Altering sub-cellular location for bioimaging by engineering the carbon based fluorescent nanoprobe

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In recent years, numerous classes of carbon-based nanomaterials, such as carbon nanotubes (CNTs), carbon dots (CDs), graphene and its derivatives, graphene quantum dots (GQDs) and fullerene, have been deeply explored for potential applications in the biological fields, e.g., bioimaging [1–5], biosensing [6,7], drug nanocarrier [8–12], etc., owing to their unique and alluring physical and chemical properties. Among them, GQDs are a subject of interesting and promising research with many advantages such as strong signal strength, resistance to photobleaching, tunable fluorescence emissions, high sensitivity and biocompatibility [13–35]. Compared with those semiconductor QDs, GQDs have remarkable superiority in low toxicity, excellent biocompatibility, low cost, and abundance of original materials in nature [36]. High quality GQDs have a wide range of applications, such as light emitting diodes (LEDs) [37,38], solar cells [39,40] and photocatalysis [41,42], aside from biological fields. Up to now, various GQDs with different photoluminescent (PL) colors have been synthesized by two dominating approaches including top-down and bottom-up methods. The top-down method refers to cutting bulk carbon materials into nanoscale-carbon materials by necessary physical and chemical processes [43]. Although this method is feasible to produce GQDs in the laboratory, the tedious and time-consuming experimental procedures limit their large-scale preparation. Furthermore, these GQDs display weak absorption band, and thereby, their fluorescent quantum yields (QY) are usually quite low with low brightness when applied in bioimaging [44,45]. The poor optical property is ascribed to the

highly defective structures, which are inevitably uncontrollable in the cutting processes from the top-down methods. On the contrary, the bottom-up method involves the formation of GQDs from molecular organic precursors [46], and these routes can effectively obtain controllable morphology and uniform GQDs.

Although several excellent synthetic methods have emerged, some fundamental issues like luminescence mechanism are still controversial. Currently, the luminescence mechanism mainly includes surface/edge state, quantum confinement effect of conjugated π -domains, and producing fluorescent molecules during the reaction [47]. In fact, the luminescence mechanism of some carbon-based fluorescent nanoprobe may be dominated by the molecular state, in which the luminescence center relies on an organic fluorophore [48]. Some basic phenomena could support this molecular state theory. For example, fluorescent components can quickly penetrate out of the dialysis bag during dialysis [49]. However, the relationship between fluorescence and GQDs self-structure is still not very clear.

Herein, we present a mass production of carbon-based fluorescent nanoprobe with sulfonic groups (S-GQDs), which is built by a facile molecular fusion route under eco-friendly hydrothermal conditions using pyrene ($C_{16}H_{10}$) as the carbon precursor (Fig. 1a). As reported in our previous work, positively charged amino-GQDs (A-GQDs) could easily pass across cell membranes to cell cytoplasm [44]. They have strong fluorescence, but show high toxicity *in vitro* and *in vivo*. While negatively charged sulfonic-GQDs, which have been reported as

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Figure 1 (a) Schematic illustration of the large-scale preparation steps of S-GQDs. (b) TEM image of S-GQDs (size distribution inset). Scale bar: 20 nm. (c) HRTEM image of S-GQDs. Scale bar: 1 nm; the lattice parameter is 0.24 nm. The inset is an FFT image of a corresponding area (selected by the yellow square). (d) The FT-IR spectra of S-GQDs. (e) The XRD pattern of S-GQDs. (f) XPS full survey of S-GQDs. The high resolution XPS of C 1s (g), O 1s (h), and S 2p (i).

graphene-based tumor cell nuclei-targeting fluorescent nanoprobes (GTTNs) [50], cannot pass through the cell membrane even after co-incubation for 48 h under normal culture conditions. However, the specific capability of GTTN targeting to cancer *in vivo* and further targeting to cancer cell nuclei was found due to the difference of cell membrane permeabilities between tumor cells and normal cells. Based on this phenomenon, we proposed a new targeting mechanism, named as the cell membrane permeability targeting (CMPT) mechanism [50]. Compared

with our previous work, the S-GQDs in this paper are expected to be a new fluorescent probe owing to its low toxicity, transmembrane transport, cellular distribution in lysosome, excellent biocompatibility and high PL QY.

