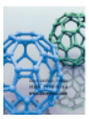
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SHORT REPORT

In vitro cytotoxicity of GO–DOx on FaDu squamous carcinoma cell lines

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Abstract: We have synthesized graphene oxide (GO) nanosheets using modified Hummer's method and conjugated it with doxorubicin (DOx), an anticancer drug. Drug release kinetics from GO–DOx conjugate indicated the drug release at acidic pH. MTT assay performed on FaDu hypopharyngeal cancer cell lines revealed that the GO–DOx nanoconjugate inhibited cell proliferation more efficiently compared with pure DOx. Preliminary results indicate the potential of designed GO–DOx drug conjugate for head and neck cancer.

Keywords: graphene oxide, doxorubicin, nanomedicine, head and neck cancer

Introduction

Cancer is a leading cause of death in developed and in developing countries. Among various deadly cancers, the incidence of head and neck cancer (HNC) in the world is on rise. Among HNCs, oral cavity cancer is the second most common in India and accounts for 86% of the world's oral cancer cases (National Institute of Public Health in February 2011). Modern modalities to treat HNC are radiation, chemotherapy, surgery, antibody-blocking therapy, or a combination of these therapies. In spite of technological developments, the available therapies for HNC suffer significant limitations. Recent advances in nanotechnology have led to multifunctional nanoparticle probes for molecular and cellular imaging, nanoparticle drugs for targeted therapy, and integrated nanodevices for early cancer detection and therapy. Nanotechnology offers a wealth of tools such as novel therapeutic devices, including drug- and gene-delivery vectors, photodynamic, photothermal, magnetothermal probes, and radiation enhancers for clinicians.

Graphene oxide (GO) is a chemically converted derivative of graphite, consisting of oxygen functional groups in the basal plane giving rise to multifunctionalities. It posses unique physical and chemical properties that enable its potential biomedical applications (biosensors, drug delivery, and cancer therapy). GO is considered a good scaffold for high drug loading through π–π stacking with functional groups such as –OH, –COOH, and –CHO and for its two-dimensional structure. Furthermore, GO has been reported to exhibit high photothermal effects under the irradiation of low-power near-field infrared (NIR) radiations.[1]

Capitalizing on the unique properties of GO, we envisioned to design a multifunctional nanomedicine platform capable of dual chemo and photothermal therapy of cancer. Doxorubicin (DOx) is a well-known chemotherapy drug, which is used in the variety of cancer treatments such as breast, ovarian, bladder, lung, thyroid, stomach cancers, and advance esophageal squamous cell carcinoma.[2]

In this study, we conjugated DOx with GO nanosheets and studied their in vitro cytotoxicity on the FaDu cell line. Furthermore, systematic in vitro studies for toxicity

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evaluation of GO–DOx were performed on the hypopharyngeal cancer cell line (FaDu cells [ATCC, Manassas, VA, USA]). The cell morphology, viability, mortality, and membrane integrity of the FaDu cells were determined after 24 h of the treatment of cells with GO–DOx. MTT assay revealed that the GO–DOx nanoconjugate inhibited cell proliferation more efficiently compared with pure DOx. The intracellular localization and uptake of GO–DOx have been visualized by transmission electron microscopy and confocal microscopy. Preliminary results indicate the potential of designed GO-DOx drug conjugate for HNC.

Materials and methods

All the reagents used in this study were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Synthesis

Graphene oxide

GO sheets were synthesized using the modified Hummers method.[3] Highly exfoliated graphite powder (Bay Carbon Inc, Bay City, MI, USA) (0.5 g) was taken as a precursor and added to 23 mL of concentrated H_2SO_4, in a conical flask under ice-bath. After complete mixing, $NaNO_3$ (0.5 g) and $KMnO_4$ (1.5 g) were added to the mixture. This resulted in an exothermic reaction. The resulted solution was heated to 35°C and stirred for 1 h. Hundred milliliters of Millipore water was added to this solution and was continually stirred for another 2 h at 35°C. Finally, the reaction was quenched with the addition of 27% H_2O_2 (1 mL). The resulting mixture was then centrifuged at low rpm for 10 min for the removal of unreacted graphite. The supernatant consisting of GO sheets was collected for further washing and consequent centrifuging at 12,000 rpm. The sample was washed with water until the pH of the solution reached 7 and dried to collect the GO nanosheets.

