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Self-Assembled Graphene–Dextran Nanohybrid for Killing Drug-Resistant Cancer Cells

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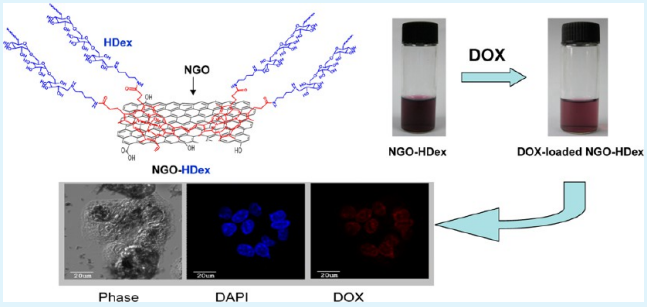
Supporting Information

ABSTRACT: A nanohybrid based on nanoscale graphene oxide (NGO) and dextran has been designed and employed for effectively killing drug-resistant MCF-7/ADR cells. This graphene-based nanohybrid was readily prepared through $\pi-\pi$ interaction of NGO and hematin-terminated dextran (HDex), being denoted as NGO–HDex. It revealed an improved stability in physiological conditions as compared to native NGO. Besides, NGO–HDex could efficiently load doxorubicin (DOX), an anticancer drug, with drug loading capacity of 3.4 mg/mg NGO and liberate the drug with a pH-dependent profile. Cell viability assay indicated that the NGO–HDex displayed lower cytotoxicity against MCF-7/ADR cells as compared to native NGO. DOX-loaded NGO–HDex, however, revealed more efficient killing effect in the cells than free DOX because the nanohybrid caused a higher amount of DOX accumulated in the cells. The results of this study highlight that the NGO–HDex has high potential for killing drug-resistant cancer cells.



KEYWORDS: *graphene oxide, doxorubicin, dextran, hematin, drug resistance*

1. INTRODUCTION

Traditional chemotherapy drugs have been widely employed for effective clinical cancer therapy. However, their anticancer efficacy is often compromised by the development of drug resistance in cancer cells because of the activity of multidrug resistance (MDR) transporters.[1] It has been found that nanoscale drug carriers are capable of circumventing this activity, offering an efficient intracellular drug delivery and thus causing improved anticancer efficacy.[2] For that reason, a few nanocarriers such as nanomicelles and nanoparticles have been widely invesigated for anticancer drug delivery.[2,3] In recent years, nanosized graphene oxide (NGO) as a new drug delivery system has received much attention.[4−10] Compared with traditional drug delivery carriers like nanomicelles, NGO takes advantages of facile loading of hydrophobic drugs and high drug loading capacity.[8,11] For example, Chen et al. prepared a NGO–DOX nanohybrid with a high doxorubicin (DOX) loading capacity of 2.35 mg/mg NGO.[11] Further, Wu et al. found that DOX-loaded NGO system was effective to enhance the accumulation of DOX in drug-resistant MCF-7/ADR cells.[12] More recently, Zhi et al. indicated that positively charged NGO-nanocomposites were capable of mediating the codelivery of DOX and miRNA to overcome multidrug resistance of MCF-7/ADR cells.[13] However, native NGO usually has poor collodial stability and is prone to aggregration in physiological conditions, due to its electrostatic or nonspecific interactions with ions/proteins.[7,14,15] Further studies indicate that NGO can cause adverse oxidative stress and dysfunction in the cells in vitro and that NGO–protein aggregates also induce severe damage in pulmonary capillary in vivo.[16,17] Thus, further modification of NGO is higly required to improve the stability and biocompatibility of NGO.

There have been different approaches to obtain functionalized NGO with colloidal stability in the presence of salts and serum. One of the most used approaches is chemical conjugation of NGO with water-soluable biomaterials. For example, Dai et al. prepared poly(ethylene glycol) (PEG)-modified NGO using EDC/NHS conjugation chemistry.[8,18] By the same method, Liu et al. coupled NGO with dextran to yield dextran–NGO conjugates.[19] This covalent method can offer functionlized NGO systems that have collodial stability in physiological conditions and also low cytotoxicity as compared to native NGO. As an alternative method, nonconvalent modification of NGO is more facile to generate NGO hybrid via hydrophobic interactions or $\pi-\pi$ stacking of NGO with water-soluble amphiphilic polymers or aromatic molecules. For example, Dong et al. fabricated pluronic F127/NGO nanohydrid through hydrophobic interactions of NGO and pluronic F127.[5] Similar approach was also employed by Fan et al., who functionalized NGO with poly(N-isopropylacrylamide) (PNIPAAm) grafted dextran.[20] Another work from Shi et al. showed that water-soluble graphene can be easily prepared through

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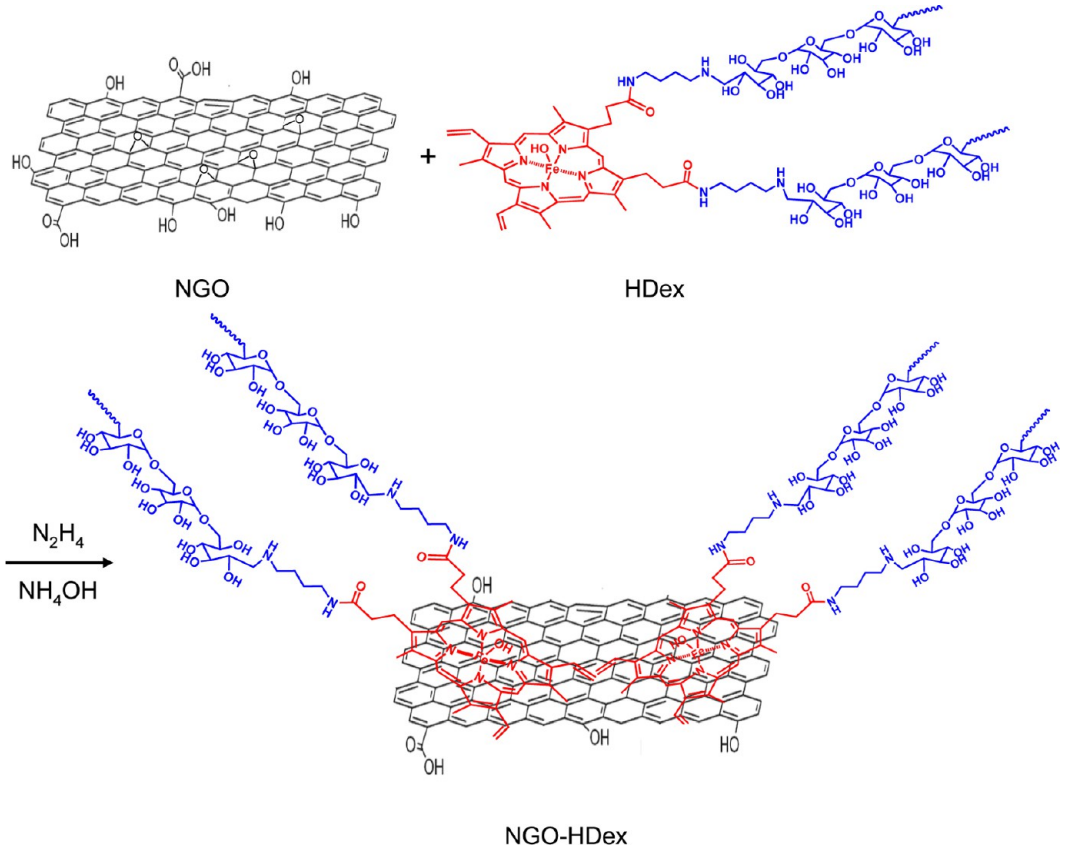

