were condensed with positively charged cresyl violet-azide (CV-N_3) via electrostatic interaction to obtain QDS-N_3 complexes. Meanwhile, the H_2S imaging photo was obtained via the ratio of red fluorescence to green fluorescence. Most importantly, this functional nanoprobe showed almost no cellular toxicity.

2 Experimental

2.1 Instruments and reagents

HT7700 transmission electron microscope (Hitachi, Japan), FluoroMax-4 spectrophotometer (Horiba Jobin Yvon, French) equipped with a plotter unit and a quartz cell, F50 ELISA (Tecan, Switzerland) and Ti-E inverted microscope (Nikon, Japan) were used in this study. Dynamic light scattering (DLS) particle size analysis as well as zeta potentials were measured using a Zetasizer Nano-ZS90 (Malvern, UK) zeta and size analyzer.

Methacrylic acid (MAA, ≥ 98.0), styrene (St, ≥ 99.5%), 2,2′-azobis(isobutyronitrile) (AIBN), oleic acid (Analytical reagent), $ZnCl_2$ (≥ 98.0%), $MnCl_2·4H_2O$ (≥ 99.0%), NaOH (Analytical reagent), $Na_2S·9H_2O$ (Analytical reagent), $Na_2EDTA·2H_2O$ (Analytical reagent) and chloroform (≥ 99.0%) were purchased from Sinopharm Chemical Reagent Co., Ltd. Cresyl violet was obtained from Sigma-Aldrich. All the chemicals were analytical grade and used as received without further purification. Ultrapure water (> 18.2 MΩ cm) was prepared with a Milli-Q filtration system (Millipore, USA).

2.2 Experimental methods

2.2.1 Synthesis of cresyl violet-azide

$NaNO_2$ solution (1.0 mmol) was dropwise added to cresyl violet (1.0 mmol) in 2 M HCl aqueous solution at 0–5 ºC. After stirring for 20 min, NaN_3 solution (2.0 mmol) was added slowly. Subsequently, the reaction mixture was stirred for 24 h at room temperature. The as-prepared brown precipitate was filtrated and recrystallized from acetonitrile. After that, the brown powder was dried in vacuum drying oven and kept for further use.

2.2.2 Synthesis of QDs nanosphere

The oil phase QDs were prepared based on the previous researches[31]. Then the amphiphilic copolymer was prepared as follows[32]. In brief, St (5.0 g), MAA (46.0 mg), and AIBN (96.0 mg) were added into chloroform (35.0 mL). Herein, St and MAA were used as monomers and AIBN as initiator. The mixture solution was then transferred into Teflon lined autoclave and heated at 100 ºC for 10 h. Then, methanol was added to precipitate the white products. The copolymer was purified by washing with chloroform, precipitating with methanol, and then centrifuging. This purification cycle was repeated at least twice. The amphiphilic copolymer was dried in 70 ºC for 3 h and kept for further use. Finally, the as-prepared QDs (15.0 mg) and amphiphilic copolymer (50.0 mg) were dispersed into chloroform to obtain transparent solution. Thereafter, the mixture solution was transferred into NaOH aqueous solution (pH 10) with ultrasonic treatment. The QDs nanospheres were obtained via removing chloroform by evaporating. The obtained QDs nanospheres product were centrifuged. The precipitate was redispersed into ultrapure water, and stored for later use. The final concentration of the colloidal solution is ca. 19.4 mg mL^{-1}.

2.2.3 Synthesis of QDS-N_3 ratiometric probe

By simply mixing and stirring, QDS-N_3 ratiometric probe was obtained via electrostatic interaction between the negatively charged QDs nanosphere and positively charged CV-N_3. And then, QDS-N_3 product was centrifuged for three times. The precipitate was redispersed into ultrapure water.

2.2.4 Cell viability test

Mammalian cell lines (HeLa and MCF-7) were cultured in Dulbecco's modified eagle medium supplemented with 10% of heat-inactivated fetal bovine serum and 1% antibiotics penicillin/streptomycin (100 U mL^{-1}) in a humidified incubator with 5% CO_2 at 37 ºC.

