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Graphene Quantum Dots Based Fluorescence Turn-On Nanoprobe for Highly Sensitive and Selective Imaging of Hydrogen Sulfide in Living Cells

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Hydrogen sulfide (H₂S), being an important gaseous signaling molecule, has been gaining increasing attention for its involvement in a wide range of physiological processes. Herein, we develop a novel fluorescence turn-on nanoprobe for selective and sensitive detection of H₂S based on graphene quantum dots (GQDs) conjugated with (2,4-dinitrophenoxy)tyrosine (DNPTyr). Taking advantage of its high fluorescence quantum yield, biocompatibility, photostability, and ease to be uptaken by cells, the GQD-based fluorescence probe is further employed for real-time monitoring the triggered dynamic change of intracellular H₂S level in live cells.

Introduction

Hydrogen sulfide (H₂S), a colorless, flammable and water-soluble gas, has been concerned for its toxicity in the past centuries.¹ In the recent years, increasing evidence recognizes H₂S as the third endogenously produced gaseous signaling molecule, along with the other two well-known gasotransmitters: nitric oxide (NO) and carbon monoxide (CO).^{2–3} Endogenous H₂S, the simplest biothiol, is generated physiologically in many tissues in humans through both enzymatic and non-enzymatic processes.^{4–5} By interacting with cysteine residues or heme iron centers of the downstream proteins, H₂S plays critical roles in a wide range of physiological processes, including regulation of vasodilation in blood vessels,⁶ neurotransmission in the brain,⁷ insulin secretion,⁸ anti-inflammation,⁹ anti-oxidation,¹⁰ anti-apoptosis,¹¹ and angiogenesis.¹² Abnormal level of H₂S is related to many diseases including Alzheimer's disease, diabetes, liver cirrhosis, Dow's syndrome, aging, and cancer.^{13–16} For example, excessive gaseous sulfur-containing compounds including H₂S are over-produced in cancer tissues, causing the odorous breath from cancer patients. Thus, determination of H₂S in living systems is essential to elucidation of the biological functions of H₂S, as well as to diagnosis and understanding of related diseases.

Due to its high reactivity, rapid metabolism and low concentration,

the traditional analytical methods such as gas chromatography, colorimetric, electrochemical analysis and sulfide precipitation are inadequate for detection of endogenous H₂S at cell level.^{17–20} By comparison, fluorescence imaging which offers high sensitivity, spatiotemporal resolution, and direct visualization, is preferable for H₂S detection in living systems. Thus research efforts have been carried out to devise organic fluorophores for H₂S detection based on the reactive capabilities of H₂S, including nucleophilic addition, copper-sulfide precipitation and nitro-, azide- or hydroxyl amide-reduction.^{21–23} However, these probes suffer from the problems of poor photostability, cytotoxicity, insufficient sensitivity, low selectivity, difficult synthesis, high costs or delayed response. To better study the underlying molecular mechanism of H₂S involved signaling transduction, it is imperative to develop effective fluorescence probes to overcome these issues for selective, sensitive and real-time imaging of H₂S in the context of physiological events at single-cell or subcellular levels.

Graphene quantum dots (GQDs), which are nanometer-sized and atomically-thin planar graphitic carbon structures, have recently emerged as novel zero-dimensional inorganic fluorophores for optical sensing and bioimaging, because of unique combination of several key merits including, excellent solubility, biocompatibility, highly tunable photoluminescence (PL), excellent photostability, good cell-permeability, ease to be functionalized, and low cost.^{24–39} It has recently been shown that PL of GQDs can be significantly quenched due to photoinduced electron transfer (PET) to the attached electron-withdrawing groups.⁴⁰ Inspired by this, we hope to design a GQD-based fluorescence turn-on nanoprobe for H₂S based on monitoring recovery of PET-quenched PL signals upon H₂S attacking. The key to achieve this is to functionalize GQD with chemical moieties which not only possess electron-withdrawing property but also carry a cleavage site specifically for H₂S over other thiols and possible anion interferents.

In this work, we developed a fluorescence turn-on probe for H₂S

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based on GQDs functionalized with (2,4-dinitrophenoxy)tyrosine (DNPTYR). DNPTYR carries the electron-withdrawing dinitrophenoxy groups to quench GQD PL through PET, and subsequent the dinitrophenoxy group specifically cleavable by H_2S to allow recovery of GQD PL (Figure 1). The GQD-DNPTYR fluorescence probe is able to selectively detect H_2S at a concentration as low as 2 nM. Furthermore, we demonstrate its use for real-time imaging of the dynamic change of intracellular H_2S level in response to stimuli.

