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Graphene quantum dot based charge-reversal nanomaterial for nucleus-targeted drug delivery and efficiency controllable photodynamic therapy

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ABSTRACT

Graphene quantum dots (GQD), the new zero-dimensional carbon nanomaterial, has been demonstrated as a promising material for biomedical applications due to its good biocompatibility and low toxicity. However, the integration of multiple therapeutic approaches into a nano-sized platform based on the GQD has not been explored yet to our best knowledge. In this report, we regulate the generation of reactive oxygen species (ROS) when using the GQD as a photosensitizer by varying the doping amount of nitrogen atoms to achieve efficiency controllable photodynamic therapy (PDT). On the other hand, charge-reversal (3-Aminopropyl) triethoxysilane (APTES) was employed to conjugate on the surface of GQD for nucleus targeting drug delivery for the first time. The treatment outcome of produced ROS and nucleus-targeting drug delivery was investigated by fluorescence imaging. The results demonstrated that the N-GQD-DOX-APTES in dual roles as a drug carrier and photosensitizer could achieve nucleus-targeting delivery and strong ROS production simultaneously. This approach provides a promising strategy for the development of multifunctional therapy in one nano platform for biomedical applications.

KEYWORDS: Graphene quantum dots; nitrogen doped graphene quantum dots; charge-reversal; nucleus-targeted drug delivery; photodynamic therapy

1. INTRODUCTION

Designing a nano-sized drug carrier to enhance the preferential accumulation of cancer drug in cancer tissues or cells is a promising approach to cancer treatment [1, 2]. A variety of materials, such as polymers [3], metal nanoparticles [4, 5], quantum dots [6, 7] and carbon material [8, 9] et al., have been reported to serve as drug carriers. In order to enhance cancer treatment, there is a tendency in the literature recently in the development of nuclear-targeted drug carriers because some anticancer drugs rely on targeting at nuclear DNA [10, 11]. Several types of functionalized nanoparticles have been constructed for nucleus-targeting drug delivery against resistant tumors [12, 13]. In the design of nucleus-targeting drug carriers, charge-reversal polymer materials have received increasing attention due to its property of charge shift between positive and negative with pH dependence [14]. It is well known that a negatively charged drug carrier exhibits prolonged circulation time in blood due to the interaction with negatively charged proteins [15]. In contrast, once in the acidic tumor microenvironment, a positively charged drug carrier shows increased capacity to be internalized by tumor cells *via* the negative surface of cell membrane [16]. Thus, a charge-reversal polymer can improve the selectivity toward the target nucleus and increase the accumulation of delivered drug in cell nuclei [17]. 3-(aminopropyl) triethoxysilane (APTES) is a biocompatible charge –reversal agent, which has attracted much attention because it can be easily grafted onto the target substrate under mild reaction conditions [16]. In addition, being covered by APTES can enhance the stability of anti-tumor drug loaded on the drug carrier [16].

Photodynamic therapy (PDT) is a clinically approved therapeutic modality used for the treatment of neoplastic diseases [18, 19]. In PDT, a photosensitizing agent under the illumination of light at proper wavelengths produces reactive oxygen species (ROS) such as singlet oxygen by

energy transfer from light excited photosensitizer molecules to oxygen molecules [20]. The generated ROS can be used to selectively damage a target tissue region since light illumination can be precisely controlled [18]. Although many photosensitizing agents based on porphyrinic molecules yield good clinical outcomes in cancer treatment, the hydrophobicity of porphyrin derivatives limits its effective delivery in tumors [21]. To alleviate this problem, mesoporous silica nanostructure materials [22], metal-based nanoparticles [23] and certain carbon materials [24, 25] have been developed as photosensitizing agents carriers to improve the efficacy. However, the poor water solubility, potential toxicity and side effects of these carriers' materials raise concerns about biosafety [24]. One innovative way to address this problem is to use a new biocompatible drug carrier that can reach the target effectively and the drug carrier itself can serve as a photosensitive agent, thus making it available for loading extra drugs for enhanced treatment.