As shown in Fig. 1a, mass production of S-GQDs could be achieved by hydrothermal reaction from the precursor pyrene powder which was transformed to 1,3,6-trinitropyrene by hot HNO_3 . The obtained 1,3,6-trinitropyrene was re-dissolved in a $\text{Na}_2\text{S}_2\text{O}_3$ solution and blended with ultrasonic treatment. The mixed suspension

was transferred to a Teflon-lined autoclave and heated at 200°C for 12 h. During the heating, small graphite-like fragments could fuse into larger GQDs structures. After being cooled to room temperature, the resulting black colloidal solution was obtained and purified.

Fig. 1b and c show the transmission electron microscopy (TEM) image and high-resolution TEM (HRTEM) image of individual crystallized S-GQDs. The size distribution of S-GQDs is shown in Fig. 1b (inset), revealing that the size of S-GQDs is mainly distributed in the range of 2–5 nm with a mean diameter of approximately 3 nm. Besides, Gaussian distribution is suitable for characterizing the size distribution. The full-width at-half-maximum (FWHM) of the fitted Gaussian curve (Fig. 1b inset) is 1.05 nm, indicating the regular distribution feature of the GQDs [51]. The selected area of HRTEM image by the box has a fast Fourier transform (FFT) image shown in Fig. 1c (inset), demonstrating the distance between the lattice fringes is 0.24 nm, which agrees well with the lattice fringes of graphene [1120] [52,53]. The S-GQDs were functionalized by the chemical groups, whose characteristic signals are shown in FT-IR (Fig. 1d), C=O at 1617 cm^{-1} , C=C at 1450 cm^{-1} , C–OH at 1125 cm^{-1} , O–H at 3464 cm^{-1} , SO_3 at 1041 cm^{-1} and C–S at 618 cm^{-1} , indicating that S-GQDs are co-functionalized by –OH and SO_3 and endowed with relatively

good solubility in aqueous solution (AS). XRD analysis (Fig. 1e) conveys the fact of the lattice spacing (0.39 nm) and diffraction center (about 22°) in line with graphite [002]. To study the elemental constituent of the S-GQDs, XPS measurements (Fig. 1f–i), particularly high-resolution C 1s, O 1s and S 2s spectra, were conducted. As shown in Fig. 1f, XPS full survey of S-GQDs indicates the main elements of C, O, and S contained in S-GQDs. High-resolution C 1s spectrum is shown in Fig. 1g, whose peaks are fitted to the main C–C feature, C–OH and C–S, which indicates the carbon skeleton of S-GQDs and functional groups. Other notable features originate from the O–H bond and SO_3 in Fig. 1h. The strong signals of $\text{SO}_3/2p_{1/2}$ and $\text{SO}_3/2p_{3/2}$ (Fig. 1i) are ascribed to the sulfonic-group of S-GQDs.

To detect the optical properties and explore the fluorescent mechanism of S-GQDs, we characterized the UV-vis absorption spectrum of S-GQDs in AS and found an absorption band at ca. 240 nm (Fig. 2a), which was assigned to the $\pi \rightarrow \pi^*$ transition of aromatic sp^2 domains [54]. Besides, a photograph of the S-GQDs illuminated under UV light (365 nm) is exhibited. The bright blue-green fluorescence of GQDs is clearly seen with the naked eye (inset of Fig. 2a). Similar to the GQDs prepared by a hydrothermal graphene oxide reduction method, a typical absorption peak at ca. 290 nm is observed. Then a near-

Figure 2 (a) UV-visible absorption (ABS), photoluminescent emission (PL) and photoluminescent excitation (PLE) spectra of S-GQDs. (b) PL spectra of S-GQDs excited at different excitation wavelengths varying from 360 to 460 nm. (c) Photostability of S-GQDs over a long period of time from one day to a month. (d) PL spectra of dialysate and remaining solution after S-GQDs dialysis. (e) Solvent-dependent PL intensity of S-GQDs collected at 373 nm excitation. (f) Solvent-dependent PL peaks of S-GQDs collected at 373 nm excitation.

