Simultaneous Delivery of antimiR-21 and Doxorubicin by Graphene Oxide for Reducing Toxicity in Cancer Therapy

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ABSTRACT: Graphene oxide (GO) has been studied by many researchers for its potential drug-delivery value. In order to reduce the side effects of anticancer drugs by decreasing the dosage and maintain the therapeutic effects, a dual drug-delivery system that used GO as a carrier and simultaneously loaded with antitumor drugs and antimiR-21 was rationally designed for the cooperative treatment of tumors. Results obtained from our studies have found that MDA-MB-231 cells were inhibited in low Dox dose. The outcomes of confocal microscopy indicated that Dox and antimiR-21 could be released rapidly in cancer cells, which is good for killing cancer cells. In addition, qRT-PCR further demonstrated that miR-21 was silenced by antimiR-21. Consequently, GO has a great potential to codeliver chemotherapeutic drugs and gene drugs in cancer combination therapy for reducing toxicity.

INTRODUCTION

The incidence of cancer in the world is increasing annually, which is a serious threat to people’s health.1,2 In addition, from the data provided by research, we can predict that there will be 20 million cancer patients in 2030 across the world.3 The existing small molecular anticancer drugs not only cause damage to normal cells or tissues but also restrict their application because of poor water solubility. Therefore, changing the administration method of anticancer drugs and synergistic treatment of multiple drugs are both effective ways for tumor treatment. As a drug-delivery system, nanocarriers have attracted much attention of many researchers.4–6

The emergence of nanotechnology is conducive to the development of cancer treatment and new dimension of therapeutics, where the combination of diagnosis and treatment can improve the health-care management of clinics.7,8 Nanoparticles with a specific lateral size (50–200 nm) have an enhanced permeability and retention effect on tumor tissues, which can infiltrate into and gather inside the tumor tissue for a better treatment effect.9–11

Graphene oxide (GO), a novel 2D nanomaterial prepared from natural graphite, has recently attracted significant attention because of its intriguing electronic, mechanical, thermal, and optical properties.12–14 Because of the small size and large surface area-to-volume ratio, high stability, good biocompatibility, and easy surface modification properties, GO has been widely explored for biological and biomedical applications including bioimaging,15–18 biosensors,19–23 and drug or oligodeoxynucleotides delivery.24–26 Several aromatic anticancer drugs, such as camptothecin and doxorubicin (Dox), were loaded onto GO through π–π stacking for delivering into the cancer cells in the previous study.27

On the other hand, microRNAs (miRNAs) are recognized as potential biomarkers of cancer, which regulate their post-transcriptional repression by binding to mRNAs of protein-coding genes, causing the regulation of target mRNA degradation and protein expressions.28–31 In particular, miR-21 has recently received much attention by many research groups because of its over-expression in a variety of cancer types including brain, liver, colon, and breast cancers.32,33 Several targets of miR-21 have been experimentally validated, including PDCD4, PTEN, BCL-2, and RECK.34 Most importantly, silencing of miR-21 could impede its ability to inhibit tumor suppressor genes, which further inhibits the invasion, proliferation, and migration of breast cancer cells. In addition, it has been shown that inhibiting the function of miR-21 is helpful to overcome the multidrug resistance of cancer cells. Although there have been studies using GO to deliver small interfering RNAs to inhibit the function of miR-21 (silence miR-21),35 it is well known that siRNA is expensive, unstable, and easy to be degraded. Therefore, it is necessary to seek a low-priced and steady delivery system. Here, a complementary strand of miR-21 (cDNA21 or antimiR-21),...
which is cost-efficient and less susceptible to degradation is applied to the study for silencing miR-21.