As small pieces of graphene, graphene quantum dots (GQD) have received significant attention in biosensing and bio-imaging, owing to their tunable photoluminescence [26, 27] and good biocompatibility as well as photo-stability [28, 29]. Interestingly, GQD has been successfully used as a drug delivery system for the pH-sensitive release of anti-cancer drug [30], attributing to the fact that such small-size (<50 nm) drug carriers are more likely to accumulate in tumors [31]. More importantly, several studies have shown that GQD might be potential candidates as a novel photosensitizer agent to produce ROS species such as singlet oxygen ($^1\text{O}_2$) via the photo-excited process for PDT to kill nearby cells [20, 32]. However, pure GQD exhibits limited PDT efficiency owing to its low ROS generation yield [33]. How to improve the photocatalytic performance of GQD is still a significant and challenging task.

In this work, we report a charge reversal N-GQD-DOX-APTES designed for the nucleus-targeting delivery of anti-tumor drugs for multi-functional cancer treatment to address the above

challenge in the photocatalytic performance of GQD and the gap in the biosafety and nucleus targeting efficacy of the currently available PDT drug carrier. The charge reversal agent APTES is grafted onto the N-GQD-DOX to take advantage of the small size of N-GQD and the charge reversal property of APTES thus increasing drug uptake by cell nuclei, so that the cancer drug, DOX, can directly target on nuclei for improved treatment. Moreover, the ROS generation by GQD is significantly enhanced without using additional photosensitizers by optimizing the amount of nitrogen source bound to GQD, which was confirmed by ROS measurements. The results of the cell study show that N-GQD-DOX-APTES improve both nucleus-targeting drug delivery and PDT efficacy compared to free DOX and N-GQD-DOX. Therefore, this nanocarrier could be further developed to achieve dual therapies especially for tumors resistant to a single type of treatment.

2 MATERIALS AND METHODS

2.1 Instrumentation

UV-Vis spectroscopy and Photoluminescence (PL) spectra were carried out using a Microplate Reader system (SpectraMax M5, First Street San Jose, California, USA). The morphology of N-GQD were investigated by High-resolution TEM (HRTEM) measurements a transmission electron microscope (JEOL JEM2100F JOEL, Tokyo, Japan). A portable Raman spectroscopy system (InnoRam-785S, B&W Tek, Newark, Delaware, USA) coupled to a video microscope accessory (BAC151A, B&W Tek, Newark, Delaware USA) was used to measure all Raman spectra. The surface charges of materials in PBS at different pH values were analyzed in a Nano ZS Zetasizer (Malvern Instruments, Malvern, UK). A multi-label plate reader (PerkinElmer Victor 3, Thermo Fisher Scientific, Massachusetts, USA) was used to measure cell viability using the MTT assay. Fluorescence images were captured using an inverted fluorescence microscope

(Zeiss Axiovert S100, Carl Zeiss, Germany). An ultrasound sonicator (Elmasonic, Elma E30H, Elma, Wetzikon, Switzerland) was used to disperse DOX and N-GQD.

2.2 Reagents

Citric acid (CA), dicyandiamide (DCD) and 2', 7'- Dichlorofluorescein diacetate (DCFDA), Doxorubicin hydrochloride (DOX), Disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), (3-Aminopropyl) triethoxysilane (APTES) and Dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Dihydroethidium (DHE) was ordered from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Silver nitrate (AgNO_3), Ethanol and Sodium hydroxide (NaOH) were ordered from Alfa Aesar (Heysham, Lancashire, UK). Triton X-100 Detergent was purchased from Bio-Rad (Laboratories, Hercules, USA). Hydroxylamine hydrochloride (HH) was ordered from MP Biomedicals (Solon, Ohio, USA). PBS buffer was filtered using a 0.22- μm filtration system prior to use. Nanopure water ($18.3 \text{ M}\Omega \cdot \text{cm}$) was used throughout the experiments. Dialysis membrane bag (MWCO = 500 D) was purchased from MYM Biological Technology Company Limited (Hyderabad-29, Telangana, India). All reagents were of analytical grade and used as received.

2.3 Cell lines and cell culture

Breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC) (Virginia, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA). DMEM were supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, USA), 100 units/ml penicillin and 100 mg/ml of streptomycin (Gibco, USA). Cells were cultured in a 37 °C incubator with 5% CO_2 prior to use in the experiments.

2.4 Preparation of GQD and N-GQD

GQD were synthesized by pyrolyzing CA [34]. Briefly, 2 g of CA heated to 200 °C and

maintained for 30 min. The obtained colloidal solution dissolved in 100 mL of NaOH (10 mg mL^{-1}). After the pH of the obtained GQD solution was adjusted to nearly neutral, the stock solution was collected after centrifuging treatment at 10000 rpm for 10 min [34, 35].