The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability. MCF-7 cells were seeded in a 96-well microplate with 10,000 cells per well with five parallel wells. The microplates were incubated at 37 ºC for overnight. Subsequently, cells were treated with different concentrations (0–200 μg mL^{-1}) of QDS-N_3 ratiometric probe at 37 ºC for 24 h. After the medium

was removed, microplate wells were washed three times with PBS. And then, 10 μL of sterile-filtered MTT stock solution in PBS (4.0 mg mL^{-1}) was added to each well. After incubation for 3 h, the absorbance at 490 nm was measured on microplate reader.

2.2.5 Cell imaging analysis of H_2S in living cells using QDS-N_3 ratiometric probe

HeLa and MCF-7 cells were seeded in a 8-well Chambered Cover Glass (Cellvis, USA) at 40000 cells per well at 37 ºC for overnight. Then, the original cell culture media were replaced with the QDS-N_3 ratiometric probe (8.0 μg) in 200 μL of serum-free DMEM culture media for each well. After incubation for 3 h, cells were treated with Na_2S (100 μM) in PBS at room temperature for 1.5 h. After removing the medium, the cells were washed with PBS for 3 times. All fluorescence images were acquired using Ti-E inverted microscope (Nikon, Japan).

3 Results and discussion

3.1 Synthesis and Characterization of QDS-N_3 ratiometric probe

In this work, a FRET ratiometric probe based on quantum dot-cresyl violet was developed for imaging hydrogen sulfide in living cells. As we all know, in the aromatic diazonium salt, the π electrons on the diazo group can overlap with the π electrons on the benzene ring, which increases the stability. Thus, aromatic diazonium salt prepared and reacted under an ice bath condition may act as an intermediate to further synthesize the azide compound. Organic azides have been widely used in biochemistry as a bioorthogonal functional group. They are compatible with living cellular systems. The fluorescent azide-based probes can be synthesized by rapid and easy preparation with aminecontaining fluorophores. As shown in Fig.1A, the primary amine on cresyl violet (CV-NH_2) reacted with $NaNO_2$ (under HCl medium) at 0–5 ºC to obtain the intermediate aromatic diazonium salt. Upon the addition of NaN_3, diazo group could be easily substituted by azide group to form cresyl violet-azide (CV-N_3). And then, via a facile ultrasonication emulsion strategy, the mixture chloroform solution containing amphiphilic copolymer and hydrophobic QDs was successfully transferred into the oil-in-water (O/W) micelle. The negatively charged spherical nanocomposite with QDs embedded in the polymer matrixes were successfully fabricated after the evaporation of chloroform. Finally, we obtained QDS-N_3 ratiometric probe via electrostatic interaction between the negatively charged QDs nanospheres with positively charged CV-N_3 (Fig.1B). These complexes can be taken up by the cell through endocytosis. Meanwhile, the H_2S imaging in living cell can be achieved via the ratio of red fluorescence to green fluorescence (Fig.1C).