In cancer treatment, chemotherapy drugs are not perfect. The main problem with chemotherapy drugs is not invalid, but the side effects are too strong. Chemotherapy drugs generally have a powerful killing effect on cancer cells; however, they still cause serious damage to normal cells which is induced by their lack of targeting. GO has been widely reported to display great potential for drug delivery. Anticancer drugs or nucleic acids can be easily delivered into cells using GO to carry them through the cell membrane. Hence, in order to reduce the side effects of anticancer drugs by decreasing the dosage and maintain the therapeutic effects, cDNA21 combined with the anticancer drug was used to treat cancer. A dual delivery system based on GO which was simultaneously loaded with anticancer drug was used to treat cancer. A dual delivery system based on GO which was simultaneously loaded with cDNA21 and Dox was rationally designed for cancer synergetic therapy in our investigation. It is our expectation that the outcomes provided in this study could offer valuable information to stimulate the development of cancer synergetic therapy.

The fundamental principle of our strategy to design the dual-functional drug delivery system, Dox−GO−cDNA21, is displayed in Scheme 1. As shown in Scheme 1, FAM-cDNA21 (fluorescent group FAM labeled cDNA21) and Dox were loaded onto the GO surface by π−π stacking and hydrogen bonding interaction. The GO-loaded FAM-cDNA21 and Dox were efficiently sent into the cytoplasm under the action of endocytosis of the cancer cell. In the cytoplasm, acidic conditions were induced by the lysosome, Dox was released from the surface of GO and entered the cell nucleus to produce anticancer effects. On the other hand, because of the presence of miR-21 in the cancer cytoplasm, FAM-cDNA21 was pulled out of the GO surface, and it tended to form FAM-cDNA21/miR-21 heteroduplexes, which results in the silence of miR-21. Because miR-21 was silenced, the proliferation and migration of cancer cells were suppressed. Additionally, single-stranded DNA could be adsorbed on the surface of GO via π−π stacking interactions, and then, the fluorescent groups carried by the DNA was quenched by fluorescence resonance energy transfer. However, DNA/RNA heteroduplexes cannot be adsorbed by GO. Fluorescence of FAM-modified oligonucleotide will be gradually recovered because it was hybridized with miR-21 to form heteroduplexes and was removed from the GO surface. As a consequence, cDNA21 could propel resultful collaboration with Dox to trigger the dual anticancer function even in the condition of low dosage of Dox. It was speculated by us that the constructed method is suitable for using in cancer synergetic therapy for the purpose of reducing the dosage of anticancer drugs.

### RESULTS AND DISCUSSION

#### Preparation and Characterization of GO.
In order to control the size of GO suitable for endocytosis, which requires the average diameter of particles less than 200 nm. GO was prepared from graphite powder according to the improved Hummers method and sonicated in water to produce well-exfoliated GO. Transmission electron microscopy (TEM) and dynamic light scattering were applied to determine the morphology and size distribution of GO. As shown in Figure S1, different-sized GO were obtained using the sonication cutting approach, and the particle size of GO actually decreases with the increase of ultrasound time. From Figure 1a−d, it is known that the average diameter of GO reaches 172 ± 12 nm after 1 h of sonication. In addition, the results of TEM also showed a significant decrease in the diameter of GO (Figure 1c,d). Consequently, the GO, after 1 h of ultrasonic treatment, was chosen for subsequent experiments.

#### Preparation and Characterization of GO.
With the aim of estimating the optimal dosage of GO for the Dox−GO−cDNA21 system (nucleic acid sequences in Table S1), the fluorescence quenching capability of the prepared GO was evaluated by mixing FAM-cDNA21 (250 nM) with different concentrations of GO. The fluorescence of FAM gradually declined with the growing concentration of GO, as shown in Figure 2a, until almost complete quenching at the concentration of 50 μg/mL and no further remarkable fluorescence decrease were observed. Therefore, the abovementioned concentration of GO was chosen and used in this study. To
demonstrate that Dox was loaded onto GO−cDNA21, the fluorescence spectra and UV−vis spectra were applied. Compared to the blank GO, the results of Figure 2b,c show that Dox−GO−cDNA21 has totally different UV−vis absorbance and fluorescence signals. The UV−visible spectrum indicates a significant increase in absorbance at 230 and 480 nm, while the fluorescence spectrum demonstrates a significant decrease in Dox fluorescence at 590 nm. Furthermore, it can be seen from Figure 2d that the concentration of Dox is linear with the fluorescence intensity when the concentration is in the range of 19.53−625 ng/mL. In light of the standard curve of Figure 2d and formula 1, the drug-loading efficiency can be calculated to be 39.55 wt %.

