Reduced Graphene Oxide Nanosheet for Chemo-photothermal Therapy

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ABSTRACT: The protein-functionalized reduced graphene oxide (rGO) nanosheet is of great interest in stimuli-responsive drug delivery and controlled release applications. We developed doxorubicin (DOX)-loaded bovine serum albumin (BSA)-functionalized rGO (DOX-BSA-rGO) nanosheets. To investigate the reduction of BSA-functionalized GO nanosheets and drug loading efficiency, we used X-ray photoelectron spectroscopy (XPS) and UV–visible spectrophotometer analysis. DOX-BSA-rGO nanosheets exhibited dose-dependent cellular uptake without any cytotoxic effect. We also demonstrated near-infrared (NIR)-induced chemo-photothermal therapy of brain tumor cells treated with DOX-BSA-rGO nanosheets. Therefore, this DOX-BSA-rGO nanosheet could be a powerful tool for chemo-photothermal therapy applications.

INTRODUCTION

Chemotherapy has widely been used to treat tumor cells. However, conventional chemotherapy still has various limitations, such as hair loss, nausea, vomiting, and heart damage.1,2 To overcome these limitations, anticancer drug-loaded functional nanoparticle-mediated tumor therapy has previously been developed.3,4 In particular, photothermal therapy could kill the tumor cells in a localized specific region and minimize the damage of neighbor tissues around tumor cells.5 Near-infrared (NIR) laser irradiation deeply penetrates into the tissues without any cell damage due to its long wavelength (650–900 nm).6 Carbon- or gold-based nanomaterials, which could highly absorb NIR wavelength, have previously been used for photothermal therapy applications.7–9 Despite their potential, the major limitation is the localized therapy, which can result in incomplete ablation and tumor recurrence, because the heating is only generated within the specific region that was exposed to NIR laser irradiation.

Graphene, which is the thickness of a carbon atom layer, has recently emerged as a promising material for photothermal therapy applications. Due to its excellent physical and chemical properties (e.g., high electrical conductivity, strength), graphene has widely been used for biosensor, drug delivery, and molecular imaging applications.10,11 Compared to conventional nanomaterials containing sphere and core–shell structures,12–14 two-dimensional (2D) graphene nanosheets have great potential for high drug loading efficiency and conjugation of proteins, drugs, and fluorescent probes.15–19 Nevertheless, the bare graphene or graphene oxide (GO) is required for surface modification process to improve its biocompatibility.20–22 Recently, protein-based reduced GO (rGO) has been developed as a drug delivery carrier, photothermal agent, and bioimaging probe.23,24 Pegylated graphene has previously been employed for in vivo photothermal therapy. Although pegylated graphene showed high efficiency of tumor passive targeting and therapy, it required a larger amount (20 mg/kg) of samples and optimal graphene concentrations for improving the cell viability.25,26

Bovine serum albumin (BSA), known as a reduction agent under alkaline conditions, is one of the amphiphilic adhesive polymers that can bind hydrophilic and hydrophobic materials.27,28 BSA is used as a reduction binder that can load drugs. External stimuli (e.g., pH) have also been reported to regulate the controlled release of drugs.29–32 Recently, carbon-based materials showing high absorption at NIR wavelength have been used for photothermal therapy and controlled drug release.30–32 In particular, graphene-based photothermal therapy has been used to reduce tissue damage around tumor cells.33 It has been known that chemo-photothermal therapy shows higher efficiency of therapy compared to single treatment of chemotherapy or photothermal therapy.34 Therefore, an anticancer drug-loaded biocompatible graphene nanomaterial needs to be developed for chemo-photothermal therapy applications. In this paper, we developed DOX-loaded BSA-functionalized rGO (DOX-BSA-rGO) nanosheets for chemo-photothermal therapy of brain tumor cells.

