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Direct noncovalent conjugation of folic acid on reduced graphene oxide as anticancer drug carrier

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ABSTRACT

Conjugate of reduced graphene oxide with folic acid (rGO/FA) was prepared through a completely noncovalent functionalization method. rGO/FA conjugate was soluble in physiological media with high dispersion stability and enabled the accumulation of hydrophobic doxorubicin (DOX) and CdSe quantum dot (QD) as an anticancer drug and a fluorescent tag, respectively. rGO/FA conjugate loaded with DOX showed specific targeting to MDA-MB 231 cells, excellent drug-release efficiency and cytotoxicity *in vitro*. Considering the simplicity and extendibility of noncovalent functionalization methods, rGO/FA conjugate can be widely utilized for the designing of new graphene-based nanocarriers.

Introduction

Carbon nanomaterials such as graphene oxide (GO) and reduced graphene oxide (rGO) with two-dimensional (2D) structural feature and high surface area have been intensively surveyed for the design of new carbon-based nanocarriers or biomedicines [1–4]. For the practical application of graphene-based materials for the drug delivery and cancer treatment, high dispersion stability of graphene-based materials has to be provided through bio-functionalization process [5–8]. To endow solubility in physiological media, covalent functionalization methods has been widely utilized for the preparation of bio-functionalized GO or rGO. For example, covalent conjugation of folic acid (FA) with either GO or rGO has been attempted by amidation reactions with either carboxylic acid-functionalized GO or amino-functionalized rGO, where graphene/FA conjugates show high specificity and affinity for overexpressed folate receptors on the surface of several cancer cells [9,10]. While plentiful organic reactions can be widely utilized for the functionalization of GO or rGO through covalent functionalization methods [11–13], non-covalent functionalization methods [14] have several additional advantages such as minimizing chemical reaction and purification steps. Therefore, it is highly interesting to investigate noncovalent bio-functionalization methods for graphene-based materials. In typical electrical and optoelectrical applications of rGO, the approach of π–π interaction is the most frequently utilized method because π-rich molecules spontaneously conjugate on π-rich rGO plate [15–17]. Photoluminescence (PL) quenching behavior of incoming fluorescent molecules after conjugation on rGO plate can be used as a validation tool for the successful π–π interaction [18,19]. However, the approach of π–π interaction has certain limitations because most biomolecules do not show fluorescence and hence, the incorporation of fluorescent tags is required [20]. Hence, another widely applicable method must be unveiled for the functionalization of graphene-based materials with various nonfluorescent biomolecules.

Recently, several experimental studies have revealed that several aliphatic polymers, such as polyacrylic acid, poly(*N*-isopropyl acrylamide) [21], polyacrylamide [22], poly(*N*-vinyl pyrrolidone) [23], poly(4-vinyl pyridine) [24], polyethylene glycol [25], pluronic [26,27], aliphatic dendrimer [28], and cellulose derivatives, such as hydroxypropyl cellulose [29], carboxymethyl cellulose [30], and lignin [31] can formulate stable assemblies (or conjugates) with rGO in aqueous media through noncovalent

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interaction between them. This noncovalent approach has been successfully utilized for the formulation of rGO/chitosan assembly showing antibacterial activity [32] and rGO/heparin conjugate demonstrating blood compatibility [33]. These experimental studies have been supported by molecular simulation studies that demonstrate the spontaneous and noncovalent binding of biomolecules such as cholesterol and L-leucine on graphene plate through van der Waals interaction [34,35].

All these previous experimental and theoretical works on noncovalent functionalization methods for graphene-based materials through van der Waals interaction rather than π–π interaction prompt us to investigate the designing of graphene-based biomedicines through van der Waals interaction between π-poor bioactive molecules (or polymers) and π-rich rGO. In this study, noncovalent conjugation of FA on rGO is attempted through van der Waals interaction, where FA plays a role of selective targeting moiety toward MBA-MB 231 human breast cancer cells having folate receptor surface expression. Meanwhile, most of the previous studies have employed covalent conjugation of FA either on GO or rGO [36,37]. Further loading of anticancer drugs such as doxorubicin hydrochloride (DOX) and fluorescence tags such as CdSe quantum dot (QD) on rGO are also attempted through π–π interaction and van der Waals interaction, respectively (Fig. 1). Therefore, graphene-based nanocarrier for targeted delivery of anticancer drug on cancer cell is firstly demonstrated through completely noncovalent approaches in this study.