In Vitro Drug Release Experiments. In the Dox release study of Dox−GO−cDNA21 in vitro, the release behavior of drug-loaded samples in different pH buffers was tested separately. As shown in Figure 3a, the cumulative release rates of Dox are not very high in different pH conditions. This could be ascribed to the strong hydrogen bond and π−π stacking interactions between Dox and GO. Moreover, compared with the neutral environment, the acidic environment is more conducive to the release of Dox. The cause of this phenomenon may be due to the fact that π−π bonds can be interrupted in the acidic environment, and hydrogen bonds between drug molecules and GO are weakened at low pH, which result in preferable release of the loaded Dox. This result revealed that the Dox would be better released when Dox−GO−cDNA21 is in the acidic condition which is induced by the lysosome in the cancer cell. In order to confirm the combination of cDNA21 and miR-21, the hybridization experiment of FAM-cDNA21 with miR-21 were performed. The fluorescent signal of FAM was enhanced with the increment of miR-21 concentrations from 0 to 100 nM (Figure 3b), which demonstrates that the fluorescent signal is associated with the concentration of miR-21. It was verified by the result that cDNA21 could hybridize and silence miR-21 in cancer cells. In addition, to investigate the stability of Dox−GO−cDNA21 against damage by intracellular enzymes, different enzymes including DNase I, T4 DNA ligase, EXO I, EXO III, T4 DNA ligase, DNA polymerase, and RNase H were individually added to Dox−GO−cDNA21. It is observed from Figure S2 that almost no fluorescent signal was detected under different enzyme environments. This result indicates that GO has a good protective effect on nucleic acids, which means that the drug-loading system has excellent stability against different enzymes in cells.

Confocal Fluorescence Imaging. With the purpose to affirm whether Dox−GO−cDNA21 could readily enter cancer cells, Dox and FAM-cDNA21 were employed as the fluorescent probe for intracellular imaging, as shown in Figure 4a−f. In the images of confocal fluorescence, the fluorescence of FAM-cDNA21 (Figure 4c) and Dox (Figure 4d) was captured after the MDA-MB-231 cell was treated with Dox−GO−cDNA21, which means the drug was indeed taken up into the MDA-MB-231 cell. More importantly, Figure 4c,d indicated that FAM-cDNA21 and Dox could be released from the surface of GO, which fully confirmed the feasibility of our strategy.
In Vitro Cell Cytotoxicity Assays. Prior to cytotoxicity assessment of Dox–GO–cDNA21, it is necessary to investigate the cytotoxicity of GO to confirm the nontoxic behavior of GO, as reported in the literature. Herein, the MDA-MB-231 cell was selected because of the high expression of miR-21. The cell was treated with GO in the concentration range of 0–250 μg/mL for 48 h before conducting cell viability measurements by MTT assay. It can be seen in Figure S3 that GO exhibited negligible cytotoxicity at concentrations below 80 μg/mL as cell viability remained relatively high. Subsequently, the MDA-MB-231 cellular cytotoxicity effect toward Dox–GO–cDNA21, which including different concentrations of Dox and cDNA21, was also investigated through MTT assay (Figure 5a). The results indicated that cDNA21 and Dox produce synergistic inhibition on the MDA-MB-231 cell. Meanwhile, at a certain concentration of Dox (0.4, 0.8, and 1.2 μg/mL), the cell viability decreased gradually as the concentration of cDNA21 increased; that means increasing the concentration of cDNA21 while reducing the concentration of Dox can also inhibit the growth of the MDA-MB-231 cell. According to the result of the MTT assay shown in Figure 5b, when the concentration of cDNA21 reached 250 nM, the inhibitory effect of the drug-loading system on the MDA-MB-231 cell was not significantly decreased with reduction in the dosage of Dox. Cell viability without dramatic changes in the concentration of Dox between 0.4 and 1.2 μg/mL is clearly observed.