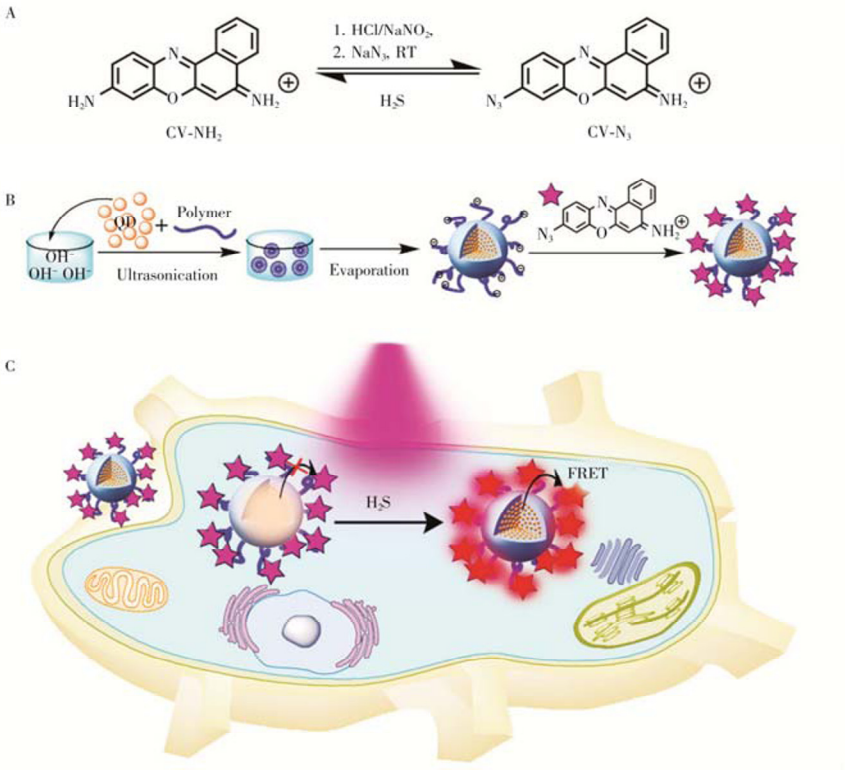

Fig.1 Schematic illustration of a FRET ratio fluorescent probe based on quantum dot-cresyl violet (QD-CV) for monitoring H_2S in living cells. (A) Synthesis of CV-N_3; (B) Synthesis of negatively charged quantum dot nanosphere; (C) QDS-N_3 enters the living cell through endocytosis for monitoring H_2S

Transmission electron microscope (TEM) (Fig.2A) and dynamic light scattering (DLS) (Fig.2B) results indicated that the as-prepared oil-phase QDs were small and uniform clusters with the average size of (10.3 ± 1.2) nm, and such small size made them easier to coat. The synthesized composite QDS-N_3 probes were spheres and their surfaces were smooth with an average diameter of about 120 nm (Fig.2C and Fig.2D), whose size could ensure the fluency of materials in blood circulation.

To the best of our knowledge, the zeta-potential test is a reasonable technique for rapid characterization of adsorption efficiency. Therefore, zeta-potential of our nanocomposites before and after the adsorption of CV-N_3 was measured. As shown in Fig.3, the synthesized QDs nanosphere was negatively charged and the zeta potential was about –5.6 mV at pH 7.2. After simply mixing and incubating with CV-N_3 overnight at room temperature, the QDS-N_3 ratiometric probe was positively charged and the zeta potential was about 35.3 mV, which indicated that the QDs nanosphere had successfully combined a large number of positively charged CV-N_3 via electrostatic interaction. Meanwhile, the fluorescence peak didn't change but the intensity was quenched about 30% (Fig.3A), which could be attributed to the high frequency vibration of hydrophilic chemicals including H_2O[26]. However, the emission of QDs was still naked-eye-visible and strong enough for cell imaging. It is well known that the phospholipid bilayer is the basic scaffold of the cell membrane. The phosphate groups extend toward extracellular cause cell membrane surface is negatively charged. Therefore, the positive QDS-N_3 complex is not only susceptible to contact with the cell membrane but also beneficial for improving cellular uptake.