Figure 1. Synthesis of NGO−HDex hybrids.

noncovalent $\pi-\pi$ stacking of graphene and pyrenebutyrate.[21] This nonconvalent method was also used to decorate carbon nanotubes[22,23] and fullerenols.[24,25]

The purpose of this study is to generate a NGO-based carrier with collodial stability for the delivery of DOX in drug-resistant MCF-7/ADR cells. In this system, dextran and hematin were employed to functionalize NGO. Dextran is a biocompatible natural polysaccharide with excellent water solubility and often used to improve the stability of carbon nanomaterials.[19,20,26] Hematin is a Fe(III) compound from decomposition of hemoglobin and considered to be biocompatible since it has already been used clinically to treat porphyric attacks.[27] This nanocarrier was obtained by one-pot reduction of NGO in the presence of hematin-dextran conjugate (HDex), as illustrated in Figure 1. It is thought that NGO can self-assemble with HDex through $\pi-\pi$ stacking of NGO and hematin residue in the HDex. Besides, water-soluble dextran makes the NGO−HDex possess an improved stability in physiological conditions. Further, DOX is loaded in NGO−HDex for killing drug-resistant MCF-7/ADR cells. Herein, DOX loading capacity of NGO−HDex and the drug release behavior were examined. The cytotoxicity of NGO−HDex and DOX-loaded NGO−HDex against MCF-7/ADR cells was evaluated in vitro.

2. MATERIALS AND METHODS

2.1. Materials. Dextran (MW = 5000) was purchased from Seebio Co. Ltd. (Shanghai, China). N-Boc-1,4-diaminobutane sodium cyanoborohydride ($NaBH_3CN$), hematin, deuterium water (D_2O), deuterated DMSO (DMSO-d_6), and hydrazine solution (80% aq.) were purchased from Adamas-beta Inc. (Shanghai, China). N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from GLS Biochem. Ltd. (Shanghai,China). Sulfuric acid (H_2SO_4, 98%), phosphorus pentoxide (P_2O_5), hydrogen chlororide (HCl, 37%), sodium chlororide (NaCl), potassium persulfat ($K_2S_2O_8$), potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2, 30% v/v), and natural graphite powder ($\lesssim$30 μm, with purity >99.85 wt %), all commonly used chemicals and solvents unless otherwise mentioned, were purchased from Sinopharm Chemical Reagent co., Ltd., China. RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin, trypsin were obtained from Gibco Invitrogen Corp.

2.2. Synthesis of NGO. Nanographene oxide (denoted as NGO) was prepared by a modified Hummers method.[28,29] The graphite (3.0 g) was mixed with $K_2S_2O_8$ (2.5 g) and P_2O_5 (2.5 g), to which 12 mL of H_2SO_4 was added. After being stirred at 80 °C for 4.5 h, the suspension cooled to room temperature and was poured into 500 mL of deionized water. The residues were then filtered and washed with deionized water to remove excess acid. After drying, the residues were added into 120 mL of H_2SO_4 and $KMnO_4$ (15 g) was added slowly during vigorous stirring. The temperature of the solution was kept below 10 °C. The reaction was then performed at 35 °C for 2 h. With the addition of 250 mL of deionized water, the mixture was stirred for another 2 h. To the mixture was added 500 mL of deionized water and 20 mL of H_2O_2 (30% v/v). After filtration, the residues were washed by HCl (10% v/v) and then deionized water. The residues were dispersed in water and sonicated (180 W) for 60 min to generate homogeneous solution. The as-prepared NGO was then sonicated (180 W) for 60 min. Finally, the mixture was purified by dialysis against deionized water (MWCO 25000). The resulting products were filtered with a nylon membrane (0.22 μm) and stored at 4 °C.

2.3. Synthesis of Aminated Dextran. Aminated dextran (denoted as Dex-NH_2) was synthesized by first reacting with N-Boc-1,4-diaminobutane and sodium cyanoborohydride, and then deprotection using TFA. Typically, N-Boc-1,4-diaminobutane (0.8 g) and dextran (2 g) were dissolved in deionized water (10 mL). After stirred for 2 h under nitrogen protection, $NaBH_3CN$ (0.8 g) was added in portions and the mixture was allowed to react at room temperature for

3 d. After neutralized with 2 M HCl solution to pH 7, the solution was ultrafiltrated against water (MWCO 1000). Boc-aminated dextran (denoted as Dex−C_4−Boc) was obtained as white foam after freeze-drying. Yield: 1.7 g (85%). ^{1}H NMR (D_2O): δ 1.3−1.4 (Boc, −C(C$\underline{H}_3$)), 1.4−1.7 (−NH−CH_2−$C_2\underline{H}_4$−CH_2−NHBoc), 3.0 (−NH−CH_2−C_2H_4−C$\underline{H}_2$−NHBoc), 3.2−4.1 (dextran glucosidic protons), 5.0 (dextran anomeric proton).

Next, Dex−C_4−Boc (1.7 g) was treated with 1.5 mL of TFA in 15.5 mL of deionized water. The reaction was conducted for one night under nitrogen protection. After neutralized with 4 M NaOH to pH 7, the mixture was ultrafiltrated against water (MWCO 1000). Dex−NH_2 was obtained as white foam after freeze-dried. Yield: 1.5 g (88%). ^{1}H NMR (D_2O): δ 1.5−1.6 (−NH−CH_2−C_2H_4−C$\underline{H}_2$−NH_2), 3.0 (−NH−CH_2−C_2H_4−C$\underline{H}_2$−NH_2), 3.2−4.1 (dextran glucosidic protons), 5.0 (dextran anomeric proton).