qRT-PCR Analysis of miR-21. In order to further confirm that miR-21 in the MDA-MB-231 cell was silenced, qRT-PCR has been applied in our investigation. Treated with GO–cDNA21 and Dox–GO–cDNA21, qRT-PCR was performed from the total RNA extracted from the MDA-MB-231 cell to evaluate miR-21 inhibition. The result of qRT-PCR consists with the abovementioned other results. As shown in Figure 6, the both type-treated cells displayed 99% decrease of the miR-21 level compared with the control, which indicates that the expression of miR-21 is inhibited.

CONCLUSIONS

In conclusion, a cancer combined therapeutic system Dox–GO–cDNA21 was designed and prepared in our study for the codelivery of anticancer drugs and nucleic acids into cancer cells to reduce the side effect of the high concentration of Dox. Through synergistic delivery of 250 nM of cDNA21, the dosage of Dox can be reduced by approximately two times without weakening the efficacy of the drug. The results of our investigation indicated that the delivery system still shows potent anticancer effects even under the condition of low-dose anticancer drugs. In addition, fluorescence observations of confocal microscopy demonstrated that Dox–GO–cDNA21 can quickly enter MDA-MB-231 cells to release drugs. Moreover, the result of qRT-PCR further confirmed that miR-21 was silenced by the antimiR-21 which is delivered into the cancer cell using the designed system. We anticipate that the accomplishment of this research will be to provide beneficial information to the design of the drug-delivery system for cancer therapy and also inspire applications in the treatment of other diseases.

MATERIALS AND METHODS

Chemicals. GO was prepared from graphite powder according to the improved Hummers method. Meilun Biotechnology Tech Co., Ltd (Dalian) provided doxorubicin hydrochloride (Dox). magnesium chloride hexahydrate (MgCl₂·6H₂O), potassium chloride (KCl), and sodium chloride (NaCl) were acquired from China National Pharmaceutical Group Corp. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Beijing Solarbio Science & Technology Co., Ltd. All enzymes used in the experiment (including DNase I, EXO I, EXO III, etc.) were procured from New England Biolabs (Beijing) Ltd. The reagents used in the experiment were of analytical grade, which were used as...
received. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and phosphate-buffered saline (PBS) were purchased from Sigma. DNA and RNA used in the experiment were both supplied by Shanghai Sangon Biotechnology. The sequence of the nucleic acid can be seen in Table S1.

Characterization. High-resolution TEM image was obtained using a JEM-2100 electron microscope. The Malvern Zetasizer Nano-ZS was used to study the particle size distribution. The microplate reader (TECAN Infinite M200 Pro) was used to detect the UV absorbance of formazan in the MTT assay. Confocal imaging studies were performed using a Zeiss LSM880 laser scanning confocal microscope.

Quantitation of GO. To facilitate the research of the drug-delivery system, cDNA21 was labeled as FAM. The FAM-cDNA21 was controlled to 250 nM, and GO was gradually added to quench the fluorescence of FAM-cDNA21, which aims at determining the amount of GO. The amount of GO required is determined when the fluorescence of FAM-cDNA21 is just completely quenched.

Preparation of Dox–GO–cDNA21 Complexes. Dox (10 mg) was first dissolved in dimethyl sulfoxide (DMSO) (1 mL) and diluted to 100 μg/mL. cDNA21 (final concentration of 250 nM) and GO (final concentration of 50 μg/mL) were then mixed in the Tris-HCl buffer to load the cDNA21 onto GO. Next, the diluted Dox (final concentration: 20 μg/mL) was added to the above prepared mixture and stirred overnight in the dark at room temperature so that the Dox is fully loaded on the GO. To obtain the purified product, the mixture was centrifuged at 8000 rpm for 20 min. After centrifugation, the supernatant was collected, and the fluorescence intensity of Dox was measured. In addition, the precipitate was redispersed with ultrapure water to obtain the final product. In this experiment, we use the following formula to calculate the drug-loading efficiency.