According to our previous work [36, 37], N-GQD were prepared by the hydrothermal treatment of the mixture of CA (2.0 g) and different amount of DCD (0.5, 1, 2, 3, 4 g) in 5-mL nanopure water. The mixture was heated to 180°C and maintained at this temperature for 12 hours. The product was dispersed into 100-mL Nanopure water then the N-GQD stock solution was collected after centrifuging treatment at 10000 rpm for 10 min.

2.5 Preparation of Raman substrate

In order to investigation the Raman characterization of the GQD and different of mass ratios ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}}$) N-GQD, we wrapped the GQD and N-GQD on the silver nanoparticle surface according to our reported method. Briefly, 4 mL GQD and N-GQD samples (0.2 mg mL^{-1}) was added into 1 mL AgNO_3 (10 mg mL^{-1}) solution before adding 5 mL solution of HH (0.03 mM) and NaOH (0.15 mM) that contained 33- μL Triton X-100. Then mixture solution was sonicated treatment for 30 min. Finally, the GQD and N-GQD samples covered on Ag NPs were purified by performing 10-min centrifugation at 10000 rpm twice to remove unused agents [37].

2.6 DOX loading

First, 10-mg DOX was dissolved in 200- μL DMSO as the stock solution. Then 20- μL DOX stock solution were dispersed in 10-mL PBS (pH = 7.4), followed by mixing with 5-mg N-GQD in 10- mL PBS (pH 7.4) under 400-rpm stirring for 24 h. The solution was dialyzed for 24 hours to remove unbound DOX and obtain drug loaded N-GQD (N-GQD-DOX). The absorbance peak at 480 nm was used to quantify the concentration of N-GQD-DOX in UV-Vis absorption

spectroscopy.

2.7 Preparation of N-GQD-DOX-APTES

Briefly, each 10-mL dialyzed N-GQD-DOX sample added to two-neck round-bottom flask (25 mL) containing a Teflon-coated stir bar was placed on a magnetic stirrer. The sample was slowly heated to 50 °C in a water bath. After the temperature was stabilized, we added various volumes (10, 20, 40, 60 and 80 μ L) of 100 μ L/mL APTES aqueous solution into the N-GQDs-DOX sample to create a series of APTES concentrations (0.42, 0.84, 1.68, 2.52 and 3.36 μ M), which was then stirred at 400 rpm for 24 h at 50 °C. The resulting N-GQD-DOX modified by APTES is abbreviated as N-GQD-DOX-APTES.

2.8 DOX release in Vitro

N-GQD-DOX or N-GQD-DOX-APTES complex prepared under pH = 7.4 was re-suspended in PBS buffer at various pH values (5.7, 6.5, and 7.4) at 37 °C for 48 hours. Briefly, 10-mL N-GQD-DOX or N-GQD-DOX-APTES were dialyzed against 50-mL PBS buffer using a 500 Da molecular dialysis bag. At the specific time, 2-mL dialysate solution was placed in a 24-well plate to record fluorescence intensity at 590 nm (λ_{exc} = 488 nm) of the released DOX.

2.9 Measurement of cell viability using MTT assay

MTT assay was used to measure the metabolic activity of MDA-MB-231 cells. In this MTT assay, the total number of living cells was measured using the activity of NAD(P)H-dependent cellular oxidoreductase that reduces the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into insoluble formazan. After the treatment of MDA-MB-231 cells with free DOX, N-GQD-DOX or N-GQD-DOX-APTES for 12-h, the cells were evaluated for cell viability. After incubation for 12-h, 10- μ L sterilized MTT solution with a concentration of 5-mg/ml was added to the 96-well plates. After 3-h incubation, 100- μ L 10% SDS and 0.1% HCl acting as

the solubilizing solution was added to each well to dissolve formazan. Then 12-h later, absorbance was measured at a wavelength of 595 nm using the plate reader (PerkinElmer Victor3, Thermo Fisher Scientific, Massachusetts, USA) [38].