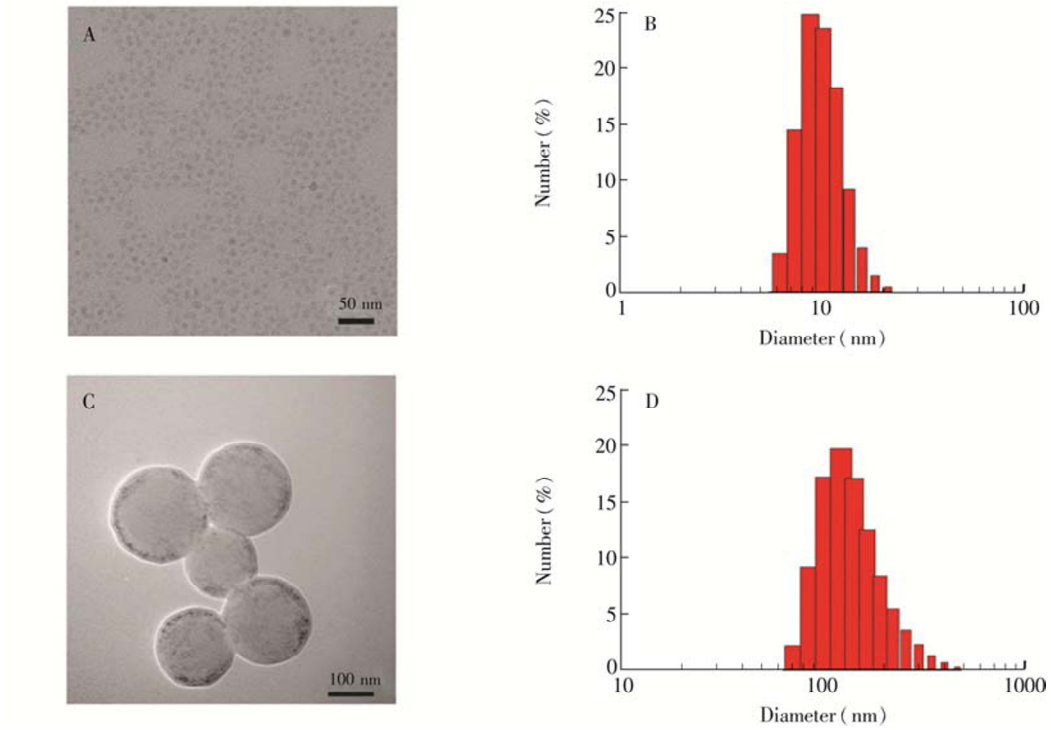

Fig.2 (A) TEM image of oil phase QDs; (B) DLS analysis of oil phase QDs; (C) TEM image of QDS-N_3; (D) DLS analysis of QDS-N_3

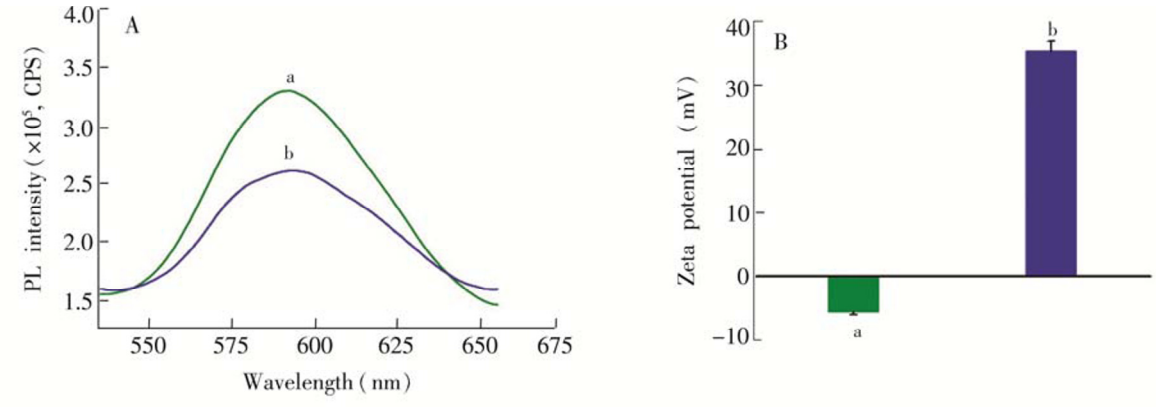

Fig.3 (A) Fluorescence spectra of quantum dot nanosphere (a) and QDS-N_3 (b) (λ_{ex}: 365 nm), and (B) their Zeta potential, respectively

QDS-N_3 complex was dispersed in buffer solution with pH 4.5–10.0 and incubated at 37 ºC for 24 h to investigate the effect of pH on its property. The observed particle size and fluorescence spectrum didn't change (Fig.4A and Fig.4B), indicating the good stability of complex over a wide pH range. Meanwhile, the good stability of QDS-N_3 complex was also checked by dispersing in the DMEM culture media. As shown in Fig.4C, after incubation in DMEM media at 37 °C for 3 h, the shape and size of the nanospheres were well retained, which indicated that the QDS-N_3 complex was stable under complex environments. These results proved good biocompatibility of QDS-N_3 complex and could be further used in cell imaging.

3.2 Mechanism of ratiometric probe for H_2S detection

At the molecular level, H_2S acts as both a good reducing agent and a good nucleophile. H_2S detection could be achieved via chemoselective reduction of azides to amines by reactive sulfur species to achieve a shift in fluorescence emission. In order to clarify the principle of QDS-N_3 ratiometric probe in detection of H_2S, the mechanism was studied in this work. As shown in Fig.5, under the premise of maintaining the same excitation peak (365 nm), detailed optical studies indicated that the QDS-N_3 had maximum emission at 591 nm, because the absorption band of CV-N_3 had almost no overlap with emission spectrum of quantum dot and CV-N_3 couldn't be excited at 365 nm. Upon the addition of H_2S, azide was reduced to amine to produce CV-NH_2. At this time, the excitation wavelength of quantum dots lied in the absorption range of CV-NH_2. The energy of quantum dots was transferred to CV-NH_2, and the probe emitted red fluorescence (620 nm).