Fig. 1 Schematic illustration of the fluorescence turn-on nanoprobe of H_2S based on (2,4-dinitrophenoxy)tyrosine functionalized GQD (GQD-DNPTYR).

Results and discussion

Similar to the previously reported,⁴¹ GQDs were synthesized using pyrene ($\text{C}_{16}\text{H}_{10}$) as the carbon precursor via a facile hydrothermal treatment in sodium bicarbonate (NaHCO_3) solution, followed by neutralization of the excess acid and dialysis. The absolute quantum yield (QY) of the obtained GQDs was measured to be ~31.43% (at pH 7.4), indicating the good potential of these bright GQDs for fluorescence imaging or sensing. Evidently, the produced GQDs are uniform, and of good crystallinity with ~0.245 nm lattice spacing which corresponds to (1120) lattice fringes of graphene (Figure 2a). The GQDs have an average diameter of ~2.93 nm (± 0.18 nm, 100 samples) with a narrow size distribution (Figure 2c). As revealed by the atomic force microscopy (AFM) image, the average thickness of GQDs is ~0.68 nm (± 0.25 nm, 120 samples) (Figure 2b and d), indicating that they are mostly single-atom-thick.

GQD dispersion appears light-yellow under daylight and emits green-yellow fluorescence under 365 nm UV-illumination (inset of Figure 3a). The emission peak is excitation independent, indicating good homogeneity (Figure 3a). The maximum emission intensity from GQDs is achieved at 534 nm while being excited at 480 nm. Thus, this pair of emission and excitation wavelengths is employed for the following experiments. The DNPTYR molecule is covalently conjugated on GQDs via the reaction between the carboxyl group on the GQD and the amino group on DNPTYR. The linkage of DNPTYR causes a significant PL quenching of GQD (Figure 3b and inset of Figure 3b). Specifically, 76.5% PL quenching is observed, because the photon-excited electron goes to electron-withdrawing dinitrophenoxy moiety of DNPTYR instead of relaxing back to HOMO level and giving out emission. Consistently, the measured QY of GQD-DNPTYR decreases to ~5.88%. In order to confirm such photo-

Fig. 2 (a) TEM image of produced GQDs. Inset shows graphitic lattice spacing of GQD resolved under high-resolution TEM. (b) AFM image of GQDs. The inset shows the height profile along the red line. (c) Diameter and (d) Height distribution of GQDs obtained from TEM (100 samples) and AFM (120 samples) measurements, respectively.

induced electron transfer phenomenon (PET), a control experiment is carried out using 4-hydroxyphenylalanine (tyrosine) instead of DNPTYR to conjugate with GQD. Different to DNPTYR terminated with a dinitrophenoxy moiety, tyrosine carries a phenolichydroxyl moiety. As expected, conjugation of tyrosine causes a negligible change in emission spectrum of GQDs or the fluorescence color of GQD solution under 365 nm UV-illumination (Figure 3b), indicating that the PL properties of GQDs are well-preserved after tyrosine conjugation in the absence of PET.

Abundant oxygen-containing functional groups formed on these GQDs, which, together with the small size of GQDs, make GQD aqueous dispersion very stable (no aggregation is observed after months). After conjugation of DNPTYR with carboxyl-bearing GQD, carbonyl peak from COOH group on GQD at 1730 cm^{-1} diminishes while CO-NH peak at 1644 cm^{-1} originated from the amide bond between GQD and DNPTYR appears in the Fourier transform infrared spectroscopy (FTIR) spectra (Figure 3c). Due to the abundant oxygenated functional groups (e.g. $-\text{COOH}$), the zeta potential of bare GQDs is highly negative (-29.21 ± 2.20 mV, $n = 10$). After the conjugation of neutral DNPTYR molecules, the zeta potential of GQD-DNPTYR positively shifted to -7.17 ± 0.54 mV ($n = 10$), indicating that the carboxyl groups on GQDs are largely consumed to form amide bonds with DNPTYR molecules. Dynamic light scattering (DLS) measurements show that the hydrodynamic diameter of GQD-DNPTYR is slightly larger than that of bare GQD, indicating that the conjugation of DNPTYR is successful, and DNPTYR conjugation does not induce aggregation of GQDs despite a decrease of particle charge (Figure S1 in SI).