visible light absorption at about 398 nm also appears. Both of them can be ascribed to the fluorescent molecules existing at edges or in the carbon core, which trigger strong fluorescence of the obtained S-GQDs [48], and the relative PL QY of the S-GQDs is about 22% (Fig. S1a). To further explore the optical properties of the as-prepared S-GQDs, a detailed PL study was carried out with different excitation wavelengths (Fig. 2b). Generally, the excitation wavelength dependence of the emission wavelength and PL intensity change is a common phenomenon observed in carbon-based fluorescent materials, which means PL peaks would shift to longer wavelengths when the excitation wavelength increases and the PL intensity would decrease rapidly simultaneously [55]. However, the as-synthesized S-GQDs exhibit an emission wavelength nearly independent of excitation wavelength (Fig. 2b) in the range of 360–460 nm. The PL maximum is well confined, nearly irrespective of excitation wavelength. The maximum excitation wavelength is 373 nm and the maximum emission wavelength is 480 nm. The reason for this phenomenon is according to fluorescent substances [43]. The strongest PL maximum appears at 480 nm upon 373 nm excitation, which is consistent with their PL excitation (PLE) spectrum with a strong excitation peak at 373 nm (Fig. 2a). The 398 nm PLE peak also has a corresponding absorption band in UV-vis. The PLE spectrum clearly demonstrates that the observed luminescence from the GQDs is directly correlated with the two new transitions at 290 and 373 nm rather than the commonly observed $\pi \rightarrow \pi^*$ transition [54]. The PL lifetime of the GQDs is 4.932 ns as shown in Fig. S1b.

The photostability of the GQDs was examined by monitoring the change in the PL intensity under continuous radiation using a 100 W Xenon lamp (Fig. S1c). After irradiation for 3 h, S-GQDs show a high resistance to photobleaching as observed by the PL intensity and emission brightness of the S-GQDs (inset Fig. S1c), indicating that the S-GQDs are extremely photostable during the relatively short time period. However, the fluorescence would tempestuously reduce when S-GQDs were stored for a long time, which suggests that fluorescent molecules are of significance to the fluorescent mechanism (Fig. 2c). According to the dialysis process, we found that the fluorescent intensity of the dialysate was stronger than that of the remaining solution (Fig. 2d), in accord with the fluorescent molecules mechanism. The pH-dependence of PL was also tested in a wide range of pH values as shown in Fig. S2a. Under alkaline and neutral conditions, the S-GQDs emitted relatively strong PL. Whereas the PL intensity became rapidly reduced

under acidic conditions because the chemical groups of the fluorescent molecules coupled with S-GQDs were protonated. Thus, the hydrophilic groups were severely broken, leading to aggregation of GQDs. Then these GQDs became inactive in PL [54]. It should be mentioned that there were strong fluorescent intensities at pH 5–12, indicating enormous potential application *in vivo*. All the facts implied that the emission was characterized by the exciton-state of molecules/chemical groups rather than quantum effect [45].

Fig. S2b shows the dependence of PL intensity on the concentration of S-GQDs colloidal solution. Different concentrations were tested with the absorbance at 373 nm. At low concentrations (with absorbance less than 0.2), a linear relationship is observed. But with increasing absorbance and concentrations, the slope of PL intensity shows a sharp slowdown [51,56] (intrinsic colour variation of various concentrations and the corresponding fluorescent brightness are displayed in the inset of Fig. S2b). We could foresee that the PL intensity would eventually reach a plateau, because high concentrations of GQDs induce the self-absorption quenching of fluorescence. As shown in Fig. 2e, the PL of GQDs decreases in solvents from dimethylformamide (DMF), tetrahydrofuran (THF), AS, D-hanks (buffer solution) to DMEM (culture medium). The S-GQDs in the solvent with strong polarity owned the strongest PL. Whereas, the peaks of maximum PL exhibit negligible solvent-dependent behaviours (Fig. 2f) [54]. Besides, it is distinctly observed that the fluorescence intensity of S-GQDs decreased in these solvents under UV exposure (Fig. 2e inset), proving that these solvents apparently influence the fluorescence of S-GQDs. It is easily understood that ions or solvent molecules attaching to the edges or carbon cores of GQDs prompt the change of marginal fluorescent molecules or photoluminescence related functional groups rather than the impact of conjugated system. Based on these, we could speculate that fluorescent molecule-conjugated GQDs have a pivotal effect on the photoluminescence mechanism of the obtained S-GQDs. When they were excited within a certain excitation wavelength range, those electron-donating chemical groups on the fluorescent molecule-conjugated GQDs enhanced the radiative recombination of localized electron-hole pairs [48,54].