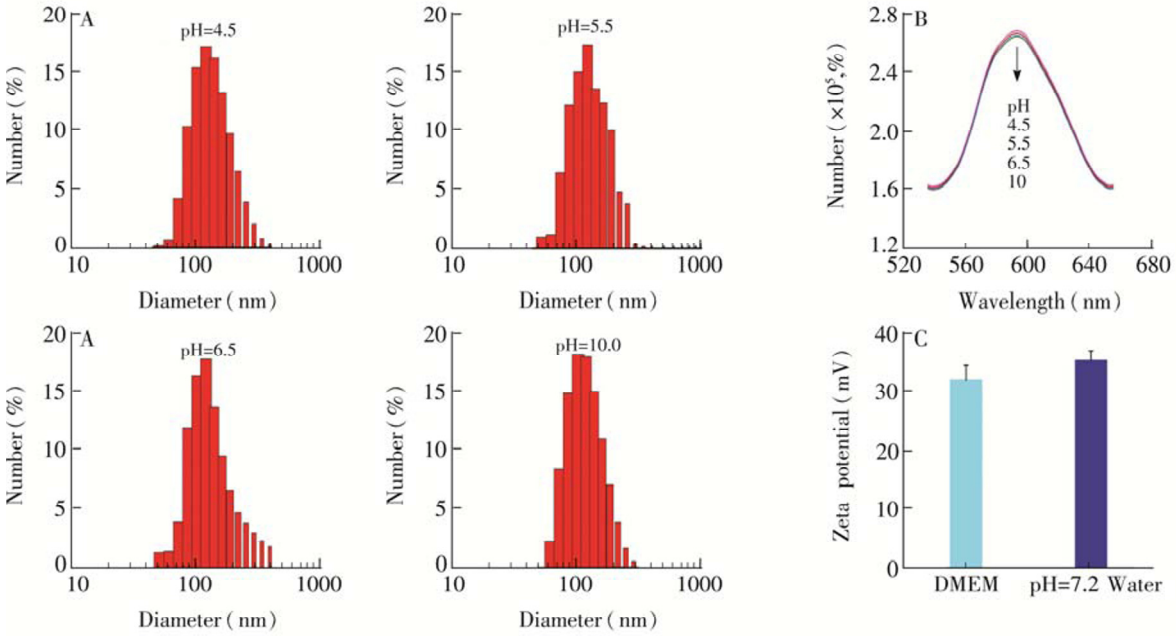

Fig.4 Effect of pH on (A) DLS and (B) fluorescence of NCs-PEG, respectively; (C) Zeta potential of NCs-PEG in culture media