2.4. Synthesis of Dextran−Hematin Conjugates. Dextran−hematin conjugates (denoted as HDex) were synthesized by a coupling reaction of Dex−NH_2 with hematin using EDC/NHS as coupling agents. Aminated dextran (1.5 g) was dissolved in 10 mL of deionized water. Hematin (95 mg) was dissolved in 10 mL of DMSO, in which EDC (69 mg) and NHS (42 mg) were added. After 2 h, Dex−NH_2 solution was slowly added and the mixture was stirred under nitrogen for 2 d. After that, the solution was neutralized with 0.1 M NaOH to pH 7 and dialyzed first against 50 mM NaCl solution and then deionized water (MWCO 3500). HDex was obtained as a black foam after freeze-drying. Yield: 1.6 g (55%).

2.5. Synthesis of NGO−HDex Hydrids. The hematin-conjugated dextran-functionalized graphene oxide hydrids were prepared similarly as previously reported.[30] HDex (55 mg) was dissolved in 10 mL of deionized water, to which 10 mL of the homogeneous nanographene oxide solution (0.5 mg/mL) was added. After the addition of 0.1 mL of ammonia solution and 15 μL of 50% hydrazine solution, the mixture was vigorously stirred for a few minutes, and then reacted under nitrogen at 60 °C for 3.5 h. The solution was then dialyzed against water (MWCO 25000) and filtered with a nylon membrane (0.22 μm) to obtain the graphene hydrid (denoted as NGO−HDex) dispension.

2.6. Characterization. ^{1}H NMR (500 MHz) spectra were recorded on a Bruker Avance 500 MHz spectrometer. The signals of solvent residues were used as reference peaks for the ^{1}H NMR chemical shift and were set at δ 4.79 for water. The degree of substitution (DS) of hematin, which is defined as the number of dextran chains per hematin molecule, was determined using UV−visible spectrophotometer (U-3010, Hitachi, Japan). The concentration of hematin was calculated by determining the absorption at 386 nm of HDex solution. The calibration curve was derived from a series of hematin solutions. The concentration of NGO was calculated by determining the absorption at 260 nm of diluted NGO−HDex solution. The weight percentage of HDex in NGO−HDex hybrid was determined by gravity in percentage.

X-ray powder diffraction (XRD) patterns were recorded using a D/MAX-2500X diffractometer (Rigaku, Japan), equipped with a rotating anode and with a Cu−Kα radiation source (λ = 1.54178 Å). The morphology of NGO and NGO−HDex was observed by an atomic force microscope (AFM, SPA- 300HV). AFM samples were prepared by drop casting the sample suspension in water onto freshly cleaved mica surfaces, and dried under room temperature. The particle size distribution and zeta potential of NGO and NGO−HDex (0.1 mg/mL in water) was determined by Nanosizer (Zetasizer 3000 HS, Malvern, UK).

2.7. Drug Loading and Release. Loading of DOX was done by simply mixing DOX of various concentrations and NGO−HDex solution. In a typical example, 0.5 mL of NGO−HDex with a NGO concentration of 0.14 mg/mL was first mixed with 0.5 mL of 0.2 mg/mL DOX solution (stock solution of an initial concentration of 1 mg/mL diluted with PBS) and sonicated for 0.5 h. After stirred for 24 h at 37 °C in the dark, the suspension was centrifuged at 16 000 rpm for 30 min and the residues were washed with 1 mL of PBS (0.1 M, pH 7.4) twice in order to achieve complete removal of unloaded DOX. The upper layer after each centrifugation was collected and the DOX concentration was measured with a UV−visible spectrophotometer (U-3010, Hitachi, Japan) at the wavelength of 480 nm using a standard DOX calibration curve generated from a series of DOX solutions with determined concentrations. The DOX loading capacity (DL) and DOX embedding efficiency (EE) of NGO−HDex was calculated according to eq 1 and 2, respectively,

$$\mathrm{DL(mg/mg)} = \frac{M_{\mathrm{DOX}} - M_{\mathrm{DOX}}'}{M_{\mathrm{NGO}}} \quad (1)$$

$$\mathrm{EE(\%)} = \frac{M_{\mathrm{DOX}} - M_{\mathrm{DOX}}'}{M_{\mathrm{DOX}}} \times 100\% \quad (2)$$

where M_{DOX} is the initial amount of DOX added, M_{DOX}' is the total amount of DOX in the supernatant after loading, and M_{NGO} is the amount of NGO in NGO−HDex added.

To investigate the release profile of DOX-loaded NGO−HDex sample, we added 1 mL of PBS solution to each sample and the mixtures were incubated at 37 °C with constant shaking in the dark. At regular time intervals, the samples were taken out and centrifuged at 16 000 rpm for 30 min. The DOX concentration in the upper layer was determined as described above. Cumulative DOX release (%) was calculated as follows: $(M_t/M_0) \times 100\%$, where M_t is the amount of DOX released from NGO−HDex at time t and M_0 is the amount of DOX initially loaded onto NGO−HDex.

2.8. In Vitro Cytotoxicity Assay. MCF-7/ADR cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). The cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) at an atmosphere of 5% CO_2 at 37 °C. MCF-7/ADR cells were maintained with free DOX at a concentration from 0.2 to 1 μg/mL with increasing propagation time.

To investigate the cytotoxicity of NGO and NGO hybrid, the cell viability of MCF-7/ADR cells cultured with NGO or NGO−HDex was evaluated by CCK-8 assay (Dojindo Molecular Technologies, Inc.). MCF-7/ADR cells were plated in the 96-well plates (4000 cells per well) and incubated for 24 h. Sterilized NGO or NGO−HDex stock solutions at different concentrations were respectively added to the cells in the culture medium. The final test concentration was 5, 10, 20, 40, 60, 80, and 100 μg/mL, respectively. The cells cultured in NGO or NGO−HDex free medium were taken as the control. After 48 h incubation, the cells were washed with D-Hanks buffer solution, treated with 200 μg/mL of CCK-8 solution and incubated for another 1 h at 37 °C. The optical density (OD) of each well at 450 nm was recorded on a Microplate Reader (Thermo, Varioskan Flash). The cell viability (% of control) is calculated according to eq 3

$$\text{cell viability (\%of control)} = \frac{\mathrm{OD_{test}} - \mathrm{OD_{blank}}}{\mathrm{OD_{control}} - \mathrm{OD_{blank}}} \times 100\% \quad (3)$$

where $\mathrm{OD_{test}}$ is the optical density of the cells exposed to NGO or or NGO−HDex sample, $\mathrm{OD_{control}}$ is the optical density of the control sample and $\mathrm{OD_{blank}}$ is the optical density of the wells without MCF-7/ADR cells.