We also investigated the surface charge of S-GQDs in AS and culture medium by zeta potential measurements. As shown in Fig. S2c, the S-GQDs are negatively charged in AS (average value of -38 mV and absolute value >30 mV) and show a rough trend that the S-GQDs

become more negatively charged when the pH value changes from 5 to 12. Referring to the FT-IR spectra, the negative surface charge of S-GQDs is ascribed to the presence of abundant hydroxyl, carboxyl and sulfonic acid groups. From the view of colloidal theory [56], a zeta potential more than -30 mV is necessary for the stable dispersion of a general colloidal system. Hence, the S-GQDs should be better dispersed in AS than in culture medium. The complex component in culture medium made the unstable S-GQDs agglomerated because of the interactions between them. To investigate the stability of S-GQDs, we did a simple sedimentation experiment for different times and measured their zeta potentials as shown in Fig. S2d. As a result, the S-GQDs become unstable because of the decreased zeta potential in AS as the settling time increases. However, the zeta potentials in culture medium, which are smaller than in AS, do not show noticeable changes over time due to the interactions between S-GQDs and proteins or other complex components in the medium. In other words, the S-GQDs aggregate rapidly in the culture medium and then remain relatively stable as time goes on.

Endowed with superb optical properties such as high QY, the S-GQDs were considered to serve as promising biofluorescent probes. We discussed the difference of the cellular behaviours among the three GQDs, negative S-GQDs, GTTN and positive A-GQDs. Bioimaging experiments were performed by a confocal fluorescence microscope as shown in Fig. 3. Bright blue fluorescence was observed in cellular cytoplasm of S-GQDs and A-

GQDs, while the GTTN only bounded and adsorbed on the cell membrane surface without entering the cells after 48 h incubation (Fig. 3a). As shown in Fig. S3, both S-GQDs and A-GQDs entered the cells through the membrane even within 10 min. It is revealed that engineering the nanoparticles through the synthetic route and raw materials could change the biotoxicity and cellular distribution. Indeed, many nanoparticles have been found to enter cells through endocytosis, a fundamental process that eukaryotic cells engulfs substance *via* the membrane deformation [57]. Lysosome is an important organelle, which is tiny sacs filled with enzymes, playing vital roles in cellular degradation, autophagy, immunological stress, etc. [58]. Most cell uptake endocytic routes of nanoparticles converge upon the lysosome, which is regarded as the most common intracellular site of nanoparticle sequestration and degradation [59]. In addition to the endo-lysosomal pathway, recent evidence suggested that many nanoparticles were found in lysosome, which may cause *in vivo* and *in vitro* toxicity [58,60–65]. We found that both S-GQDs and A-GQDs can enter the cell, and locate in lysosome (Fig. 3b) which can be indicated by Pearson's correlation of lysosome colocalization after co-incubation with S-GQDs, GTTN and A-GQDs for 48 h (Fig. S4a). The Pearson's correlation of S-GQDs and lysosome was almost 0.6 after 1 h incubation and increased as the incubation time prolonged (Fig. S4b). S-GQDs exhibit very good biosecurity (i.e., nontoxicity) even with high dose (300 mg L^{-1}) and long incubation time (48 h) (Fig. 4a, d) while A-GQDs are toxic to cells when the co-

Figure 3 Confocal images of S-GQDs, A-GQDs and GTTN in 4T1 cell *in vitro*. (a) Cell membrane co-localization. (b) Lysosome co-localization. Cells were co-incubated with 100 mg L^{-1} S-GQDs, 30 mg L^{-1} A-GQDs or 100 mg L^{-1} GTTN for 48 h. Left, graphene-based nanoparticles (405 nm excitation); middle, cell membrane stained by CellMask Orange plasma membrane stain (561 nm excitation) or cell lysosome stained by LysoTracker Deep Red (635 nm excitation); right, merged image. Scale bar: $10 \mu\text{m}$.

Figure 4 Cell viability assays of (a) S-GQDs, (b) GTTN, and (c) A-GQDs at different concentrations for different incubation times detected by cck-8 assay. Representative light microscopic images of (d) S-GQDs of 300 mg L^{-1} (48 h, scale bar: $20 \mu\text{m}$); (e) GTTN of 300 mg L^{-1} (48 h, scale bar: $20 \mu\text{m}$); (f) A-GQDs of 50 mg L^{-1} (48 h, scale bar: $20 \mu\text{m}$). Static settled S-GQDs and GTTN for different times from 5 min to 48 h in AS and culture medium. TEM images of S-GQDs (g) and GTTN (j) after being settled for 48 h. Scale bars: 200 nm (g); 100 nm (j). UV-visible absorption peak value changes of S-GQDs (h) and GTTN (k) at 398 nm . Photoluminescent peak value changes of S-GQDs (i) and GTTN (l) at 480 nm as the settling time increases.

incubation concentration increased to 50 mg L^{-1} after 24 and 48 h incubation (Fig. 4c, f). GTTN has low toxicity but still shows adverse impact on cells with the very high dose of 300 mg L^{-1} after 48 h incubation (Fig. 4b, e). S-GQDs, A-GQDs and GTTN are good fluorescent nanoprobes with different sub-cellular locations.