H_2S can dissolve in aqueous solution, primarily as the monoanionic form, HS^- .⁴² Therefore, NaHS is usually used as a mimic of the dissolved H_2S . GQD-DNPTYR dispersion (0.02 mg/mL) displays weak fluorescence in PBS buffer solution (pH 7.0), while addition of NaHS elicits a significant fluorescence turn-on response (Figure 4a). By contrast, NaHS doesn't cause PL change of bare GQDs (Figure S2 in the SI). The H_2S

Fig. 3 (a) Photoluminescence (PL) spectra of a GQD suspension (in PBS buffer solution) under different excitation wavelengths (as indicated by the figure legend). Inset shows photographs of GQD solution under daylight (left) and 365 nm UV (right). (b) PL spectra of GQD, GQD-tyrosine and GQD-DNPTYR solution **with the concentration of 0.05 mg/mL, respectively**, at an excitation wavelength of 480 nm. Inset shows photographs of GQD (left), GQD-tyrosine (middle) and GQD-DNPTYR (right) solution under 365 nm UV. (c) FTIR spectra of DNPTYR, GQD, and GQD-DNPTYR.

triggered fluorescence recovery indicates that H_2S acts as an effective thiolating agent to cleave the dinitrophenoxy ester in DNPTYR, whereby causing loss of electron-withdrawing dinitrophenoxy moiety and subsequent termination of PET process (Figure 1). **The PL recovery after the addition of 10 μM NaHS is also confirmed by the observation that the particle QY increases from ~ 5.88 to $\sim 21.15\%$.** As demonstrated in Figure 4a

and b, the fluorescence response of GQD-DNPTYR to addition of 10 μM NaHS is time-dependent, and the PL increase reaches a steady state after ~ 10 min (Figure 4b). Therefore, for all the following experiments, a 10 min reaction time is used. Our GQD probe is faster than other colorimetric or fluorimetric probes (comparison provided in Table S1 in SI).

PL increase of GQD-DNPTYR in response to NaHS is dose-dependent (Figure 4c). A trace amount of NaHS (2 nM) can be detected with a signal-to-noise ratio (S/N) of 4.3, and the PL enhancement shows an excellent linear response up to ~ 1500 nM ($\sim 72.24\%$ PL recovery as compared to GQD-DNPTYR at this concentration) (Figure 4d). The theoretical limit of detection (LOD) is calculated to be 0.89 nM based on $\text{LOD} = 3 \times \sigma/m$, where σ is the standard deviation of the response at the lowest tested concentration (2 nM here) and m is the slope of the concentration-dependent response (fitted line in Figure 4d). Our H_2S sensor outperforms the previously reported methods (comparison table S2 in SI). The high sensitivity achieved by our sensor is important because H_2S level in our body is low, e.g., 15 nanomolar level in brain and liver tissue.⁴³

H_2S is endogenously produced by decomposition of cysteine catalyzed by primary two pyridoxal-5'-phosphate-dependent enzymes,⁴ such as cystathionine- γ -lyase (CSE, mainly located in vascular muscle cells, liver and heart), and cystathionine- β -synthase (CBS, predominantly found in brains, kidney, and pancreas).⁴⁴⁻⁴⁵ Knowing that the reaction between L-cysteine and 2-mercaptoethanol catalyzed by enzyme CBS produces S-hydroxyethyl-L-cysteine and H_2S , the resulting H_2S level reflects CBS level.⁴⁶ Therefore, GQD-DNPTYR can be used for CBS assay. Varying concentrations of CBS (0–40 nM) are added together with cysteine (10 μM) and mercaptoethanol (10 μM) into GQD-DNPTYR solution. As shown in Figure 4e, PL of GQD-DNPTYR increases with increasing concentration of CBS. Detection limit as low as 0.05 nM (S/N = 3.6) and

Fig. 4 (a) Time-dependent (0–20 min) PL spectra of 0.02 mg/mL GQD-DNPTYR in PBS (pH 7.0) containing 10 μM NaHS. (b) The increase of fluorescence intensity in term of reaction time. (c) PL spectra of 0.02 mg/mL GQD-DNPTYR in PBS (pH 7.0) incubated with different concentrations of NaHS. (d) PL recovery efficiency vs. NaHS concentration. (e) PL spectra of 0.02 mg/mL GQD-DNPTYR in PBS (pH 7.0) incubated with different concentrations of cystathionine- β -synthase (CBS) in the presence of cysteine (10 μM) and mercaptoethanol (10 μM). (f) PL recovery efficiency vs. CBS concentration. The error bars indicate the standard deviation from 3 measurements.

maximum linear response up to 30 nM are achieved (Figure 4f). We show here that H₂S sensitive GQD-DNPTYR can be employed to detect pyridoxal-5'-phosphate-dependent enzymes.