Experimental

Materials and instruments

FA, hydrazine monohydrate, DOX, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), and CdSe QD (maximum emission at 543 nm) were purchased from Sigma-Aldrich (Korea) and used without further purification. Penicillin–Streptomycin, fetal bovine serum (FBS), and RPMI-1640 medium were purchased from Gibco BRL (Carlsbad, CA). GO was synthesized from natural graphite by modified Hummers' method and aqueous GO solution was freshly prepared just before use [38]. UV–vis spectra were obtained from UV–vis spectrometer of Hewlett-Packard. PL spectra were obtained from L550B luminescence spectrometer of PerkinElmer. Dynamic light scattering (DLS) data were obtained from particle size analyzer (ELS-Z) of Otsuka Electronics Corporation (South Korea) with temperature controller. Fourier transform infrared (FT-IR) spectra were obtained from IR100/IR200 spectrometer of Thermoelectron Corporation. Raman analysis was done from LabRAM high resolution UV/VIS/NIR dispersive Raman microscope of Horiba John Yvon. Atomic force microscopy (AFM) images were obtained from XE-100 atomic force microscope of PSIA. The binding energy is accurate within ± 0.1 eV. Confocal laser scanning microscopy images were obtained from LSM510 confocal laser scanning microscope of Carl Zeiss Corporation equipped with a 543 nm He–Ne laser.

rGO/FA conjugate

1 mg of FA was dissolved into 10 ml of deionized water having 4 drops of hydrazine monohydrate and simply mixed with aqueous GO solution (1 mg in 10 ml of deionized water), resulting in solution of GO/FA mixture. Then, chemical reduction of GO/FA mixture with 4 drops of hydrazine monohydrate was performed at 80 °C for 4, 8, or 12 h. To remove excess hydrazine and any nonconjugated FA from rGO/FA conjugate, ultracentrifugation was performed at 12,000 rpm, forming precipitation of rGO/FA conjugate, which is readily dispersible again in aqueous media. Further dialysis using molecular weight cut-off (3500 Da, MWCO) membrane produced optically clear solution of rGO/FA conjugate with dispersion stability more than 6 months.

DOX loading and release behaviors of rGO/FA conjugate

A total of 0.5 mg of DOX in 1 ml of dimethyl sulfoxide (DMSO) was added to 10 ml of rGO/FA conjugate solution (0.05 mg/ml) in phosphate buffered saline (PBS) at pH 7.4 under vigorous stirring for 24 h under dark condition. Unbound DOX and DMSO were removed by ultracentrifugation at 12,000 rpm. The DOX concentration of upper layer after ultracentrifugation was calculated from a calibration curve, which is obtained by measuring the optical absorbance at 480 nm in UV–vis spectra of several DOX solutions with known concentrations. Subtraction of the DOX concentration of upper layer from the original concentration of DOX added into the solution of rGO/FA conjugate provides DOX-loading efficiency of rGO/FA conjugate. The release behavior of DOX from rGO/FA conjugate was evaluated by dialysis. A total of 20 ml of DOX-loaded rGO/FA conjugate solution is sealed in MWCO (3500 Da) membrane and immersed into PBS at pH of either 7.4 or 5.0 at 37 °C under incubator shaker (horizontal shaking at 75 rpm). PBS was periodically replaced with fresh one. Amount of released DOX from DOX-loaded rGO/FA conjugate was obtained by measuring optical absorption at 480 nm in UV–vis spectrum of solution inside of MWCO membrane.

In vitro *cytotoxicity measurement*

Madin–Darby canine kidney epithelial cells (MDCK cell line) and human breast cancer cells (MDA-MB 231) were cultured in RPMI medium supplemented with 10% FBS, 100 U/L penicillin, and 100 μg/ml streptomycin. During incubation of the cells for 3 days in a humidified 5% CO_2 containing balanced air incubator at 37 °C, medium was replaced for several times. The cytotoxicity of above cells was measured using MTT assay method. 200 μl of the

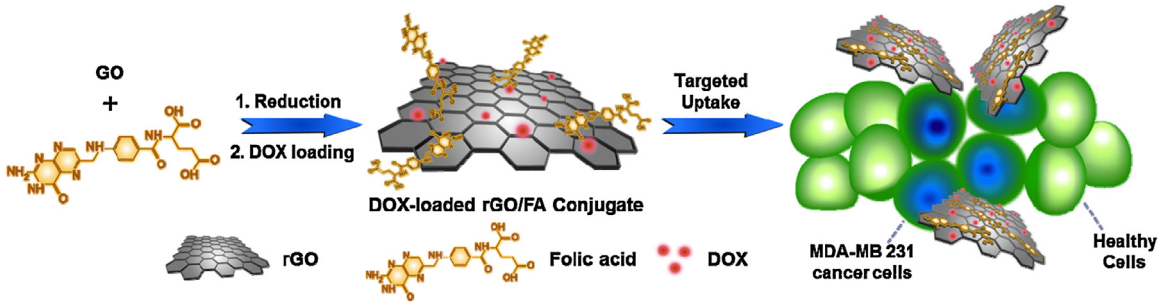

Fig. 1. Schematic illustration for targeted uptake of DOX-loaded rGO/FA conjugate into MDA-MB 231 cancer cell.