\[ W_t^\% = \frac{C_0V_t - C_tV_t}{C_0V_t} \times 100\% \]

where \( W_t^\% \) represents cumulative release dose (%), \( C_t \) represents concentration of the drug in the buffer solution taken at time \( t \), \( V_t \) represents volume of the buffer solution at time \( t \), \( C_t \) represents the concentration of Dox initially added, \( V_t \) represents the volume of the initially added Dox, \( C_0 \) represents the concentration of the drug in the supernatant obtained after centrifugation, \( V_t \) represents the volume of the supernatant obtained by centrifugation after drug loading.

Stability of Dox–GO–cDNA21. To investigate the stability of Dox–GO–cDNA21 against damage by intracellular enzymes, different enzymes including DNase I, T4 DNA ligase, EXO I, EXO III, T4 DNA ligase, DNA polymerase, and RNase H were individually added to Dox–GO–cDNA21.

In Vitro Cytotoxicity Determination. The MTT assay was used to study the in vitro cytotoxicity of GO materials to the MDA-MB-231 cells. MDA-MB-231 cells were inoculated into 96 well plates at a density of 4 × 10^4 cells/well and cultivated in an incubator (37 °C, 5% CO_2) for 24 h. After that, different concentrations of the GO material were added to each well and further cultured in the medium for 48 h. After incubation, 100 μL of fresh medium was added to replace the original medium, and then, 20 μL of MTT solution (5 mg/mL) was added and cultured for 4 h. After the termination of cultivation, the medium in the wells was carefully sucked, and 100 μL of DMSO was added to each hole to dissolve the formazan. Subsequently, the absorbance of each pore at 492 nm was measured using a microplate reader. Here, we use the following formula to calculate the cumulative drug release.

\[ OD_{treated} = \frac{OD_{treated} - OD_{control}}{OD_{treated} - OD_{control}} \times 100\% \]

where \( OD_{treated} \) and \( OD_{control} \) in the formula, respectively, represent the absorbance values of samples and the positive control. The relative cell viability was calculated by OD values measured on the basis of four independent parallel samples.

Laser Confocal Imaging. MDA-MB-231 cells were seeded in confocal dishes and incubated for 24 h. Dox (1.2 μg/mL)–GO (50 μg/mL)–cDNA21 (250 nM) were incubated with the MDA-MB-231 cells in DMEM. After 6 h incubation, MDA-MB-231 cells were washed with 1 mL of PBS five times, each time for 3 min. Then, the cell nucleus was stained with 1 mL of 4’,6-diamidino-2-phenylindole (DAPI) solution for 10 min. After staining, the MDA-MB-231 cells were washed with 1 mL of PBS five times, each time for 3 min. The cells after staining were stored in a 4 °C refrigerator for subsequent laser scanning confocal microscopy observation. The phagocytic effect of the MDA-MB-231 cells on the Dox–GO–cDNA21 complex was observed by laser scanning confocal microscopy.

qRT-PCR Analysis of miR-21. We designed a qPCR experiment to detect the expression of miR-21. First, MDA-MB-231 cells were lysed with the TRIzol reagent, and the total RNA was extracted and collected. Then, the first strand of miRNA cDNA was synthesized by polyadenylation of the total RNA. The expression of miR-21 was detected by qPCR with miR-21 forward primers and universal reverse primers provided by the kit.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01010.
Changes in the diameter and polydispersity index of GO sheets after ultrasound treatment; stability of DOX–GO–cDNA21 under different enzyme environments; and relative cell viability at different concentrations of GO.

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**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**
We are greatly appreciating Top-notch Academic Programs Project of Jiangsu Higher Education Institutions (PF2015B146), Scientific Research Foundation of Wuxi City for the Returned Overseas Chinese Scholars, and 2018 Innovative Research Team of Jiangsu Province for support this research work.

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