GO–DOx conjugate

Well-characterized GO is used for drug loading. DOx 0.175 mg/mL is mixed with 1 mg/mL of aqueous solution of GO to form a GO–DOx conjugate. The mixture was kept stirring for overnight under dark environment.[4] The mixture was then centrifuged at 12,000 rpm to collect the sample pelleted at the bottom of the centrifuge tube. Furthermore, this pellet was re-dispersed and dried using the freeze-drying method. This drug-loaded conjugate was stored at 4°C (Figure 1).

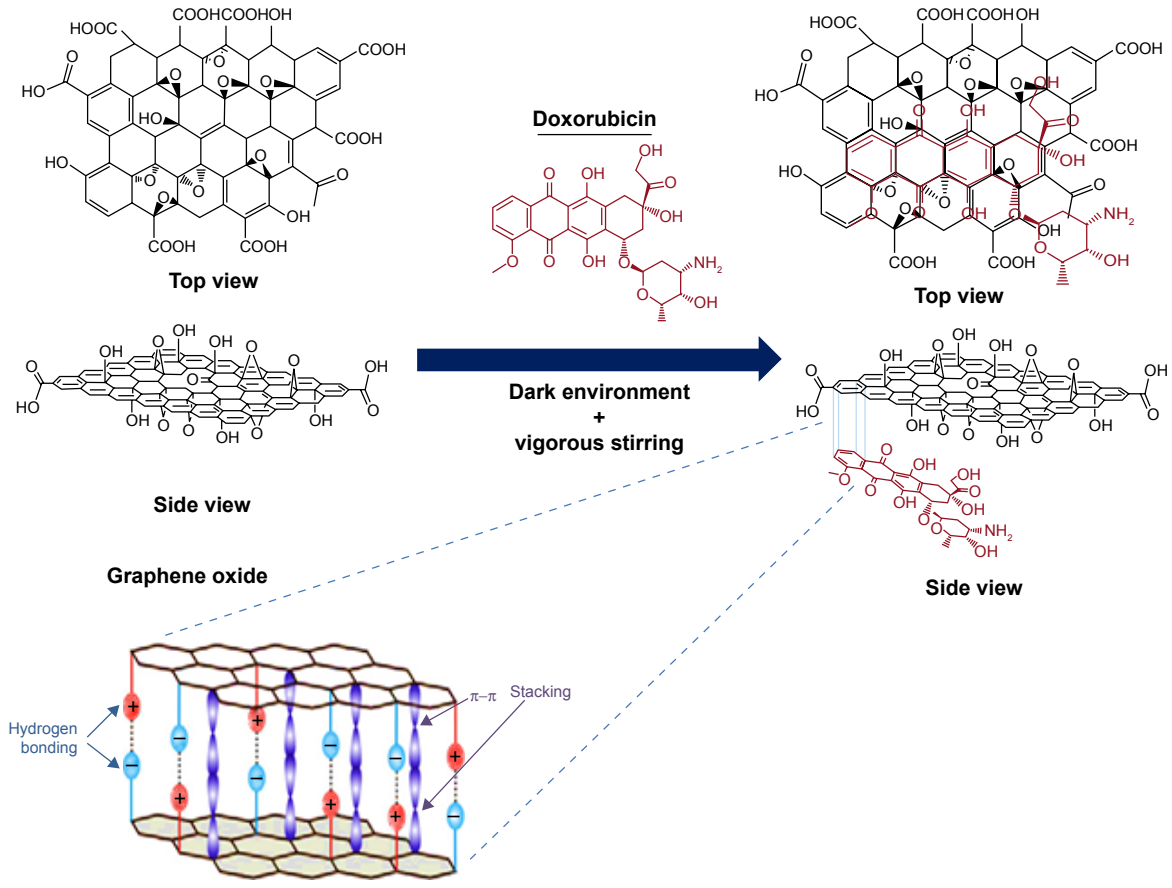


Figure 1 Schematic representation for a graphene oxide–doxorubicin conjugation.

Results and discussion

The synthesized GO consists of a graphitic domain with sp^3/sp^2 carbon having a large π-conjugated structure and –OH, –COOH, and –CHO as major functional groups. DOx is an anthracycline antibiotic with quinone ring that facilitate its noncovalent conjugation with GO through π–π stacking and other hydrophobic interactions. Furthermore, the functional groups on GO can also form hydrogen bonding with –OH and $–NH_2$ groups of DOx. The binding of DOx with GO is confirmed by Fourier transform infrared spectroscopy and photoluminescence spectroscopy (Figure 2). The presence of infrared (IR) bands at 3,005 and 2,915 cm^{-1} and quenching of luminescence of GO and DOx clearly indicate the GO–DOx conjugation.[5] The quenching in luminescence is attributed to π–π stacking of DOx and GO.

