Asian Journal of Chemistry; Vol. 27, No. 12 (2015), 4517-4522

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.19201

A Targeted Drug Delivery System Based on Reduced Graphene Oxide

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Received: 28 March 2015; *Accepted*: 8 May 2015; *Published online*: 29 August 2015; AJC-17500

Graphene sheets as drug carrier are interesting because both sides of a single sheet can be accessible for drug binding and can afford strong non-covalent binding with aromatic drugs *via* simple adsorption. Here, we linked a cell specific target molecule folic acid (FA) to PEI-PEG-PEI to form the FA-PEI-PEG-FA. The large quantity of folic acid molecules which are the hydrophobic molecules could conjugate with the sheet of chemical reduced graphene oxide (rGO) and then the system of rGO-FA-PEI-PEG-PEI-FA-rGO (NG-FA-PEI-PEG) was formed. This system could exist stable in physiological solution. Folic acid molecules allowed it to specifically target CBRH-7919 liver cancer cells with folic acid receptors. The loading and release behaviours of doxorubicin (DOX) on the functionalized graphene sheets were investigated. The doxorubicin loading ratio on NG-FA-PEI-PEG (the weight ratio of drug loaded to NG-FA-PEI-PEG) was about 30.65 %. The drug release from NG-FA-PEI-PEG was pH dependent. The results of cytotoxicity (FCM assays) clearly showed that the concentration of NG-FA-PEI-PEG as the drug delivery system should be less than 60 mg/L (Here, 10 mg/L was used as the drug delivery system). These results indicated that NG-FA-PEG-PEI might be a promising candidate for drugs delivery with the characteristics of good biocompatibility and low toxicity.

Keywords: Graphene, Drug delivery system, Folic acid, Cell imaging.

INTRODUCTION

To date, the major challenge in insoluble anticancer drug therapy is to develop a highly effective delivery system with low toxicity. Graphene (G) is known of the thinnest two-dimensional materials and one-atom-thick two-dimensional graphitic carbon system[1]. Nano graphene (NG) has special layered structure and oversized surface area and aromatic planes and therefore is capable of adsorbing aromatic compounds, which suggests potential applications as drug carriers[2-12]. But nano graphene has some disadvantages, *e.g.* not stable in physiological condition easy to aggregate and high toxicity. To overcome the problem of unstable and high toxicity and low transfection efficiency, we have has linked a cell specific targeting molecule folic acid to PEI-PEG (PEI = polyethyleneimine) which was used to enhance the stability of nano graphene solution and decrease the toxicity. Dai and colleagues[10,11] for the first time employed PEGylated (PEG = polyethylene glycol) nanoscale graphene oxide (NGO) as a nanocarrier to load anticancer drugs *via* non-covalent physisorption and studied its cellular uptake. Immediately after that, another group led by Chen[13] investigated the loading and release of doxorubicin hydrochloride on graphene oxide (GO). Zhang and colleagues[14] studied the loading controlled and targeted delivery of mixed anticancer drugs through the graphene oxide nanocarrier. All these researches were based on the graphene oxide in which the electron conjugation degree is less than the reduced graphene oxide, so it is suggested that the drug loading ratio of reduced graphene oxide should be higher than graphene oxide. The synthesis of reduced graphene oxide functionalized with good biocompatibility and physiological stability is reported here. In our strategy, FA-PEI-PEG-PEI-FA groups were introduced to nano graphene, which rendered it stable under physiological conditions[15,16] and large quantity of folic acid (FA) molecules were attached to the nano graphene for targeting specific cells with folate receptors (FR). Folic acid is a common targeting ligand used for anticancer agents, since its target molecule (*i.e.* folate receptors) is often over expressed in tumor cells[17,18]. Therefore, the folic acid has been used to test its enhancing effect on vector delivery in folate receptors-enriched tumor cells such as C6 glioma cells[19]. Folic acid has three aromatic rings and is not soluble in neutral or acid water solution, but it can be dissolved in reduced graphene oxide water solution as the π-π stacking and hydrophobic interactions with the reduced graphene oxide[10-12]. In the present study, the potency of the FA-PEI-PEG-PEI-FA which could be used as a non-viral vector was tested in the CBRH-7919 tumor cells and as a linker was adsorbed on the surface of nano graphene. The

conjugation of folic acid with PEI-PEG-PEI was used to target specific cancer cells with folate receptors and was adsorbed to the nano graphene surface (Fig. 1). Furthermore, controlled loading and targeted delivery of anticancer drugs using the nano graphene as a nanocarrier were investigated. The schematic illustration was shown in Fig. 1.