2.10 In vitro photodynamic therapy

The ability to generate the ROS is an important parameter for PDT. The presence of defects and free radicals in N-GQD indicates their potential for generating the ROS. In this study, we optimized the amount of atoms doped into GQD according to the performance of ROS generation under LED irradiation. The generated ROS level was measured using a redox-sensitive dye (DCFDA) that emits fluorescence when oxidized by various ROS. Briefly, 25- μ L DCFDA (10 mM) was added to 10-mL N-GQD in PBS (5 μ g/ mL). The resulting solution was exposed to irradiation by an LED source (LG-PS2-5, Olympus, Tokyo, Japan) with a power density of 6.8 mW/cm² and a peak wavelength of 622 nm for 20 min. Then the fluorescence intensity at 525 nm was measured when the excitation wavelength was set at 488 nm.

2.11 Detection of intracellular ROS levels using DCFDA and DHE

MDA-MB-231 cells were grown for 24 h and treated with free DOX, N-GQD-DOX or N-GQD- DOX-APTES in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Hyclone, Buckinghamshire, UK) for 12-h. One group of untreated cells served as the control. After the cells were illuminated by LED irradiation treatment for 20 min, the cells were stained with 10 μ M DCFDA (Thermo Fisher Scientific, Waltham, MA, USA) or 10 μ M dihydroethidium (DHE) (Thermo Fisher Scientific, Waltham, MA, USA) prepared in Fluoro-brite medium after 3 h. After staining for 30 min, the medium with DCFDA or DHE was removed and cells were re-suspended in fresh Fluoro-brite medium. Totally 100- μ l Fluoro-brite medium that contained one group of cells was added into a 96-well plate for measurements. The green

fluorescence images of DCFDA were immediately captured with an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 495 nm and an emission wavelength of 529 nm. Meanwhile, the red fluorescent images of DHE were captured using the same microscope at an excitation wavelength of 535 nm and an emission wavelength of 610 nm. Five images were taken in each experiment and the average fluorescence intensity value of the generated ROS was calculated from these images using ZEN software (Zeiss Axiovert S100, Carl Zeiss, Germany).

2.12 Fluorescence imaging

Intracellular and nuclear DOX accumulation after drug release in MDA-MB-231 cells were studied using fluorescence imaging. MDA-MB-231 cells were grown for 24 h and treated with or without free DOX, N-GQD-DOX and N-GQD-DOX-APTES in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Hyclone, Buckinghamshire, UK) for 24 h.[39] Hoechst staining solution (16.23 mM) was created by diluting the Hoechst® stock solution in PBS with a ratio of 1:2000. Cells were incubated in Hoechst staining solution for 10 min under a light-proof cover. Then cells were removed from the staining solution for imaging after washing for three times with PBS.

3. RESULTS

3.1 Characterization of the synthesized N-GQD

The UV-Vis absorption spectra and the PL spectra of the prepared N-GQD are shown in Figure 1(A). The absorption peak at 330 nm in the absorption spectrum of N-GQD is assigned to the $n-\pi^*$ transition of the C=O bond.[36] Furthermore, it can be observed that N-GQD exhibits a strong PL peak at 440 nm at an excitation wavelength of 350 nm. The inset of Figure 1(A) shows

the TEM image of N-GQD. The small sheets with a relatively uniform size distribution in the TEM image suggest that N-GQD are successfully synthesized and the average diameter of the N-GQD is around 4 nm. Figure 1(B-D) are discussed in the subsequent sections.

3.2 Photodynamic therapy properties of N-GQD.

Recently, GQD has been demonstrated acting as a novel photosensitizer under LED illumination and in the presence of oxygen to produce ROS [40]. In this work, we tried to optimize the PDT effect of N-GQD by changing the doping amount of nitrogen atoms to modify the surface oxygenated functional group of the GQD. Then we evaluated the GQD and related N-GQD materials in the generation of ROS using DCFDA as a probe molecule as shown in Figure 1 (B). Various mass ratios ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}}$ in the range of 0, 0.25, 0.5, 1, 1.5, and 2) were evaluated as indicated in sample labels, in which naming follows a pattern of *N-GQD-mass ratio*. It can be seen that the fluorescence intensity of N-GQD is higher than pure GQD; moreover, it increases with mass ratio and reaches the maximum at a mass ratio ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}}$) of 1 then starts to decrease. A similar trend can be observed in the UV-Vis absorption spectra, as shown in Figure 1(C). The peak at 330 nm corresponding to the n-p* transition of the C=O bond only appears in nitrogen doped GQD samples. It can be seen that the intensity of the absorption peak at 330 nm increases with the amount of nitrogen source added until the mass ratio reaches 1. These results may indicate that the ability of GQD to generate ROS can be controlled by tailoring the amount of nitrogen.