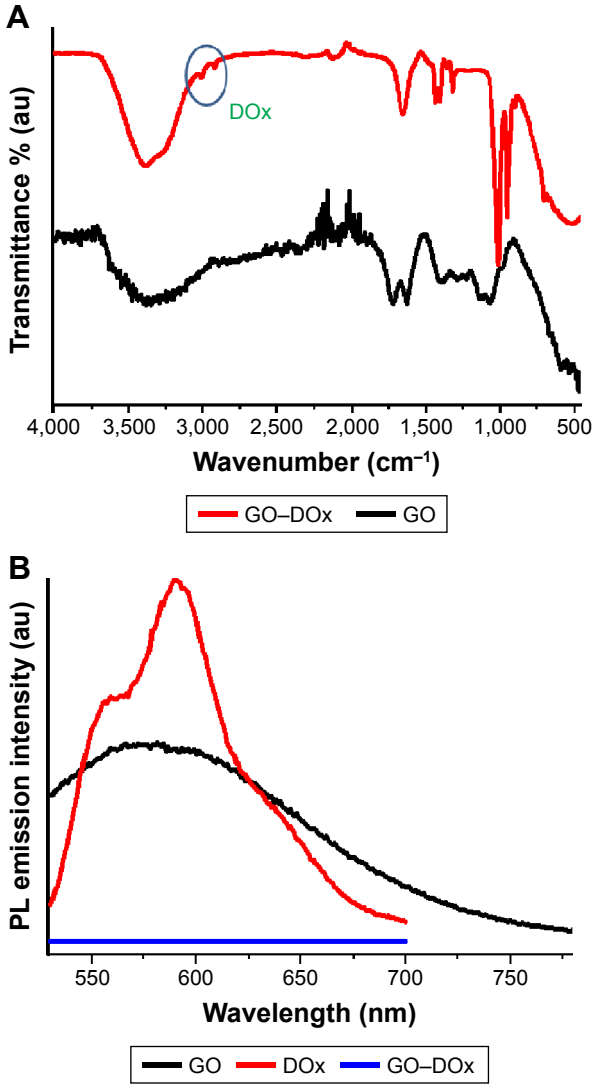


Figure 2 (**A**) FT-IR spectra showing both GO and GO–DOx conjugate and (**B**) photoluminescence emission spectra of GO, DOx, and GO–DOx conjugate.
Abbreviations: au, arbitrary unit; DOx, doxorubicin; FT-IR, Fourier transform infrared spectroscopy; GO, graphene oxide; PL, photoluminescence.

The percent drug (DOx) loading efficiency onto GO nanosheets and drug release kinetics have been investigated using UV–visible spectroscopy. The characteristic peak of DOx, λ_{max} at ~254 and ~480 nm (Figure 3) has been consumed after conjugation with GO, demonstrated that the drug has been attached to the GO platform via π–π stacking and hydrophobic interactions.

The percent loading calculated by comparing the absorbance peak intensity[6] of the samples confirms that ~90% drug is loaded to the sample.

$$\text{The drug loading efficiency, } Dl\,(\%) = \frac{\text{DOx loaded on GO nanosheet}}{\text{Initially added DOx}} \times 100$$

$$Dl\% = \frac{\text{DOx initial} - \text{DOx in supernatant}}{\text{Initially added DOx}} \times 100$$

Considering absorbance peak intensity at 254 nm,

$$Dl\% = \frac{0.43936 - 0.04335}{0.43936} \times 100$$

$$Dl\% = 90.1334\%$$

The drug release profiles from GO sheets were evaluated on the basis of the results of drug adsorption in the buffer of different pH ranging from 3 to 7 measured at specified time period with a UV–Vis spectroscopy (Figure 4). Briefly, 1 mg of the drug conjugate was dissolved in each buffer solutions of different pH followed by centrifugation at 12,000 rpm. An aliquot from the supernatant containing the drug release