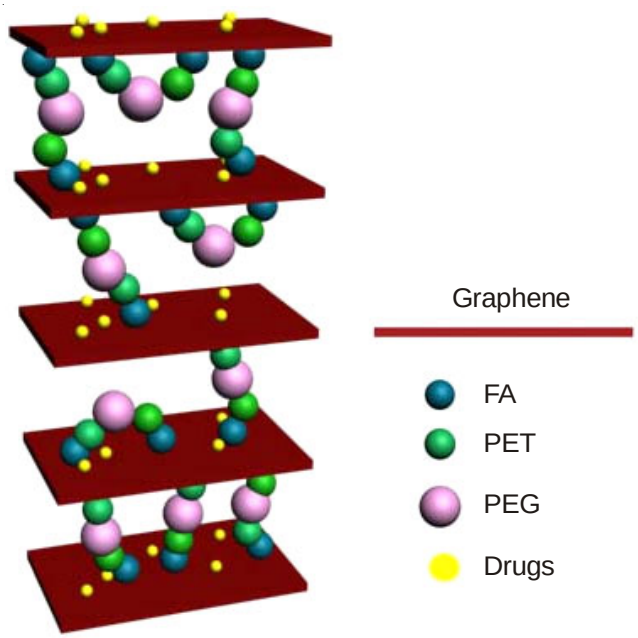


Fig. 1. Schematic illustration of NG-FA-PEI-PEG-PEI-FA-NG (NG-FA-PEI-PEG) system as the drug delivery system

EXPERIMENTAL

CBRH-7919 mouse liver cancer cells and HL7702 liver cells were gifted from Professor Xiaodong Song and Professor Jingmin Li respectively, Binzhou Medical College, China; N,N'-dicyclohexylcarbodiimide (DCC), ethylenediamine anhydrous (EDA), succinic acid (SA), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and native graphite flake were purchased from Sinopharm Chemical Reagent Co. Ltd; folic acid, poly ethylene glycol 6000 (PEG 6000,OH -PEG-OH) were purchased from Tianjin Damao chemical reagent Co. Ltd; Hyperbranched polyethylenimine 10000 (PEI-10000), doxorubicin (DOX) and Annexin V-EGFP Apoptosis Detection Kit were purchased from Sigma; DMEM culture medium and fetal bovine serum (FBS) were purchased from Invitrogen; Anodisc membrane filter (47 mm in diameter, 0.22 μm pore size; Whatman, German); Other reagents were purchased from China National Medicine Corporation.

FT-IR and UV-visible spectra were collected by using a Bruker WQF-510 FTIR spectrometer and 756PC UV spectrophotometer (Shanghai) separately. Absorbance in the WST assay was read by a Biotek Elx 800 microplate reader. Morphological analysis was performed with a Veeco DI atomic force microscope. Cell lines were cultured with a water-jacketed CO_2 incubator (German Hearcell). Sonication of the samples was carried out with a HS-3120 ultrasonicator. TEM images were obtained on a JEM-1400 (HC) transmission electron microscope. Analyses of targeted uptake of NG-FA-PEI-PEG by CBRH-7919 tumor cells were performed with Leica TCS SPE confocal laser scanning microscope (CLSM). Evaluation of the cytotoxicity of NG-FA-PEG-PEI-Res and NG-FA-PEG-PEI at different concentration was performed with COULTER EPICS XL flow cytometry (FCM).

Synthesis of nano graphene: Graphene oxide was synthesized by the oxidative treatment of purified natural graphite using a modified Hummers method[19]. Then, graphene oxide (100 mg) was dissolved in 1000 mL water and then was sonicated for 3 h with 500 W sonicator. 10 mL EDA was added and then reflux 3 h at 90 °C. The product was dialyzed for 48 h with the 0.5 % ammonia solution to remove the excess EDA or any unnecessarily things. It is the chemical reduced graphene (rGO or nano graphene) water solution (right tube of Fig. 2).