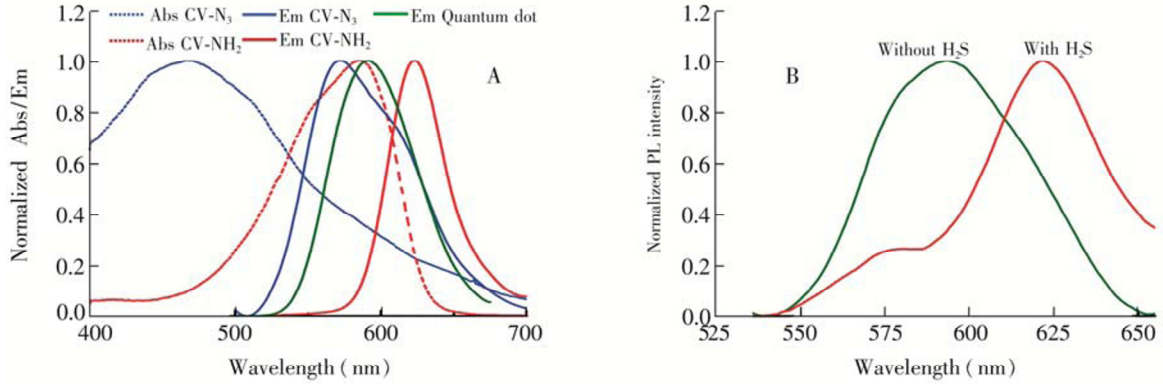

Fig.5 (A) Absorption (blue dashed line) and fluorescence emission (blue solid line) spectra of CV-N_3; Absorption (red dotted line) and fluorescence emission (red solid line) spectra of CV-NH_2; fluorescence emission (green solid line) spectrum of quantum dot; (B) The lines of green and red represent the fluorescence spectra of QDS-N_3 ratiometric probe before (green) and after (red) reaction with H_2S, respectively (λ_{ex}: 365 nm, reaction time: 1.5 h)

3.3 Response of QDS-N_3 ratiometric probe to H_2S

To illustrate the good selectivity of the developed ratiometric probe toward H_2S, the effects of other bioactive molecules on the fluorescence were further investigated and the results are shown in Fig.6A. After spiking different disturbances (100 μM NH_4^+, Cys, NO_2^-, COO^-, $S_2O_3^{2-}$; 100 μg mL^{-1} BSA, 0.3 % H_2O_2, 10 μM GSH) and target Na_2S (100 μM) into QDS-N_3 solution (330 μg mL^{-1}, acetonitrile-water, 1:9, *V*/*V*, pH 7.0) at room temperature for 1.5 h, only Na_2S induced a significant fluorescence peak at 620 nm, while other intracellular small molecules did not affect wavelength and fluorescence intensity of QDS-N_3 ratiometric probe. Under optimal conditions, the reaction of QDS-N_3 with increasing concentration of H_2S resulted in a gradual decrease in the fluorescence intensity at 591 nm, accompanied by the formation of a new red-shifted emission band at around 620 nm. The fluorescence intensity ratio of 620/591 was proportional to the concentration of H_2S in the range of 0–100 μM, and a direct fluorescent quantification method could be successfully developed for the H_2S assay in aqueous media without any preconcentration processes. These results demonstrated that selective and sensitive fluorescence detection of H_2S could be achieved based on the proposed strategy.

3.4 Cell cytotoxicity of QDS-N_3 ratiometric probe

Cytotoxicity is a major concern for *in vivo* application that determines biosafety of the probe during transport. The cytotoxicity of QDS-N_3 ratiometric probe was studied using MTT assay. As shown Fig.7, with the increase of probe concentration, the cell viability slightly decreased, but the cell survival rate remained basically above 90% at the probe concentration of 200 μg mL^{-1} after incubation for 24 h. It was worth mentioning that the QDS-N_3 ratiometric probe used here was only 40 μg mL^{-1} in the subsequent intracellular H_2S imaging. All these results indicated that the synthesized QDS-N_3 ratiometric probe was a powerful and safe agent for intercellular application.

3.5 H_2S imaging in living cells

Endogenous H_2S is synthesized from the amino acids *L*-cysteine and *L*-cystathionine by one of two enzymes (depending on cell type), cystathionine-*β*-synthase (CBS) and cystathionine-*γ*-lyase (CSE). The physiological concentration of H_2S in plasma and most tissue has been reported to be about 10–100 μM[6,12,29]. *In vivo* H_2S imaging was performed by incubating QDS-N_3 probe with human breast cancer cell line MCF-7 cells. Uptake process and intracellular movement of probe could be visualized via fluorescence of encapsulated QDs. As shown in the fluorescence imaging photo (Fig.8), after incubation with cells for 3 hours, the probe was taken up through endocytosis and distributed in the cytoplasm. The observed strong fluorescence of QDs was set as green image by Nikon analysis software, and CV-NH_2 was set as red image due to its very weak light. Upon the addition of 100 μM H_2S, red fluorescence was enhanced substantially with gradual decrease of green fluorescence. By drawing a vertical hatch on the Merge image thorough NIS-Elements Viewer 4.20 analysis software, the software could automatically read the corresponding fluorescence intensity. And then, a linear map was obtained using Origin software. The results were the same as discussed above. As a control, the cell without the probe exhibited no background fluorescence.