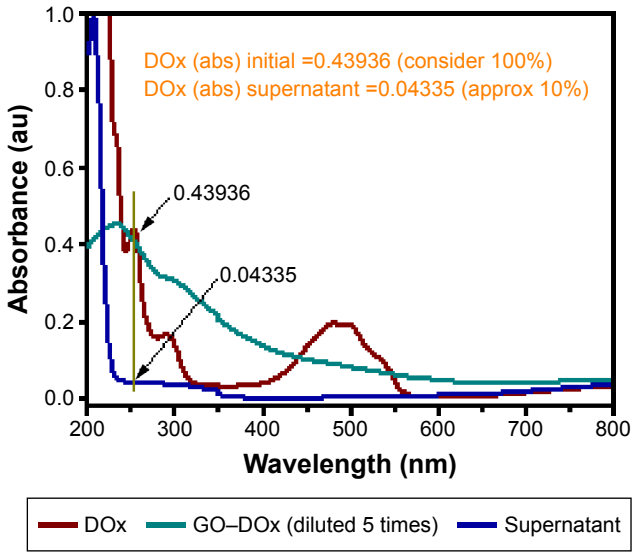


Figure 3 Percent drug (DOx) loaded onto a GO–DOx conjugate.
Abbreviations: au, arbitrary unit; DOx, doxorubicin; GO, graphene oxide.

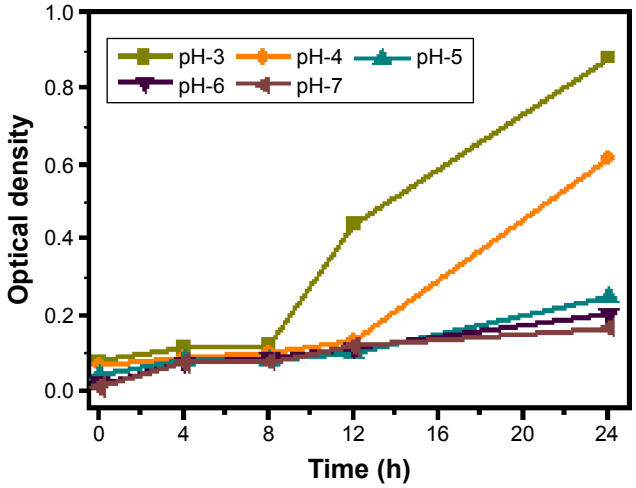


Figure 4 Drug release kinetics from a graphene oxide–doxorubicin conjugate.

medium was withdrawn at different time intervals (4, 8, 12, and 24 h). The amount of DOx in the buffer solution was quantified by monitoring the optical density at a fixed wavelength of 254 nm for drug release kinetics. Almost 88% of the cumulative drug release was observed from the nanosheets in 24 h at acidic pH.

To estimate the efficacy of the GO–DOx conjugate as a potential anticancer drug, we carried out the standard MTT assay with the FaDu cell lines from squamous cell carcinoma of pharynx. Figure 5 shows comparative cytotoxicity data for GO, DOx, and GO–DOx nanoconjugate in the FaDu cell line. To exclude the possible influence of GO nanosheets on cell viability, various concentrations of GO nanosheets (1, 2.5, 5, 10, 25, 50, 75, and 100 µg/mL) were incubated for 24 h with the FaDu cells and then cell viability was assessed. Within the tested concentration range of GO nanosheets, even as high as 100 µg/mL exhibited ~30% cell morbidity indicating less significant cytotoxic nature of GO nanosheets. The inhibitory concentration 50% of GO–DOx was found to be 14.39 µg/mL compared with 31.01 µg/mL for free DOx. The GO–DOx nanoconjugate exhibited the higher cytotoxicity compared with free DOx. The enhanced cell morbidity by the GO–DOx conjugate could be attributed to either the synergistic cytotoxic effect of GO and DOx or the reduced efflux of the drug in the nanoconjugate form compared with the free DOx.[2]

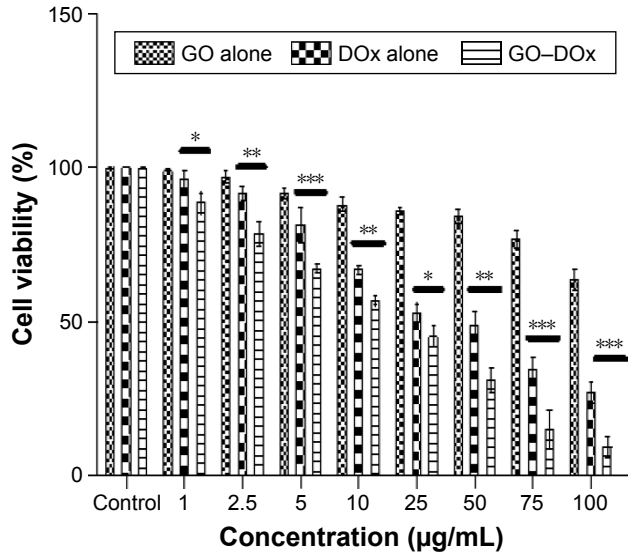


Figure 5 Cytotoxicity of DOx and GO–DOx on the FaDu cell line. *$P<0.05$; **$P<0.01$; ***$P<0.001$, significant when compared to respective controls.
Abbreviations: DOx, doxorubicin; GO, graphene oxide.