MATERIALS AND METHODS

Materials. Sulfuric acid (H2SO4, 95%), hydrochloric acid (HCl, 35%), and sodium hydroxide (NaOH, 97%) were purchased from Daejung Chemicals & Metals, Korea. Potassium permanganate (KMnO4, 99.3%), hydrogen peroxide (H2O2, 34.5%) were obtained from Junsei Chemical, Tokyo, and Samchun Chemical, Korea, respectively. Graphite, BSA, DOX hydrochloride, and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma-Aldrich,
**Synthesis of BSA-rGO Nanosheet.** GO was made by Hummer’s method. Briefly, 1 g of graphite flakes was stirred in 500 mL of 50% sulfuric acid in an ice bath, and 5 g of potassium permanganate was subsequently added. After stirring for 1 h, the mixture was warmed to 35–45 °C and was stirred for an additional 2 h. Fifty milliliters of distilled water was slowly dropped into the mixture solution, and the solution was then diluted by adding additional 150 mL of distilled water under vigorous stirring. To terminate the reaction, 10 mL of hydrogen peroxide was added and was subsequently stirred for 15 min. The solution was washed several times with 5% HCl and distilled water to remove the residual acid. Nanosized GO was obtained by further oxidation and freeze-drying. Prior to reduction of GO, graphite oxide was exfoliated to obtain GO from sonication (20 kHz, 500 W) in distilled water for 2 h. One milligram per milliliter GO was diluted in distilled water and 50 mg/mL BSA solution was subsequently added in a 70 °C water bath. The solution was adjusted to pH 12 rapidly with 1 M sodium hydroxide. After stabilization, the mixture was centrifuged at 15 000 rpm to eliminate the nonreactive BSA and NaOH. The precipitates were redispersed in distilled water and were rinsed three times.

**Characterization of DOX-BSA-rGO Nanosheet.** The morphologies of GO, BSA-rGO, and DOX-BSA-rGO nanosheets were observed by transmission electron microscopy (TEM, JEOL, JEM1010, Japan), with an accelerating voltage of 80 kV. XPS analysis was performed on K-alpha (Thermo VG, UK) and T64000 (Horiba, Ar laser at 514 nm). The characteristic peak and surface charge was analyzed by a UV–visible spectrophotometer and Zetasizer. To study the photothermal effect, the temperature of samples during NIR irradiation was measured at every 60 s using a digital thermometer.

**DOX Loading and Release from DOX-BSA-rGO Nanosheets.** The DOX-BSA-rGO nanosheet was prepared by mixing with 0.1 mg/mL of BSA-rGO. The solution was washed with phosphate buffered saline (PBS) to remove unbound DOX on BSA-rGO nanosheets and its precipitates were subsequently collected. A DOX release experiment was performed in a shaking incubator at 37 °C. For the NIR-mediated DOX release experiment, the DOX-BSA-rGO nanosheet was dispersed in PBS (pH 5) and was subsequently irradiated by NIR (808 nm, 5.5 W/cm²) light for 5 min. Supernatant was measured at 490 nm, which is the characteristic absorption peak of DOX by UV–visible spectrophotometer. The concentration of DOX released from DOX-BSA-rGO nanosheets was quantified using a calibration curve. We made the calibration curve with DOX concentrations and 1 mg/mL DOX loaded into 0.1 mg/mL BSA-rGO nanosheets for 12 h. The initial concentration of DOX was measured by a UV–visible spectrophotometer to find drug loading efficiency.

**Cell Culture.** U87MG brain tumor cells (1.6 × 10⁴ cells/well) were cultured in a 96-well plate with Minimum Essential Medium (MEM, Gibco, USA) containing 10% fetal bovine serum (FBS). The cells were split as they were 85% confluent in a well-plate. The culture medium was exchanged every 2 days.

**Cellular Uptake.** A One milligram per milliliter DOX-BSA-rGO nanosheet and 2 mg/mL FITC-dextran (10 000 Da) were dissolved in distilled water. To label FITC-dextran in the DOX-BSA-rGO nanosheet, DOX-BSA-rGO and FITC-dextran solution were stirred overnight. After overnight, the mixture was centrifuged at 14 000 rpm and was washed with PBS three times to remove unbound FITC-dextran. For cellular uptake, U87MG cells (1.6 × 10⁵ cells/mL) were seeded on a coverslip and were cultured for 24 h. After culturing for 24 h, cells were treated with FITC-dextran-labeled DOX-BSA-rGO nanosheets at various concentrations (30–100 μg/mL) for 3 h. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and were stained by using Hank’s Balanced Salt Solution (HBSS) containing Alexa fluor 594-conjugated wheat germ agglutinin (Invitrogen, CA, USA) for 30 min. The cells were then permeabilized with 1% triton X-100 in PBS for 20 min at room temperature and were subsequently counterstained with DAPI (Invitrogen, CA, USA) for 5 min. The cellular uptake was measured by a confocal laser-scanning microscope (LSM 710, Carl Zeiss, Germany).