2.9. Tumor Cell Killing Effects and Intracellular Uptake of Doxorubicin. The tumor cell killing effects of DOX-loaded NGO−HDex on MCF-7/ADR cells was investigated by determining the cell viability. MCF-7/ADR cells (4×10^3 cells per well) were seeded in a 96-well plate and incubated in complete RPMI 1640 medium containing 10% FBS at 37 °C in 5% CO_2 humidified atmosphere for 24 h. Next, the cells were washed twice with D-Hanks buffer and coincubated with complete culture medium containing either free DOX or DOX-loaded NGO−HDex at different DOX concentrations ranging from 0.1 to 10.0 μg/mL. After 48 h incubation, the cell viability was assayed by CCK-8 assay as described above. To monitor the intracellular uptake of DOX, MCF-7/ADR cells (1×10^5 cells/well) were incubated with NGO, free DOX, and DOX-loaded NGO−HDex in the medium, respectively. The equivalent DOX concentrations in the medium for free DOX and DOX-loaded NGO−HDex were kept at 10 μg/mL. After 24 h, the culture medium was removed and cells were rinsed twice with D-Hanks buffer solution. The cells were stained with DAPI for cell nucleus following the manufacturer's

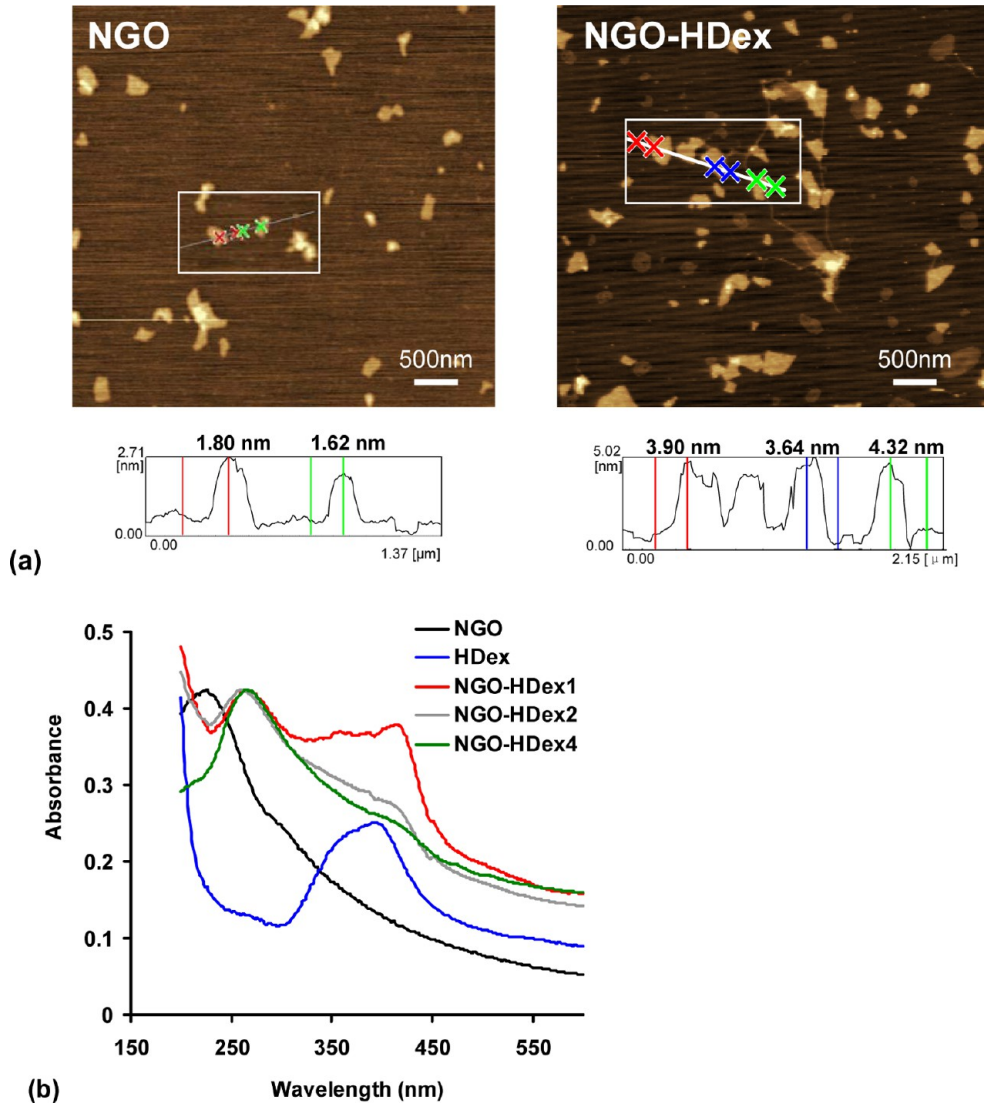

Figure 2. (a) AFM images of NGO and NGO−HDex. (b) UV−vis spectra of NGO, HDex, and NGO−HDex.

instructions (Invitrogen). The intracellular localization of DOX was visualized under a laser scanning confocal microscope (LSCM, FV1000, Olympus, Japan).

The intracellular uptake of DOX of MCF-7/ADR cells was also evaluated by a fluorescence-activated cell sorting method using a flow cytometry (FACS, FACSCalibur, BD Biosciences, USA). In brief, MCF-7/ADR cells (1×10^5 cells per well) were seeded in a six-well plate for 24 h. The cells were then washed twice with D-Hanks buffer and incubated with NGO−HDex, free DOX or DOX-loaded NGO−HDex at a DOX concentration of 10 μg/mL. After another 6 or 24 h incubation, the cells were washed twice with D-Hanks buffer, collected and resuspended in the buffer for FACS analysis. The fluorescence intensity of DOX was collected at a 488 nm excitation and with a 575 nm band-pass filter. Cells with D-Hanks buffer treatment were used as the control.

2.10. Statistical Analysis. All data are presented as mean ± standard deviation ($n = 6$). Statistical differences were analyzed using a Student's t-test. * denotes a statistical significance ($^*p < 0.05$ and $^{**}p < 0.01$) between the experimental data of two groups.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of NGO−HDex Hybrids. Hematin−dextran conjugate was prepared via a two-step procedure as described in Figure S-1 (see the Supporting Information). Amine-terminated dextran (Dex-NH_2) was obtained by the reaction of reducing terminal glucose residues in dextran with an excess of N-Boc-1,4-diaminobutane in the presence of sodium cyanoborohydride, followed by the deprotection of the Boc using trifluoroacetic acid. Complete deprotection of the Boc group was confirmed by ^{1}H NMR, showing the disappearance of the t-butyl signals at δ 1.4 (see Figure S-2 in the Supporting Information, peak 5). The degree of NH_2 end group conversion was over 95% as determined from ^{1}H NMR by comparing the integrals of signals at δ 5.0 (anomeric proton in dextran) and δ 1.5−1.6 (methylene protons in butane residue). Next, Dex−NH_2 was conjugated