Furthermore, Raman spectra in Figure 1(D) suggest that nitrogen doping amount affects the structure of GQD. In order to reduce the influence of strong fluorescence of GQD based materials on Raman spectra, the GQD and N-GQD samples were deposited on the surface of silver nanoparticles. In every Raman measurement, the sample solution was dropped into a small ring on

an aluminum foil with a diameter about 5 mm for measurements. The excitation laser power density was 9.09 W/mm² with an exposure time of 20 s for all SERS measurements. From Figure 1 (D), there are two prominent peaks of D and G bands of GQD at around 1374 and 1612 cm⁻¹ [41]. Compare with the graphene sheet, the intensity of the D band is more higher than the G band, which may due to the GQD exhibited more edge defect than graphene sheet [42]. However, with an increase in the amount of N atoms doped into GQD, the broad band centered at 1351 cm⁻¹ corresponds to the D band, which arises from the defect level, can be observed in nitrogen doped GQD samples [43, 44]. In addition, the Raman spectra of N-GQD samples show that D and G peaks are blue shifted compared with the pristine GQD, which attributed to the defect of N-GQD. According to the previous reports [44, 45], the defect density can be indicated by the intensity ratio of the D band (I_D) to G band (I_G) of the carbon based nanomaterial. As shown in the Table S1, all the intensity ratio values (I_D/I_G) of N-GQD samples are higher than the theoretical value of graphene (~ 1.0) [46]. Moreover, the ratio value ($I_D/I_G = 1.38$) of N-GQD reaches the maximum at a mass ratio ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}}$) of 1, which is consistent with the observations in the DCFDA and UV-Vis results. These results prove that the defect density of N-GQD caused by doping can effectively control the ability of N-GQD to generate ROS. Thus, N-GQD with a mass ratio ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}}$) of 1 is selected for subsequent experiments.

3.3 Characterization of drug loading and release of N-GQD-DOX and N-GQD-DOX-

APTES

The optimal N-GQD was used as drug carrier to load the DOX first. Dialysis was performed to remove unloaded N-GQD and drugs. Then purified N-GQD-DOX were protected with charge-reversal agent APTES on the surface. The UV-Vis absorption spectra of free DOX and N-GQD are shown in Figure 2(A) and (B). The characteristic peaks of DOX and N-GQD can be

found at 490 nm and 330 nm, respectively. As shown in Figure 2 (C), the absorption spectrum of N-GQD changes considerably after DOX is loaded, in which the absorption peak at 330 nm becomes weaker, and the peak at 490 nm attributable to DOX can be observed. Furthermore, a broad band centered at around 500 nm that can be attributed to DOX loaded on N-GQD is found after dialysis treatment as shown in Figure 2(D), the result is that the free drug free N on the surface has also been removed. Thus, the disappearing of N-GQD peak at 330 nm indicates that N-GQD without DOX loaded have been remove. Moreover, the red shift of the DOX peak suggests that DOX has been loaded onto N-GQD surface. Compared with Figure 2(D), the UV-Vis absorption spectra remain almost unchanged after N-GQD-DOX is modified with APTES as shown in Figure 2(E). This observation indicates that the modification with APTES has almost no effect on the absorption of N-GQD-DOX. The fluorescence peak of N-GQD-DOX-APTES shown in Figure 1(F) is much lower than that of free DOX likely due to fluorescence quenching upon drug loading [30]. Therefore, both absorption and fluorescence spectra suggest that DOX has been successfully loaded onto N-GQD surface.

The Zeta potentials of N-GQD-DOX (labeled as *a*) and N-GQD-DOX modified with APTES (labeled as *b* to *e*) are shown in Figure 3. The Zeta potential measurement of N-GQD is – 34 mV in a PBS buffer solution with a pH of 7.4 (not shown in Figure 3). This will contribute to loading positively charged drugs. In contrast, the Zeta potentials of all N-GQD-DOX-APTES samples at a pH of 7.4 as shown in Figure 3 are negative. However, when the pH value decreases to 6.5 or 5.7, the N-GQD-DOX modified with APTES at 2.52 μ M or a higher concentration exhibits charge reversion from negative to positive due the amino group of APTES protonated onto N-GQD-DOX[16]. Such a result indicates that APTES has been grafted onto the surface of N-GQD-DOX. More importantly, the pH dependent charge-reversal property of APTES would

play a key role in converting the carrier system to the positively charged state in the extracellular acidic microenvironment of tumors.[16, 47] This charge-reversal process will also improve the colloidal stability of the drug carrier system and nucleus-targeting accumulation.