**RESULTS AND DISCUSSION**

**Synthesis of DOX-BSA-rGO Nanosheets.** We synthesized DOX-BSA-rGO nanosheets (Figure 1). Briefly, the graphite oxide was exfoliated to make graphene oxide, and BSA was used for reduction of graphene oxide at pH 12 and 60 °C. Doxorubicin was finally conjugated with BSA-rGO nanosheets.

**Cell Viability Assay.** The viability of the U87MG cells cultured with 30 μg/mL BSA-rGO and DOX-BSA-rGO nanosheets was evaluated using a live/dead assay kit (Invitrogen, CA, USA). To investigate NIR irradiation-mediated viability, the cells were incubated for 4 h and were subsequently irradiated by a NIR light for 3 min at a power of 5.5 W/cm². Cells were incubated for 40 min at room temperature with live/dead assay solution containing 2 μM calcein AM (green) and 4 μM ethidium homodimer-1 (red). Fluorescent images were obtained from a confocal laser-scanning microscope. Quantitative analysis of cell viability was determined by CCK-8 assay.

**Statistical Analysis.** We performed at least three different experiments, and the data indicated the mean ± standard deviation. **p < 0.01** was considered to be statistically significant.
represented that the total ratio was increased due to the C−C and C−N peaks of DOX. To determine whether GO nanosheets were reduced and functionalized, the zeta potential and UV−visible spectrophotometer analysis were employed (Figure 3). We observed the effect of pH on surface charge of BSA protein, GO, and BSA-rGO nanosheet (Figure 3A). Interestingly, the surface charge of the BSA protein was significantly regulated by pH, indicating that the zeta potential of BSA-rGO nanosheets was 28.9 mV at pH 2 and −40.3 mV at pH 7. This amphiphilic property of BSA proteins could determine BSA functionality. The surface charges of BSA protein and BSA-rGO nanosheets were similar, because BSA proteins conjugated with rGO nanosheets. By contrast, the surface charge of GO nanosheets was negative regardless of pH, because oxygen functional groups existed at the edge and basal plane. For the UV−visible spectrophotometer, we observed the peak of GO nanosheets at 230 nm (marked in a dotted-line of Figure 3B) and 300 nm due to π → π* transition from the aromatic C=C bond and n → π* transition from the C=O bond (Figure 3B), as previously described.23 We also observed that the sp2 bond-mediated peak was shifted at 266 nm (marked as a dotted-line in Figure 3B) in the BSA-rGO nanosheet, and the peak at 300 nm was diminished due to deoxygenation. The color of solution was changed from
yellowish brown to black after the BSA-mediated reduction process. Furthermore, we observed the peak of DOX-BSA-rGO nanosheets at 490–510 nm due to DOX absorption.

**Photothermal Effect.** We observed that UV absorption of BSA-rGO or DOX-BSA-rGO nanosheets was increased at 800 nm (Figure 3B), because the π-network structure was restored to increase UV absorption. It suggested that BSA-rGO or DOX-BSA-rGO nanosheets could show photothermal effect during NIR radiation at 808 nm as previously described. To investigate whether BSA-rGO nanosheets increased the temperature caused by energy transfer from π-network restoration-mediated light absorption to heat, we applied NIR laser (5.5 W/cm², 808 nm) to BSA-rGO nanosheets for 300 s and analyzed the NIR-mediated temperature every 60 s. We observed that the temperature was proportional to concentrations of BSA-rGO nanosheets (Figure 4A). We also investigated the effect of NIR irradiation time on temperature of BSA-rGO nanosheets (Figure 4B). It represented that the temperature of BSA-rGO and DOX-BSA-rGO nanosheets was increased with irradiation time, suggesting that a BSA-rGO or DOX-BSA-rGO nanosheet would be a great candidate as a photothermal agent. By contrast, the temperature change of GO nanosheets was negligible. Although DOX-rGO nanosheets were slightly aggregated with increasing temperature, they were stable.