To further verify the versatility of QDS-N_3 ratiometric probe, HeLa cell, another common gynecological malignant tumor cell (human cervical cancer cell line) was selected as the model. QDS-N_3 ratiometric probe was co-cultured with HeLa cells. All the conditions were the same as above for MCF-7 cells. As shown in Fig.9, the results were similar to those in the MCS-7 cell. After QDS-N_3 ratiometric probe entered the cell, QDs exhibited strong fluorescence while CV-NH_2 showed no light. When 100 μM H_2S was added to the cell and cultured for 1.5 h, the green fluorescence of the QDs was weakened and the CV-NH_2 red channel showed strong fluorescence. As shown in the line map, the green and red intensity of the QDS-N_3 system

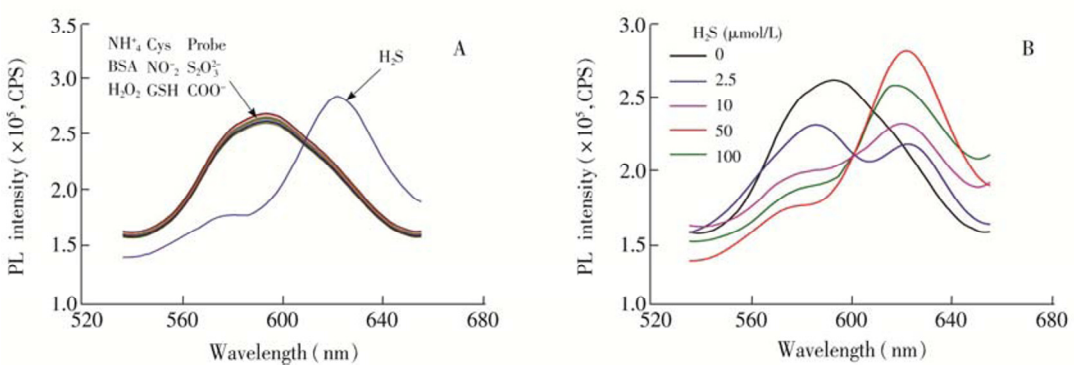

Fig.6 (A) Fluorescence spectra of target H_2S (100 μM) and different interferents (100 μM NH_4^+, Cys, NO_2^-, COO^-, $S_2O_3^{2-}$; 100 μg mL^{-1} BSA, 0.3% H_2O_2, 10 μM GSH) added to QDS-N_3 ratiometric probe solution, λ_{ex}: 365 nm, reaction time: 1.5 h. (B) Evolution of fluorescence spectra with increasing concentrations of H_2S. (QDS-N_3 ratiometric probe: 330 μg mL^{-1}, acetonitrile-water, 1:9, *V*/*V*, pH 7.0)

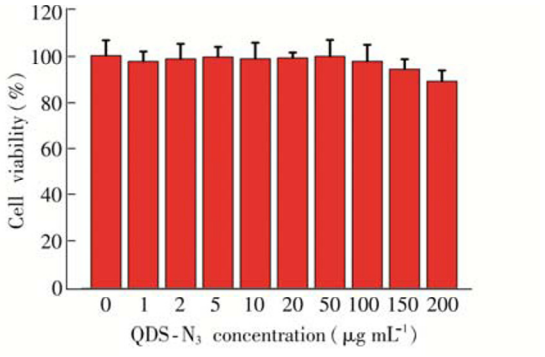

Fig.7 Cytotoxicity of different concentrations of QDS-N_3 ratiometric probe

were 190 and 2, respectively, and those in the system of QDS-N_3 with 100 μM H_2S were about 10 and 220, respectively. What's more, both red and green fluorescence intensities of control cell were zero. The above results were consistent with cell imaging results.

4 Conclusions

In summary, FRET ratiometric probe based on quantum dot-cresyl violet was synthesized via a facile strategy. By encapsulating the hydrophobic QDs and amphiphilic and negatively charged copolymer, functional probe was easily constructed. The as-prepared complexes were monodispersed nanospheres with an average diameter of about 120 nm, which had good biocompatibility and were taken up by the cell