Table 1. Synthesis and Characterization of NGO−HDex

code	NGO:HDex	HDex wt %	size (nm)	zeta potential (mV)
NGO			178.0 ± 6.73	−28.7 ± 1.48
NGO−HDex1	1:1	78	238.8 ± 19.67	−11.7 ± 1.69
NGO−HDex2	2:1	61	228.1 ± 3.1	−18.9 ± 1.57
NGO−HDex4	4:1	42	223.0 ± 12.1	−23.0 ± 1.43

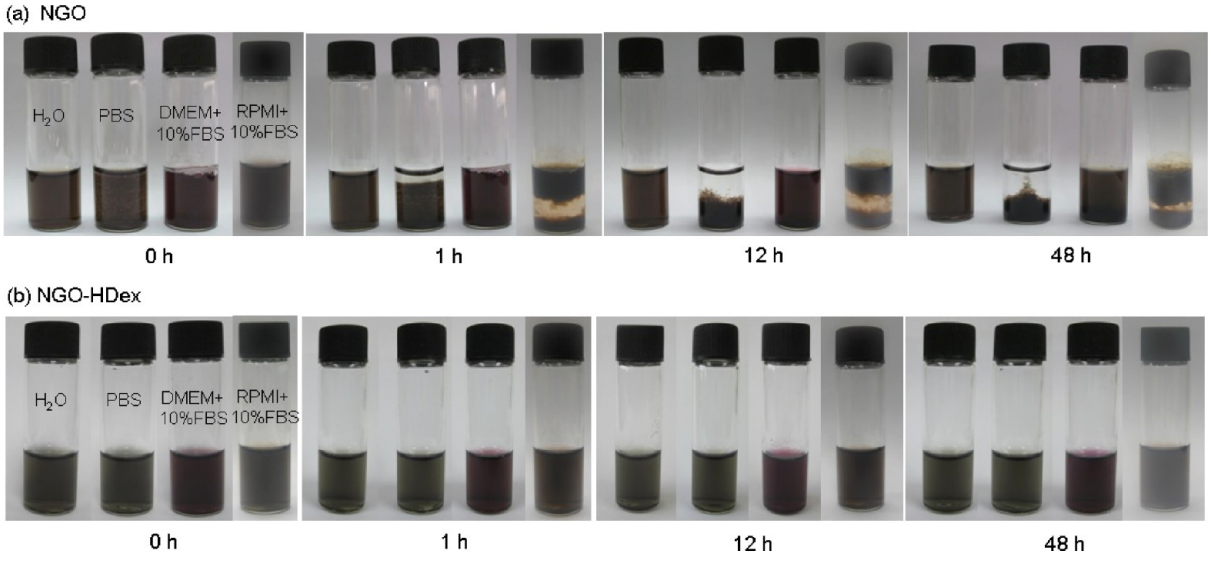

Figure 3. (a) NGO and (b) NGO−HDex dispersed in water, PBS and cell medium (DMEM+10% FBS or RMPI+10% FBS).

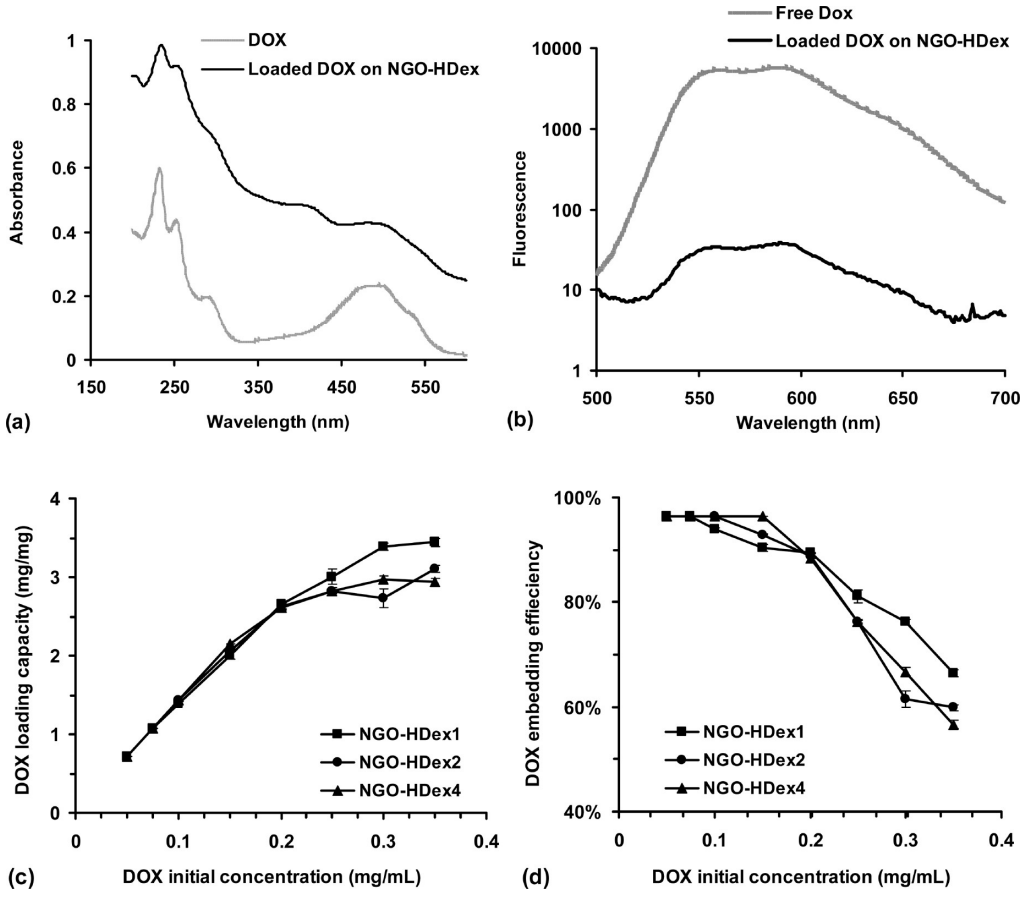

Figure 4. (a) UV−vis and (b) fluorescence spectra of DOX and DOX-loaded NGO−HDex. (c) DOX loading capacity of NGO−HDex and (d) embedding efficiency of DOX on NGO−HDex as a function of DOX initial concentrations.

with hematin using an EDC/NHS chemistry in the water/DMSO (1/1, v/v) as a mixture solvent to render both the dextran and hematin soluble homogeneously. The resulting dextran-hematin (HDex) conjugate was purified by exhausitive dialysis and obtained as black powder after freeze-drying. The degree of substitution of HDex conjugate (defined as the number of dextran chains per hematin molecule) was 1.2, as determined by UV−visible spectrophotometer.