To investigate the releasing behavior of N-GQD-DOX and N-GQD-DOX-APTES, the loading efficiency was estimated using a UV-Vis absorbance correction curve shown in Figure S1. The standard regression equation was determined by a commercial software (Origin 8.0, OriginLab, Northampton, MA, USA), which was found to be $A_{(1123\text{ nm}^{-1})} = 0.00537 \times C_{DOX} + 0.075$ ($R = 0.998$). The released DOX amount was estimated by measuring the fluorescence intensity from the dialysis solution and comparing the measured value against the fluorescence calibration curves as shown in Figure S2 and S3. It can be seen from Figure 4 (A) that N-GQD-DOX released 16% DOX while N-GQD-DOX-APTES released about 8% DOX in the first 12 h under the normal physiological condition (pH=7.4). Furthermore, after 24 h, DOX continued to be released from N-GQD-DOX-APTES; in contrast, the releasing process of N-GQD-DOX has almost ended by 24 h. After 48 h, both N-GQD -DOX and N-GQD-DOX-APTES plateaued in DOX release. Under an acidic condition (pH=5.7), as much as 42.5% DOX was released from N-GQD-DOX in 24 h and the releasing of DOX became very slow after that. In contrast, a sustained release process and a low maximum released amount of around 36.0% at 48 h as shown in Figure 4 (B) were observed for N-GQD-DOX-APTES in comparison to around 45.3% for N-GQD-DOX. This reduced release rate and sustained release process of N-GQD-DOX-APTES compared with N-GQD-DOX can probably be attributed to the protection effect induced by APTES, which covered the outside of N-GQD-DOX thus limiting the speed of DOX release from the drug carrier [33].

3.4 In vitro cytotoxicity and imaging

The cytotoxicity of N-GQD-DOX-APTES with and without LED irradiation were investigated by the MTT assay with free DOX used as the control. Briefly, MDA-MB-231 cells were incubated with 5- $\mu\text{g/mL}$ free DOX and N-GQD-DOX-APTES for 12 hours, and then cell viability was evaluated after irradiation for a series of time periods up to 20 min using an LED with a wavelength of 622 nm and a power density of 6.8 mW/cm² for 20 min.

We first investigated the influence of irradiation time on cell viability. As shown in Figure 5(A), the cell viability decreased with the LED irradiation time after incubation with N-GQD-DOX-APTES for 12 h, due to DOX released from the N-GQD drug carrier. This result can be contributed to the generation of ROS by the N-GQD that killed cancer cells [32, 48]. Next, the cytotoxic effects of N-GQD-DOX-APTES with or without of LED irradiation against MDA-MB-231 cells were tested with series of concentrations (from 0 to 10 μM). It can be seen from Figure 5(B), at a low concentration (2.5 $\mu\text{g/mL}$), free DOX shown a higher efficiency of killing cancer cells than N-GQD-DOX-APTES both with and without LED irradiation treatment likely due to the low PDT effect and the fact that free DOX can more easily diffuse into cells than DOX loaded by the carrier. However, once the concentration of N-GQD-DOX-APTES increased to a value greater than 5 $\mu\text{g/mL}$, N-GQD-DOX-APTES with LED irradiation exhibited a greater anticancer efficiency than both free DOX and N-GQD-DOX-APTES without LED irradiation. The increase in anticancer efficiency may be attributed to the extra photosensitizing effect induced by N-GQD [32].

To measure the PDT efficacy, the ROS dyes including DCFDA and DHE were used to image the generation of ROS [20]. As shown in the 2nd row of the image panel in Figure 6, strong green DCFDA fluorescence was observed in MDA-MB-231 cells incubated with 5- $\mu\text{g/mL}$ N-GQD-DOX and N-GQD-DOX-APTES both after LED irradiation for 20 min. In contrast, there

was only weak fluorescence observed in the control and free DOX images. This result indicated that N-GQD played a key photosensitizing role in ROS generation in live cells. The fluorescence intensity values were quantified and shown in Figure 6 (A). Compared with the pure N-GQD-DOX, there is a slight decrease in N-GQD-DOX-APTES fluorescence intensity, which may be attributed to the protection effect of APTES. The similar trend can be observed when another ROS dye DHE was used to measure the generation of ROS as shown in the 4th row of the image panel in Figure 6 and Figure 6 (B). These results indicated that the ROS in MDA-MB-231 cells were generated by N-GQD under LED irradiation.