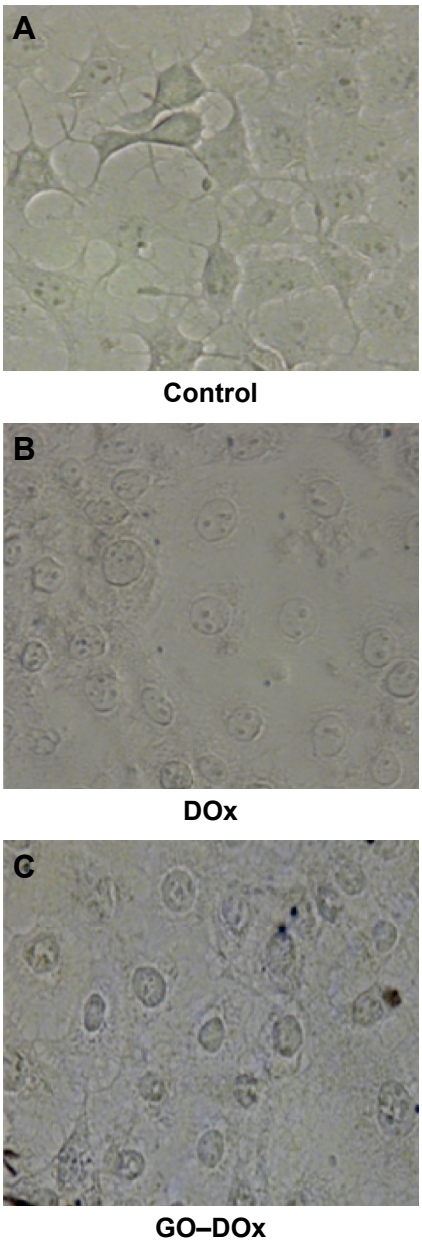


Figure 6 Morphology of FaDu cells (**A**) untreated and treated with (**B**) DOx and (**C**) GO–DOx. Phase contrast microscopy, 40× objective.
Abbreviations: DOx, doxorubicin; GO, graphene oxide.

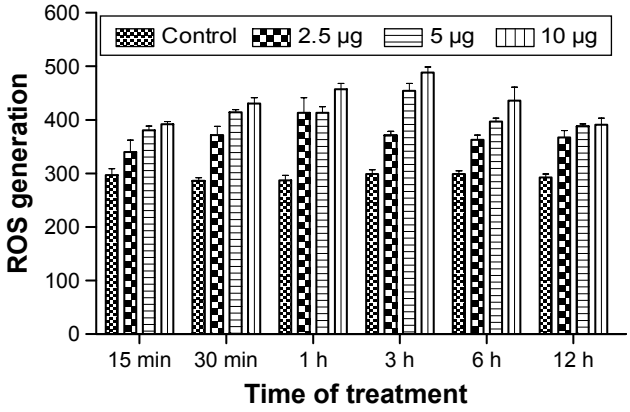


Figure 7 Reactive oxygen species (ROS) generation.

Intracellular and plasma membrane structural modifications have been widely recognized as crucial factors involved in cell injury and death. Figure 6 shows the effect of DOx and GO–DOx on the appearance of cells viewed by phase contrast microscopy. GO–DOx-treated cells showed significant morphological changes, including cell shrinkage and reduction of cellular and nuclear volume compared with untreated control cells.

Furthermore, this conjugate was studied for the reactive oxygen species (ROS) measurement (Figure 7). ROS generation implicates to be as a possible mechanism for the induction of apoptosis by various anticancer agents. Induction of oxidative stress is considered one of the principal mechanisms underlying nanomaterial. GO + DOx induced intracellular ROS formation in a dose- and time-dependent manner. After 3 h of exposure to 10 µg/mL of GO + DOx, figure shows ~twofold increase in the ROS generation, which was observed over untreated control.

Conclusion

We have successfully synthesized highly exfoliated few layered GO nanosheets by a lyophilization-assisted chemical exfoliation method. Photoluminescence emission revealed the attachment of DOx with GO nanosheets. Release kinetics showed 88% DOx release after 24 h from the GO–DOx conjugate. Compared to free DOx, the GO–DOx conjugate was found to be more effective in the FaDu cell line. The results are encouraging and pave the way for more detailed studies.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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