Previously, chemical modification of GO has been done to yield functionalized GO with the groups like caboxylic acid for further coupling with hydrophilic polymers such as dextran[19] and poly(ethylene glycol) (PEG),[4,6−8,18] or with biomolecules such as DNA.[31] However, only limited reports have appeared on the decoration of GO using a noncovalent method. For example, graphene was assembled with porphyrin-based molecules such as hemin (iron protoporphyrin)[30,32,33] or

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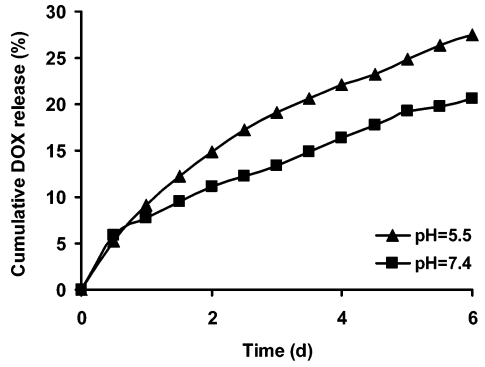

Figure 5. Cumulative DOX release from NGO−HDex1 as a function of time.

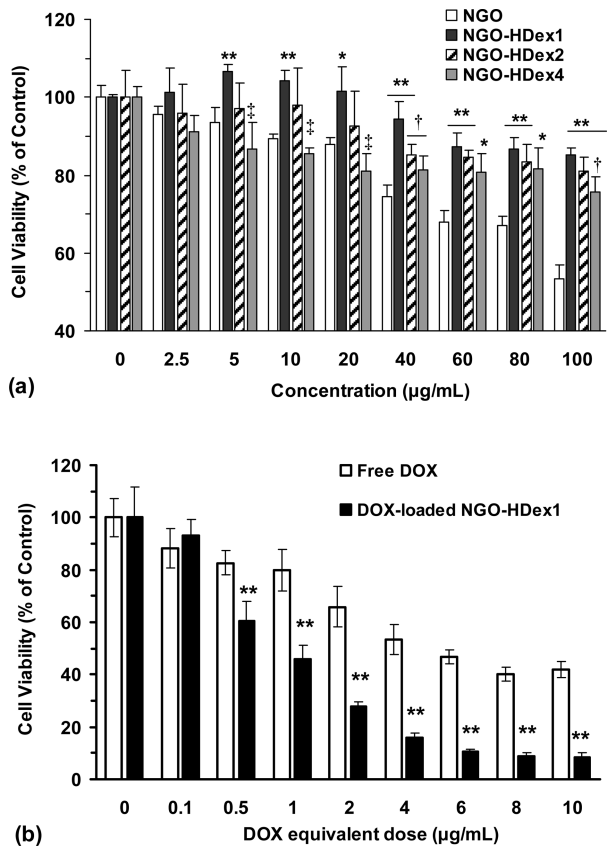

Figure 6. (a) Cell viability of MCF-7/ADR cells incubated with NGO or NGO−HDex for 48h (* $p < 0.05$ and ** $p < 0.01$ vs NGO; † $p < 0.05$ and ‡ $p < 0.01$ vs NGO−HDex1). (b) Cell viability of MCF-7/ADR cells incubated with free DOX or DOX-loaded NGO−HDex for 48 h (** $p < 0.01$ vs free DOX).

other synthetic polymers such as Pluronic F127 and PNIPAAm-grafted dextran.[20] In our study, NGO was modified by $\pi-\pi$ interaction between hematin moiety in HDex and NGO sheet in a one-pot reaction using hydrazine (Figure 1). Hematin is a Fe (III) compound from decomposition of hemoglobin and should be more cyto-biocompatible as compared to the synthetic polymers. The size, thickness and morphology of resulting NGO−HDex hybrid were determined by atomic force microscopy (AFM) (Figure 2a). It was shown that the thickness of NGO−HDex (3.6−4.3 nm) is bigger than that of native NGO,[17] implying the formation of NGO−HDex nanohybrid. In previous work, chemcial modification of NGO

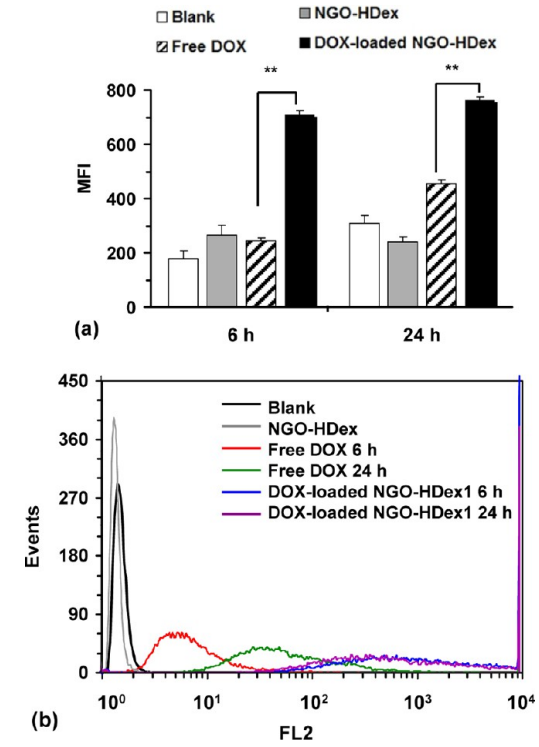

Figure 7. (a) Mean fluorescence intensity (MFI) of MCF-7/ADR cells after incubation with NGO−HDex1, free DOX, or DOX-loaded NGO−HDex1 for 6 or 24 h. (b) Flow cytometric analysis of MCF-7/ADR cells after incubation with NGO−HDex1, free DOX, or DOX-loaded NGO−HDex1 for 6 or 24 h. The equivalent dose of DOX was 10 μg/mL in cell culture (** $p < 0.01$).

with PEG also led to increased thickness compared to that of native NGO.[4] Next, UV−vis spectra of NGO, HDex, and NGO−HDex were recorded (Figure 2b). Native NGO has a single peak at 230 and 300 nm, which respectively correspond to the $\pi-\pi$ transitions of double bonds in aromatic rings and $n-\pi$ transitions of carbonyl groups. The HDex conjugate exhibits a peak at 386 nm due to the porphyrin centered $\pi-\pi$ transition. The UV spectra of NGO−HDex, however, shows a red-shift of the aromatic C═C bonds from 230 to 260 nm after hydrazine-mediated reduction of NGO, implying restoration of the $\pi-\pi$ conjugation within the graphene sheets.[14] In addtion, the NGO−HDex have all the characteristic peaks from hematin and NGO, further implying the formation of NGO−HDex hybrid. Furthermore, hematin residue in the NGO−HDex showed a red-shift soret band from 386 to 412 nm, which may be interpreted as the $\pi-\pi$ interaction as a result of the self-assembly of NGO and HDex. The crystal structure of NGO−HDex was also characterized using X-ray powder diffraction (XRD) (Figure S-3, see the Supporting Information). The refraction peak of NGO arises at $2\theta = 11°$ with an interlayer distance of 0.8 nm. However, for the NGO−HDex nanohybrid, its refraction peak disappears after the assembly of NGO and HDex, indicating an amorphous nature due to the presence of amorphous HDex.[34] Overall, all these results suggest the formation of NGO−HDex nanohybrid.