**NIR-Mediated DOX Release and Cell Viability.** We loaded DOX into amphiphilic BSA-rGO nanosheets. The loading efficiency of DOX was approximately 77%. We analyzed DOX release behavior from DOX-BSA-rGO nanosheets in a temporal manner (Figure 5). It showed that DOX at pH 5 (23% release at 6 h) was largely released from DOX-BSA-rGO nanosheets as compared to pH 7 (7% release at 6 h), because DOX was protonated to become hydrophilic at an acidic environment. The interaction between DOX and BSA protein enabled the control of DOX release from DOX-BSA-rGO nanosheets as previously described. We also investigated the effect of NIR irradiation on DOX release, showing that DOX was largely released from DOX-BSA-rGO nanosheets when NIR irradiation was applied (marked by black arrows of Figure 5). The heat caused from NIR irradiation broke the binding between DOX and BSA protein, resulting in a large release of DOX (50% at 6 h). This result demonstrated that NIR irradiation approximately twice enhanced DOX release from DOX-BSA-rGO nanosheets. We also investigated the cellular uptake using FITC-loaded DOX-BSA-rGO nanosheets (Figure 6A–C). The confocal microscopy images represented that the cellular uptake was significantly affected by concentrations of FITC-loaded DOX-BSA-rGO nanosheets, indicating that U87MG cells treated with 100 μg/mL FITC-loaded DOX-BSA-rGO nanosheets showed higher uptake efficiency. Furthermore, we observed the effect of DOX-BSA-rGO nanosheets on chemotherapy (Figure 6D). CCK-8 cell viability assay demonstrated that the viability of U87MG cells treated with DOX-BSA-rGO nanosheets (5–100 μg/mL concentration) was decreased with incubation time regardless of DOX concentrations. This was probably due to DOX released from DOX-BSA-rGO nanosheets within cell membrane.

**Chemo-photothermal Therapy of DOX-BSA-rGO Nanosheet.** We investigated the effect of DOX-BSA-rGO nanosheets on NIR-mediated chemo-photothermal therapy (Figure 7). U87MG cells were treated with 30 μg/mL BSA-
rGO nanosheets and were subsequently exposed to NIR irradiation (5.5 W/cm², 808 nm) every 3 min after 4 h incubation. The confocal microscopy images showed that NIR irradiation did not have any damage to cells (Figure 7A,D). By contrast, most cells treated with both BSA-rGO nanosheet and NIR irradiation were dead compared to neighboring cells treated without NIR irradiation, showing that the BSA-rGO nanosheet was a nontoxic material (Figure 7B,E). Interestingly, we observed that U87MG cells treated with both 30 μg/mL DOX-BSA-rGO nanosheets and NIR irradiation were largely dead (Figure 7C,F). This was probably due to chemo-photothermal therapy, showing that NIR irradiation stimulated a large release of DOX from DOX-BSA-rGO nanosheets. CCK-8 cell viability analysis demonstrated that the viability of the cells treated with both DOX-BSA-rGO nanosheets and NIR irradiation was extremely low compared to the viability of the cells without any treatment (Figure 7G). This was probably due to NIR irradiation that could allow for DOX release to neighboring cells. We also demonstrated that the viability (21.8%) of brain tumors treated with BSA-rGO nanosheets was decreased by NIR irradiation. By contrast, the brain tumors treated with both DOX-BSA-rGO nanosheets and NIR irradiation showed the lowest cell viability (1.76%) due to chemo-photothermal therapy effect.

CONCLUSIONS

We synthesized functional DOX-BSA-rGO nanosheets. A NIR-mediated drug release study showed that DOX was largely released from DOX-BSA-rGO nanosheets when NIR irradiation was applied. The heat caused from NIR irradiation broke the binding between DOX and BSA protein, resulting in large release of DOX. This result demonstrated that NIR irradiation approximately twice enhanced DOX release from DOX-BSA-rGO nanosheets. We also demonstrated that the viability of brain tumor cells treated with both DOX-BSA-rGO nanosheet and NIR irradiation was extremely low due to chemo-photothermal therapy effect. Therefore, this DOX-loaded BSA-rGO nanosheet could be a powerful tool for chemo-photothermal therapy applications.

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the Research Program through the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (MSIP) of Korea (Grant Number 2015R1A2A1A15054236). This work was supported by the BioNano Health-Guard Research Center funded by the MSIP of Korea as the Global Frontier Project (Grant number H-GUARD_2014M3A6B2060503), Republic of Korea. This research was also supported by a grant from the Marine Biotechnology Program (Grant Number 20150581,
Development of technology for biohydrogen production using hyperthermophilic archaea, funded by the Ministry of Oceans and Fisheries, Korea.

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