The selectivity of GQD-DNPTYR probe (0.05 mg/mL) for H₂S was tested by challenge from various biologically relevant species (Figure 5a). As seen, almost no fluorescence increment is triggered upon addition of even a large amount of these potential interferents, including anions (10 mM CO₃²⁻ and SO₄²⁻), metal ions (10 mM Ca²⁺ and Mg²⁺), reactive oxygen species (100 μM H₂O₂ and S₂O₃²⁻), reducing agents (small-molecule thiols: 10 mM cysteine, glutathione, and dithiothreitol). Such good selectivity of GQD-DNPTYR probe is attributable to the fact that thiolysis of dinitrophenoxyl ether is much more selective to H₂S over other free thiols and anions under physiological pH of 7.4. Hence, GQD-DNPTYR fluorescence probe is suitable for detecting H₂S in the complex intracellular environment. The cytotoxicity of GQD-DNPTYR was evaluated in MCF-7 cells using MTT assay. As shown in Figure 5b, GQD-DNPTYR doesn't cause significant cytotoxicity even at the high concentration of 0.5 mg/mL, indicating its suitability for cell imaging.

Here, we use GQD-DNPTYR nanoprobe for dynamic monitoring of intracellular H₂S in MCF-7 cells (Figure 6). After 1h incubation with 0.25 mg/mL GQD-DNPTYR, some dim green puncta appear in the cell cytosol, indicating the internalization of GQD-DNPTYR probes (Figure 6a). After NaHS (100 μM) is added to increase intracellular HS⁻, the PL intensity of GQD-DNPTYR shows a continuous enhancement over a time course of 25 min (Figure 6a and 6b). In contrast, no change in fluorescence signal is observed in the cells without being challenged by NaHS (Figure 6b). PMA (phorbol myristate acetate) which is an analog to the second messenger diacylglycerol can potentially induce reactive oxygen species in cells.⁴⁷ The generated reactive oxygen species can react with HS⁻ to decrease its level in the cell.¹⁶ As shown in Figure 6b and 6c, introducing 100 μM NaHS onto GQD-DNPTYR probes loaded MCF-7 cells causes a continuous increase of PL intensity, and subsequent addition of 10 μM PMA (at ~15 min) halts further increase in PL intensity. These experimental results demonstrate that GQD-DNPTYR is able to be employed to monitor the dynamic change of H₂S level in a cell in response to stimuli.

Fig. 5 (a) The effect of various interfering species on the fluorescence intensity of 0.05 mg/mL GQD-DNPTYR at 534 nm. (b) MTT cytotoxicity analysis of MCF-7 cells incubated with different concentrations of GQD-DNPTYR for 24 h (means ± standard deviation, n=4).

Fig. 6 Dynamic monitoring of intracellular H₂S using GQD-DNPTYR nanoprobe under confocal microscopy. (a) Confocal images (top) and bright-field (bottom) images of MCF-7 cells without incubation of GQD-DNPTYR (first line from left) or pre-incubated for 1 h with 0.25 mg/mL GQD-DNPTYR (second line from left), in the presence of 100 μM NaHS for 25 min (third line from left) or in the presence of 100 μM NaHS for 15 min following addition of 10 μM PMA for another 10 min (fourth line from left). GQDs emit green fluorescence and nuclei are stained blue by NucBlue (Molecular Probes). Scale bar = 10 μm. (b) The time course of the averaged fluorescence intensity change (2 min interval) of GQD-DNPTYR loaded MCF-7 cells (10–12) without (black square) or with 100 μM NaHS stimulation (red dots) or 100 μM NaHS stimulation 15 min following by addition of 10 μM PMA into the solution (blue triangle). (c) The statistics (mean ± standard deviation) of the fluorescence intensity of GQD-DNPTYR loaded MCF-7 cells (n = 12, taken at different field-of-view). Student's t test: * p < 0.1 vs. control. # indicates comparing pair.

Conclusions

In summary, graphene quantum dots (GQDs) with high quantum yield and carboxyl groups as chemical handles for easy functionalization are prepared using a facile and high-yield hydrothermal approach. Based on these GQDs, we have designed a novel fluorescence nanoprobe for detection of gasotransmitter H₂S. This fluorescence turn-on probe utilizes the fact that GQD's photoluminescence is highly sensitivity to photoinduced electron transfer phenomenon owing to its extremely small size and associated quantum confinement effect. Taking advantages of the brightness, biocompatibility, photostability, permeability to cell membrane, and high selectivity towards H₂S, this new GQD-based probe is employed for detection of a trace amount of H₂S and monitoring dynamic change of H₂S level in live cells in response to stimuli. It could be instrumental to investigate and understand the still poorly understood H₂S signaling.