We designed and engineered this new material through changing the synthesis route, in which sodium sulphite

(for GTTN) and hydrazine hydrate (for A-GQDs) were replaced with sodium thiosulfate as the reductant. This design greatly reduces the cytotoxicity *in vitro* (compared with A-GQDs) and allows for crossing the membrane into the cell (compared with GTTN). The results show that all the GQDs have similar sizes and shapes, and the surface charge and functional groups of S-GQDs and GTTN are also similar. So, these factors are not the cul-

pril responsible for their different cell behaviours. According to Fig. 4g, obvious particle aggregation of S-GQDs can be seen, and the particle size increases to larger than 30 nm from an average size of 3 nm (Fig. S5). As shown in Fig. S5e, obvious sediment is observed as the settling time increases, in accordance with the enlarged particle size shown in Fig. S5a–d. As shown in Fig. 4h and Fig. S5g, the ABS peak value of S-GQDs in the upper declines, while that in the bottom increases, revealing the occurrence of sedimentation as the settling time increases. It is also found that the PL intensity (Fig. 4i and Fig. S5f) and zeta potential slightly reduce in AS, which do not happen in the culture medium (Fig. S2d). As reported in our previous work, GTTN is very stable both *in vitro* and *in vivo*, due to no interaction with the proteins, and maintains its physical and chemical nature. This stability is one of the key points of GTTN targeting to tumor cell nuclei *in vivo*, whereas it may also be responsible for its difficulty entering cells in normal culture conditions. All the above results illustrate that the S-GQDs are not very stable compared with GTTN (Fig. 4j–l and Fig. S6) which will maintain its stability reflected in stable particle size, UV absorption and PL intensity. The aggregation state of S-GQDs is the key factor to enter the cells and distribute in lysosome [66]. Furthermore, for *in vivo* distribution, S-GQDs mainly distributed in liver and kidney of mice after tail vein injection of 100 mg kg^{−1} S-GQDs (Fig. S7a). And it was cleaned through renal excretion as shown in Fig. S7c, but still remained in the liver after 24h administration. FT-IR spectra of fresh S-GQDs, S-GQDs & urine and S-GQDs excreted from urine show a significant difference, revealing that the S-GQDs are not like the GTTN which is stable *in vivo* (Fig. S7b) [50]. For *in vivo* toxicity, the six parameters of serum biochemistry increased gradually after 2 h injection and all the indicators recovered to the normal ranges after 24 h injection (Fig. S8), indicating that there was no obvious damage to the kidney and liver functions after clearance of S-GQDs [67].

In conclusion, this work presented a guiding-significance example of designing carbon-based fluorescent nanoprobes to tune their physicochemical properties and biological behaviours like toxicity and cellular distribution. The obtained S-GQDs possess a series of attractive fluorescent and chemical properties such as strong absorption bands, relatively high fluorescence QY, less pH dependent surface charge in AS or medium, which provides it broad applications as fluorescent probes. This simple method of engineering carbon-based fluorescent nanoprobes could reduce cytotoxicity and

adjust cellular distribution through regulating their physicochemical properties. By this way, we could design the bio-probes according to biological needs and expand their biological applications.

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Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Experimental details and supporting data are available in the online version of the paper.

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通过碳基荧光纳米探针的设计调控其亚细胞定位和生物成像

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摘要 碳基荧光纳米探针在生物成像领域展现出诱人的应用前景。本文通过调节合成方法合成了一种磺酸基修饰的石墨烯量子点(S-GQDs)荧光探针。该探针呈现出优异的光学和理化性能,如荧光强度高、pH稳定、表面带负电等。研究表明其发光机理主要依赖荧光分子发光机制。与我们之前报道的氨基化量子点A-GQDs和肿瘤细胞核靶向探针GTTN对比,该探针具有良好的生物安全性,可以在相当短的时间内即跨膜进入细胞,而GTTN在正常的体外培养条件下无法进入细胞。为此,我们探究了产生该差异的原因。结果表明, S-GQDs与A-GQDs截然不同的合成原料导致了他们的毒性差异,而S-GQDs的不稳定性则是导致其进入细胞、与GTTN明显不同的主要原因。