Intracellular and nucleus DOX accumulation after drug release in MDA-MB-231 cells were also studied as shown in Figure 7, in which nuclei displayed blue fluorescence after labeling and DOX showed red fluorescence. In Figure 7, red fluorescence, which was attributed to DOX, for N-GQD-DOX-APTES was more intense in the nuclei regions, while weaker red fluorescence was accumulated in nuclei free DOX and N-GQD-DOX. Figure S4 in the SI also confirms that there was only weak red fluorescence observed in cell nuclei regions even at a high concentration for free DOX and N-GQD-DOX (10.0 $\mu\text{g/mL}$). We also investigated the diffusivity of N-GQD-DOX-APTES (2.5, 5.0 and 10.0 $\mu\text{g/mL}$) in nuclei as a function of concentration. As shown in Figure S5, more intense red fluorescence was observed in the nuclear regions with an increase in the concentration of the N-GQD-DOX-APTES. The enhanced nucleus targeting ability and DOX accumulation in the cell nucleus may be associated with the small size of N-GQD-DOX-APTES making it easy to be taken up by nuclei and the charge-reversal properties of the APTES [31, 33]. Thus, the above results suggested that the N-GQD-DOX modified with APTES can be used as a novel nucleus-targeting drug delivery system with high PDT efficacy.

4. DISCUSSION

Our results provide adequate evidence indicating the proposed nanomaterial, i.e. N-GQD-DOX-APTES, as a potential photosensitizer with controllable PDT efficiency and nucleus-targeted drug carrier for cancer therapy, as listed below. First, the proposed nanomaterials is an excellent photosensitizer to kill cancer cells as shown in Figure 5(A), in which the viability of MDA-MB-231 cells decreased with an increase in LED irradiation time. This treatment capability can be attributed to the ROS produced by LED illuminating N-GQD-DOX-APTES as shown by intracellular ROS images in Figure 6. Comparing the DCFDA result (Figure 1(C)), UV-Vis result (Figure 1(B)) and Raman spectra (Figure 1(D)), it can be observed that the ROS production efficiency of N-GQD is highly correlated to the defect density indicated by the intensity ratio of the D band (I_D) to G band (I_G) (I_D/I_G) because the ROS production efficiency and the defect density of the nanomaterial reach the maximum at the same mass ratio ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}} = 1$). Therefore, that the PDT efficiency, which is directly related to the ROS generation efficiency, can be controlled by varying the amount of nitrogen element doped into GQD in the proposed nanomaterial, i.e. the mass ratio ($Q_{\text{Nitrogen source}}/Q_{\text{Carbon source}}$), as shown in Figure 1(B).

Second, the proposed nanomaterial can serve as an efficient carrier for nucleus-targeted drug delivery as shown by fluorescence images in Figure 7, where N-GQD-DOX-APTES is more efficiently accumulated in cell nuclei compared with free DOX and N-GQD-DOX without grafted APTES. This capability could be attributed to two reasons. One is that N-GQD can be used as a drug carrier even without modification for the pH-sensitive release of anti-cancer drugs as shown in Figure 4. Its nucleus-targeting capability is further enhanced by grafting the charge transformation agent APTES. The Zeta potentials test results in Figure 3 show that N-GQD-DOX modified with APTES at 2.52 μM or a higher concentration exhibits charge reversion from

negative to positive when the pH value decreases from 7.4 to 5.7. This suggests that, once N-GQD-DOX-APTES reaches the acidic tumor microenvironment, the positively charged drug carrier will show an increased capacity to be internalized by tumor cells *via* the negative surface of cell membranes.

Last, the dual therapy including the PDT and drug therapy enabled by the proposed nanomaterial will increase the treatment efficiency as shown in Figure 5(B). The comparison test results in this figure show that the N-GQD-DOX-APTES with LED irradiation exhibited an anticancer efficiency significantly higher than both free DOX and N-GQD-DOX-APTES without LED irradiation when the concentration is greater than 5 $\mu\text{g/mL}$.