Experimental

Synthesis and functionalization of GQDs. GQDs derived from pyrene was obtained following a modified procedure to the previously reported.⁴¹ Primarily, the precursor, pyrene (0.5 g, Sigma-Aldrich) was refluxed with hot nitric acid (40 mL, 65%, Merck), and vigorously stirred for 12 h at 80 °C. After cooling

to room temperature, the yellowish mixture was diluted with distilled water and filtered through a membrane filter (0.22 μm , Millipore), followed by a vacuum drying to obtain a product of 1, 3, 6-trinitropyrene with a yield calculated as ca. 54%. 80 mg of the resultant powder was subsequently resuspended in a NaHCO_3 solution (0.08 M, 15 mL), followed by ultrasound sonication for 1 h. The suspension was transferred into a poly (tetrafluoroethylene) (Teflon)-lined autoclave treated by heating at 200 $^\circ\text{C}$ for 10 h. After cooling to room temperature, the resulting dark brown solution was filtered through 10 kDa molecular weight cut-off membrane to remove large particles, and further purified by dialysis for 48h to eliminate sodium ions.

To conjugate with (2,4-dinitrophenoxy)tyrosine (DNPTyr, Sigma) or tyrosine (Sigma), GQD solution (0.5 mg/mL) was first mixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (10 mM; Sigma-Aldrich) and N-hydroxysuccinimide (10 mM; Sigma-Aldrich) for 20 min in borate buffer solution (10 mM, pH 5). DNPTyr (1 mM) or tyrosine (1 mM) was subsequently added to the above mixture for reaction. After shaking for 4 h, the solution was then ultrafiltered using a filter (cut-off molecular weight of 3 kDa; Amicon Ultra-4, Millipore) to remove the unreacted molecules.

Characterizations. The morphology of synthesized GQDs was examined by transmission electron microscopy (TEM; JEM 2010, JEOL) and atomic force microscopy (AFM; MFP-3D, Asylum Research). Fourier transform infrared spectroscopy (FTIR) and photoluminescence (PL) spectroscopy were conducted with Bruker spectrometer (Vertex 70), and HORIBA fluorescence spectrometer (Fluorolog FL 1000), respectively. The zeta potentials of bare GQDs and functionalized GQDs were measured using a Malvern Zetasizer (Nano-ZS 90).

Fluorimetric Detection. The fluorescence probes for H_2S detection was carried out by the addition of different concentrations of NaHS in phosphate buffer solution (10 mM, pH 7.0, 500 μL) containing 0.05 mg/mL GQD-DNPTyr (or GQD-tyrosine of the same concentration in control experiments). Fluorescence spectra were recorded at 534 nm with excitation at 480 nm at after 10 min of incubation.

MTT Cell Viability Assay. The evaluation of cytotoxicity of GQD-DNPTyr was carried out by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide)-based cell viability assay (Sigma). MCF-7 (breast adenocarcinoma from human; American Type Culture Collection) cells were dispersed within 96-well plates and cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum at $\sim 3 \times 10^5$ cells per mL. The plates were then kept at 37 $^\circ\text{C}$ in a 5% CO_2 /95% air incubator for 12 h. The cells were sequentially incubated with different concentrations of GQD-DNPTyr (0-500 $\mu\text{g}/\text{mL}$) for another 24 h. After refreshing with the medium containing MTT reagent (0.5 mg/mL), cells were incubated for 4 h at 37 $^\circ\text{C}$. Then the remaining MTT solution was removed and DMSO was added into each well to dissolve the precipitated formazan crystal. After a brief shaking for 10 min, the absorbance was measured

at 570 nm in a plate reader (SpectraMax M5, Molecular Devices) due to the amount of solubilized formazan as the indicator of cell viability.

Cell imaging. The MCF-7 was separately seeded in two 8-well plate and cultured with fresh cell medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin; Life Technologies) at 37 $^\circ\text{C}$ in a humidified atmosphere containing 5% CO_2 and 95% air for 24 h. After 1 h incubation with GQD-DNPTyr (0.25 mg/well), the cells were washed and refreshed with buffer solution before being imaged ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) with a LSM710 confocal laser-scanning microscope (Carl Zeiss, Germany).

Conflicts of interest

There are no conflicts to declare.

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