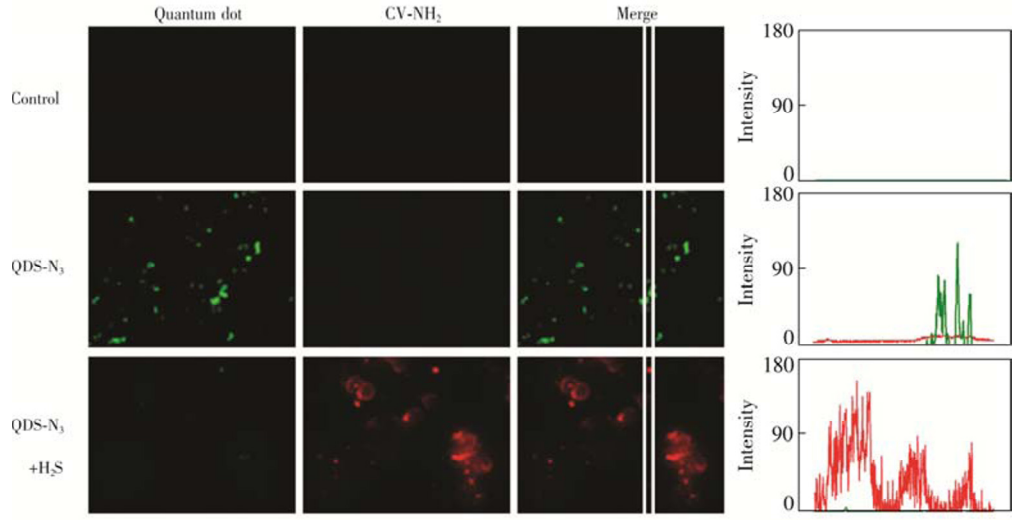

Fig.8 QDS-N_3 ratiometric probe was taken up by the MCF-7 cell through endocytosis and achieved intracellular H_2S monitoring. Green signal: quantum dot; Red signal: CV-NH_2. The photo of ratiometric imaging was obtained through the red and green intensity ratio. QDS-N_3: 40 μg mL^{-1}; H_2S: 100 μM

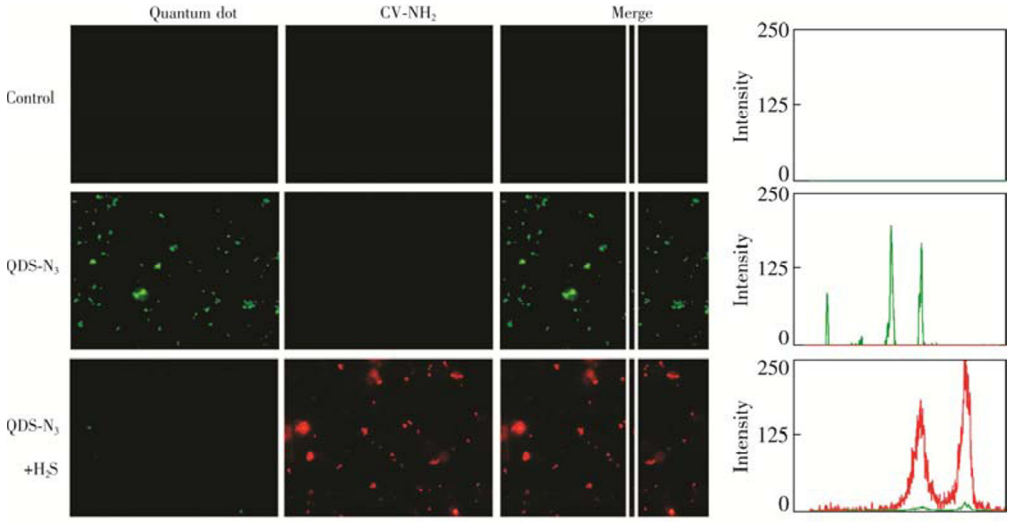

Fig.9 QDS-N_3 ratiometric probe was taken up by HeLa cell through endocytosis and achieved intracellular H_2S monitoring. Green signal: quantum dot; Red signal: CV-NH_2. The photo of ratiometric imaging was obtained through the red and green intensity ratio (QDS-N_3: 40 μg mL^{-1}; H_2S: 100 μM

through endocytosis. The efficacy of this probe for H_2S detection was highlighted by the successful intracellular imaging of H_2S in MCF-7 and Hela cell. In this ratiometric system, the quantification strategy using the fluorescence ratio of 620/591 nm allowed the elimination of interferences of excitation intensity fluctuation, intracellular environment and other factors. Furthermore, this method also offered a general protocol for preparing nanosensors for various small molecules in living cells.

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