The particle sizes and surface charges of as-prepared NGO−HDex were examined by dynamic light scattering analysis. NGO−HDex hybrids reveal larger sizes (~220−240 nm) as compared to native NGO (~178 nm).[35] This larger size is most likely due to the presence of HDex on NGO surface. Besides,

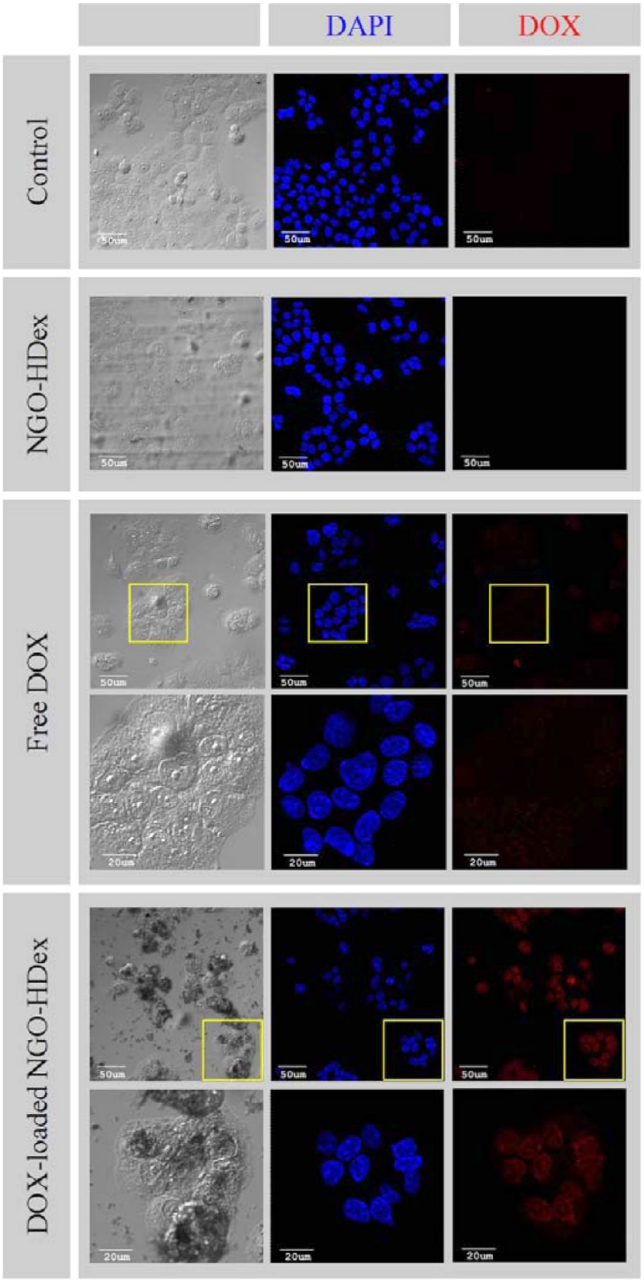

Figure 8. Confocal laser microscopic observation of MCF-7/ADR cells after incubation with NGO−HDex1, free DOX or DOX-loaded NGO−HDex for 24 h. The equivalent dose of DOX was 10 μg/mL.

native NGO has negative surface charge (−28.7 mV) because of the presence of carboxylic groups in the NGO, which is the reason why the NGO can be dispersed in aqueous solution. Instead, the NGO−HDex hydrids show more neutral surface charges (Table 1). It was found that when the content of HDex was increased from 42 to 78%, the surface charges of the NGO−HDex shifted from −23.0 to −11.7 mV, indicating that the surface of native NGO is partially shielded by neutral dextran. Neutral polymers such as dextran may improve colloidal stability of NGO because dextran can weaken serum opsonization of NGO in vivo.[36,37] As revealed in Figure 3, both native NGO and NGO−HDex can be dispersed homogeneously in water over 48 h. However, in PBS and the medium (DMEM+10% FBS or RPMI 1640 + 10% FBS), NGO experienced fast aggregation after 1 h and the precipitate was observed after 48 h. In parallel, NGO−HDex remained homogeneous in PBS or the medium without any agglomeration. Overall, this near-neutral surface charge and improved collodial stability are highly desired, in order to reduce protein absorption and avoid related side effects when NGO−HDex is applied for in vivo.[37]

3.2. Drug Loading and Release. The antitumor drug DOX is used as a model drug to evaluate the drug loading capacity (DL) of NGO−HDex nanohybrids. The experiments were done under the condition of different initial DOX concentrations with respect to the same concentration of NGO (0.07 mg/mL) in the NGO−HDex solutions after adding DOX. The stacking of DOX onto NGO−HDex was evident from the UV−vis spectroscopy. It was observed that free DOX showed the characteristic absorption peaks at 232, 252, 290, and 480 nm (Figure 4a). When loaded on NGO−HDex, DOX exhibited red-shifts of the absorption peaks from 232 and 480 nm to 235 and 492 nm, respectively. This is most likely due to the electron donor−acceptor interaction of DOX and NGO−HDex.[38] Using a fluorescence spectroscopy, free DOX displayed a maximum fluorescence emission value at 589 nm with an excited source at 480 nm (Figure 4b). However, fluorescence quenching of DOX was observed as DOX was loaded in NGO−HDex, likely because of the energy transfer or photoinduced electron-transfer effect. These results indicate successful loading of DOX in NGO−HDex. When loaded with DOX in RPMI-1640 medium containing with 10% FBS, NGO−HDex remained homogeneous suspension after 48 h, whereas native NGO precipitated after 12 h (Figure S-4, see the Supporting Information). DL was also determined by UV−vis method at absorbance of 480 nm. As shown in Figure 4(c), the DL of NGO−HDex increased when the DOX initial concentrations were increased to 0.2 mg/mL and a plateau value arrived at 3.4 mg DOX/mg NGO when an initial DOX concentration of 0.3 mg/mL was applied. Interestingly, the assembly of HDex with NGO does not compromise the DL because increasing the HDex content of NGO−HDex from 42 to 78% gives comparable DL values. The embedding efficiencies (EE) for NGO−HDex were shown in Figure 4). When the initial DOX concentration was below 0.15 mg/mL, the EE values were above 90%, indicating efficient loading of DOX in NGO−HDex.