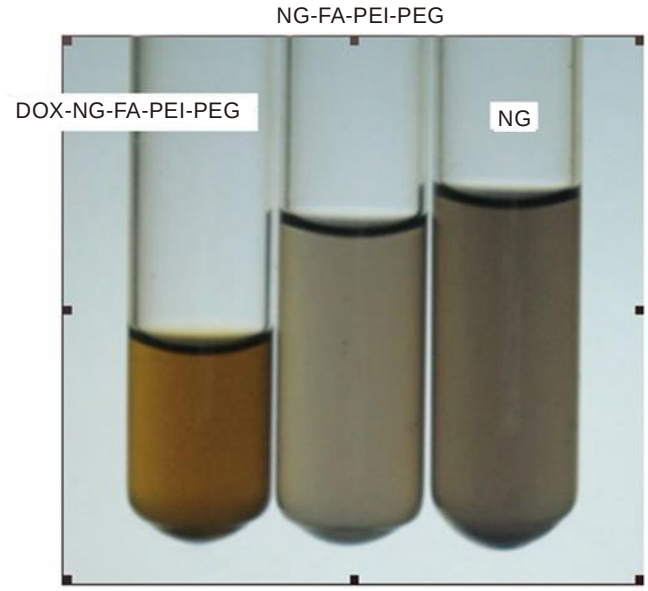


Fig. 2. Aqueous solution of nano graphene, NG-FA-PEI-PEG and DOX-NG-FA-PEI-PEG

Synthesis of FA-PEI-PEG-PEI-FA: PEG-6000 12 g and 2.5079 g succinic acid were dissolved in 40 mL anhydrous chloroform and refluxed at 70 °C for 48 h. After chloroform was removed by distillation, polymer was redissolved in 20 mL deionized water and dialyzed (MWCO: 3000 Da) against water for two days to remove small molecular succinic acid and succinic anhydride. The solid sample of COOH -PEG-COOH was obtained through freeze-drying.

0.1662 g NHS and 0.2109 g DCC was dissolved in 40 mL CH_2Cl_2 solution and 2 g COOH-PEG-COOH was added, the mixture were stirred overnight at room temperature. The precipitated 1,3-dicyclohexylurea (DCU) crystals which like needle was removed by filtration. Supernatant was mixture with 40 mL petroleum ether for 3 h, the solvent was removed by distillation. The filtrate was added to diethyl ether and cooled at 4 °C for 2 h. The precipitate was collected by filtration and dried under vacuum at room temperature. 2 g PEI and the activated PEG were dissolved in PBS (pH 7.4) and magnetically stirred for 24 h at room temperature to produce PEI-PEG-PEI (PEG-PEI)[20]. The mixture was purified by membrane dialyses (MWCO: 8000 Da)) in distilled water for 1 day and the solution was lyophilized.

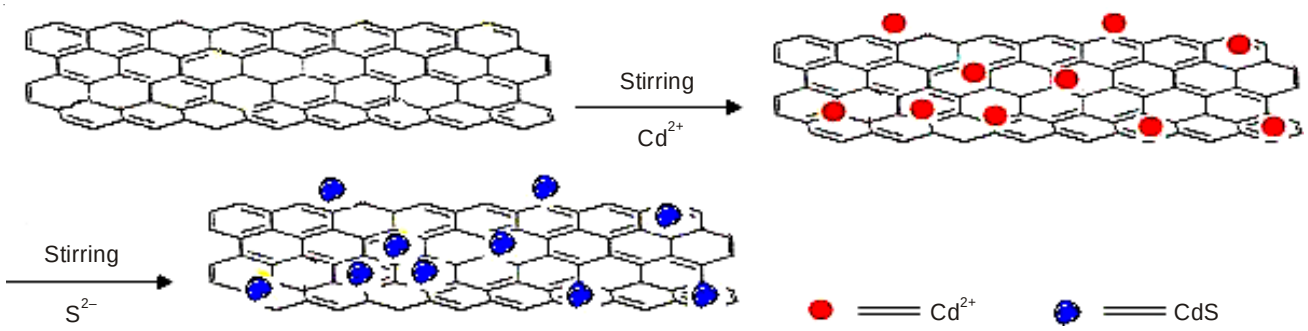


Fig. 3. Schematic illustration of CdS-NG-FA-PEI-PEG preparation (Here, FA-PEI-PEG no displayed)

0.15 g folic acid dissolved in 20 mL DMSO solution, N-hydroxysuccinimide 0.5495 g and N,N′-dicyclohexylcarbodiimide 0.3870 g was added and ultrasoniced for 24 h. The folic acid solution was mixed with the above PEG-PEI solution, stirred for 24 h. The solution was dialyzed against deionized water (MWCO: 10000 Da) for 24 h and lyophilized. FA-PEI-PEG-PEI-FA (FA-PEI-PEG) was obtained.