In summary, we report the development and evaluation of a multifunctional materials, i.e. N-GQD-DOX-APTES, as a potential photosensitizer and nucleus-targeted drug carrier for cancer therapy. Due to its graphene nature, N-GQD can generate ROS and kill cancer cells. In particular, the defects and surface functional groups of N-GQD can be tailored by changing the amount of nitrogen source to tune the ROS generation performance. In addition, the small size of N-GQD make it easy to be taken up by cells due to the size-dependent effect. More importantly, N-GQD-DOX-APTES as a drug carrier exhibited excellent nucleus-targeting capability, which could be attributed to the charge-reversal property of APTES. Therefore, this multifunctional N-GQD-DOX-APTES system has a great potential for multi-modal cancer therapy.

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CONFLICTS OF INTEREST

The authors declare to have no conflicting interests.

AUTHOR CONTRIBUTIONS

J. J. and Q. L. conceived and designed the study. J. J. performed most experiments. S. R. and A. F. conducted the cell biology experiment jointly with J. J. S. R. conducted the fluorescence imaging experiments. S. L. provides support for biological experiments. J. J. and Q. L. wrote the paper and edited the manuscript. All authors approved the manuscript.

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FIGURE LEGENDS

FIGURE 1. Characterization of the synthesized N-GQD. (A) UV-Vis absorption, photoluminescence emission (λ_{em} = 440 nm) spectra with the excitation (λ_{ex} = 360 nm) of the N-GQD (0.5 mg/mL) solution. Inset: HRTEM image of the synthesized N-GQD. (B) DCFDA fluorescence intensity of 5 μ g/mL GQD and a series of N-GQD samples after LED irradiation for 20 mins. Excitation: 488 nm; emission: 525 nm. (C) UV-Vis absorption spectra of the synthesized GQD and N-GQD samples, respectively. (D) Raman spectra of GQD and N-GQD wrap on Ag NPs substrates excited by a laser at 785 nm.

FIGURE 2. Characterization of drug loaded of N-GQD-DOX and N-GQD-DOX-APTES. UV-Vis spectra of (A) Free DOX(50 μ g/mL), (B) N-GQD (50 μ g/mL), (C) N-GQD loaded with DOX prior to dialysis treatment, (D) N-GQD-DOX after dialysis treatment for 24 h, (E) N-GQD-DOX modified with APTES and (F) fluorescence spectra of free DOX and N-GQD-DOX-APTES.

FIGURE 3. Surface charges of the N-GQD-DOX-APTES at different pH values analyzed by a Nano ZS Zetasizer. Zeta potential of N-GQD-DOX modified with APTES at various concentrations (0.42, 0.84, 1.68, 2.52 and 3.36- μ M, labeled as *a* to *e*) in PBS buffer at different pH values (7.4, 6.5, and 5.7).

FIGURE 4. Evaluation of drug delivery properties *in vitro*. Percentage of DOX retained on N-GQD for N-GQD-DOX and N-GQD-DOX-APTES at different pH values: (A) 7.4 and (B) 5.7. The percentage of the released DOX is simply the percentage of retained DOX subtracted from 100%.

FIGURE 5. Viability measurements of MDA-MB-231 cells using the MTT assay after incubation with various materials and with (or without) LED irradiation. (A) Viability of MDA-MB-231 cells incubated with 5- μ g/mL N-GQD-DOX-APTES for different irradiation time periods. (B) Viability of MDA-MB-231 cells after incubating for 12 h with a series of concentrations of free DOX, N-GQD-DOX-APTEs from 0 to 10 μ g/mL then with or without LED irradiation treatment for 20 min.

FIGURE 6. ROS generation of free DOX, N-GQD-DOX and N-GQD-DOX-APTES in MDA-MB-231 cells. Image panel on the left: fluorescence images of MDA-MB-231 cells incubated with 5- μ g/mL N-GQD-DOX-APTES with LED irradiation treatment for 20 min and stained with DCFDA (2nd row) and DHE (4th row). The corresponding phase contrast images are also shown for comparison. Quantified fluorescence results for DCFDA and DHE are shown in (A) and (B), respectively, on the right.

FIGURE 7. DOX distribution in nuclei for free DOX, N-GQD-DOX and N-GQD-DOX-APTES. Nucleus staining (first column), DOX fluorescence (second column) and merged (third column) images of in MDA-MB-231 cells after incubation with 5- μ g/ mL free DOX (first row), N-GQD-DOX (second row) and N-GQD-DOX-APTES (third row).

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

Graphical Abstract