In view of the high drug loading capacity of NGO−HDex, we also investigated drug release profile of DOX-loaded NGO−HDex using UV−Vis spectrophotoscopy (Figure 5). At pH 7.4, only about 11% of DOX released out from NGO−HDex within 2 days. This slow drug release profile is favorable for clinical anticancer chemotherapy since this may give rise to reduced toxicity of anticancer drugs to normal tissues before the drugs reach targeted tumor by passive targeting (enhanced permeability and retention effect) or active tumor targeting. Also, the effect of pH on the DOX release profile was probed. The DOX release rate was higher at pH 5.5 than pH 7.4. For example, about 20 and 28% of loaded DOX was released after 6 days at pH 7.4 and 5.5, respectively. The accelerated drug release at pH 5.5 may be attributed to partial dissociation of H-bonding between −OH and $-NH_2$ groups in DOX and the −OH and −COOH groups on NGO. The accelerated DOX release from DOX-loaded NGO−HDex under acidic conditions is highly desired for an effective cancer therapy, owing to an acidic microenvironment in solid tumor, endosomes and lysosomes.[39] This pH-dependent release profile was also observed in the NGO systems such as NGO,[11] PEG-coupled

NGO[4,18] and Pluronic F127-NGO.[5] Notably, as compared to these systems, the NGO−HDex seems to induce a smaller difference of DOX release rate between pH 5.5 and pH 7.4 (about 8% at day 6). This result is likely due to the reason that the reduction of NGO by hydrazine causes diminished amounts of −OH and −COOH groups in the NGO, which minimizes H-bonding interaction of the NGO and DOX.

3.3. Cellular Experiments. For biomedical applications, cytotoxicity of drug nanocarriers should be taken into account. Thus, cytotoxicity of native NGO and NGO−HDex against MCF-7/ADR cells was evaluated by CCK-8 assay (Figure 6a). The NGO−HDex revealed a low cytotoxicity (cell viability >90%) at a concentration up to 20 μg/mL. A mild cytotoxicity (above 80% cell viability) was detected at a high concentration of 100 μg/mL. However, at the same concentration, native NGO is cytotoxic in MCF-7/ADR cells with about 53% of the cells maintaining survival, comparable to previously reported results.[40,41] These results indicate that modification of NGO with HDex improves cyto-biocompatibility of NGO.

In view of low cytotoxicity of NGO−HDex hybrids, we examined the killing effect of DOX-loaded NGO−HDex on MCF-7/ADR cells. The NGO−HDex1 (Table 1) was chosen as a typical example because of its high DOX loading capacity and lowest cytotoxicity. Figure 6b shows the cell viability after the cells were incubated with free DOX or DOX-loaded NGO−HDex1 for 48 h. With increasing amount of DOX from 0.1 to 10 μg/mL, the cell viability of DOX-loaded NGO−HDex1 significanlty reduced to about 30% at a DOX concentration of 2 μg/mL. In parallel, at the same DOX concentration, the cell treated by DOX alone revealed higher cell survival with about 60% cells maintaining their metabolic acitivity. Even at a high DOX concentration of 10 μg/mL, the cell retained about 40% cell ability after exposed in DOX alone. This phenomenon is due to the P-glycoprotein (P-gp) function that modulates the drug efflux.[2] The half maximal inhibitory concentration (IC_{50}), defined as the DOX concentration causing 50% cell growth inhibition in a given period, was also determined from Figure 6b. The DOX-loaded NGO−HDex1 revealed lower IC_{50} value (1.02 μg/mL) than free DOX (3.86 μg/mL) against MCF-7/ADR cells, suggesting that the NGO−HDex system offers enhanced tumor cell killing ability.

To ascertain whether DOX-loaded NGO−HDex can promote DOX uptake by MCF-7/ADR cells, the cells were incubated with DOX-loaded NGO−HDex and the uptake was assessed by flow cytometry at different time intervals. The cells incubated with either DOX or NGO−HDex alone were used as a control. Because DOX is fluorescent, cell uptake is directly analyzed without introducing additional fluorescent probes. As shown in Figure 7, at 6 and 24 h, the cells incubated with DOX-loaded NGO−HDex exhibited higher mean fluorescence intensity when compared to those incubated with free DOX, suggesting that the NGO−HDex may serve as an efficient carrier for intracellular delivery of DOX. Figure 8 gives the distribution of DOX in MCF-7/ADR cells after the cellular uptake of DOX-loaded NGO−HDex, as observed by confocal laser microscopy. The cells treated with DOX exhibited weak fluorescence signal after 24 h incubation and the fluoresecence was mainly found around the perimembrane region of the cells. However, strong fluorescence signal was detected for DOX-loaded NGO−HDex, meaning that a higher amount of DOX is accumulated in the cells. Interestingly, the DOX was located not only in the cytoplasm but the nucleus. These results thus again suggest that DOX-loaded NGO−HDex is efficient as a nanocarrier for the delivery of DOX in MCF-7/ADR cells. This further reasonably explains why DOX-loaded NGO−HDex induces higher cytotoxicity in the cells than free DOX (Figure 7b).

4. CONCLUSIONS

We have demonstrated a new graphene-based nanohybrid can be obtained by noncovalent "π−π interaction" of NGO and hematin-conjugated dextran (HDex). The NGO−HDex nanohybrid exhibits improved collodial stability in salt and serum as compared to native NGO. Imporatntly, HDex-decorated NGO has improved cyto-compatibility as compared to native NGO. Anticancer drug DOX can be efficetively loaded into NGO−HDex with a high loading capacity of 3.4 mg/mg NGO. DOX-loaded NGO−HDex is more efficient than free DOX for killing drug resistant MCF-7/ADR cells. The results of this study suggest that NGO−HDex nanohybrid has high potential as a drug nanocarrier for cancer therapy.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Information on the synthesis of Dex-NH_2 and HDex conjugates. (Figure S-1); ^{1}H NMR spectra of dextran, Dex−C_4−Boc and Dex−NH_2 polymers. (Figure S-2), X-ray diffractograms of NGO, HDex, and NGO−HDex. (Figure S-3), and DOX-loaded (a) NGO and (b) NGO−HDex1 dispersed in RPMI-1640 supplemented with 10% FBS. (Figure S-4). This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

NGO, nanosized graphene oxide
HDex, hematin-conjugated dextran
NGO−HDex, graphene−dextran hybrid
DOX, doxorubicin
FBS, fetal bovine serum
XRD, X-ray powder diffraction
AFM, atomic force microscopy

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