Preparation of NG-FA-PEI-PEG: 10 mg nano graphene was added to 1000 mL water with high frequency ultrasonic for 4 h. Immediately 10 mg FA-PEI-PEG was added and then ultrasonic for 0.5 h. And then NG-FA-PEI-PEG water solution was prepared (middle tube of Fig. 2) The solid sample was obtained by freeze drying.

Preparation of CdS-NG-FA-PEI-PEG: 1 mg NG-FA-PEI-PEG was added to 100 mL water and it was dispersed into colloid by ultrasonic for 4 h. 20 drops 4.65×10^{-3} mol/L Cd^{2+} solution was added under stirring at ice bath, then 6 drops 4.588×10^{-3} mol/L S^{2-} solution was added slowly at stirring (Fig. 3). The solution is the CdS-NG-FA-PEI-PEG water solution. TEM images (Fig. 4) were acquired to check the morphology. Here, CdS quantum dots (QDs) are only used as the fluorescence imaging reagent of cells. Fig. 4 showed the dashed red circle is a piece of nano graphene and the black dots are CdS QDs on the nano graphene sheet, the size of CdS QDs is mostly less than 15 nm and the adsorbing on nano graphene sheet is perfectly for confocal laser fluorescence image.

CBRH7919 Mouse liver cancer cell uptake of NG-FA-PEI-PEG: To investigate cellular uptake of the NG-FA-PEI-PEG by means of CLSM, CdS nanoparticals was loaded on the NG-FA-PEI-PEG. CBRH-7919 liver cancer cells were incubated with CdS-NG-FA-PEI-PEG at a concentration of 5 mg L^{-1} (in term of NG-FA-PEI-PEG) for 3 h and washed with PBS. The cells were observed under CLSM (Fig. 5). Fig. 5(b) is the merge picture; the blue color is the fluorescence of CdS QDs on the NG-FA-PEI-PEG sheet which was excited by 488 nm laser[21]. It suggested that the NG-FA-PEI-PEG has successfully been uptake by the cancer cell.

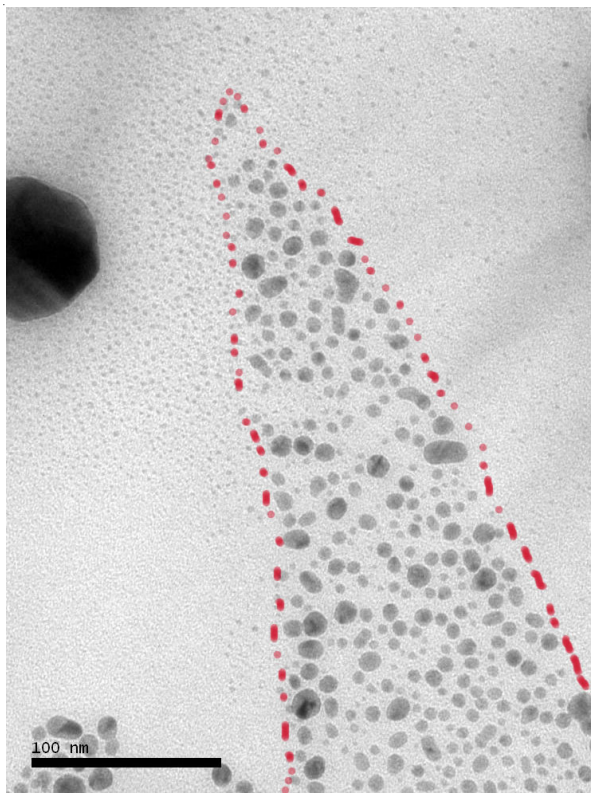
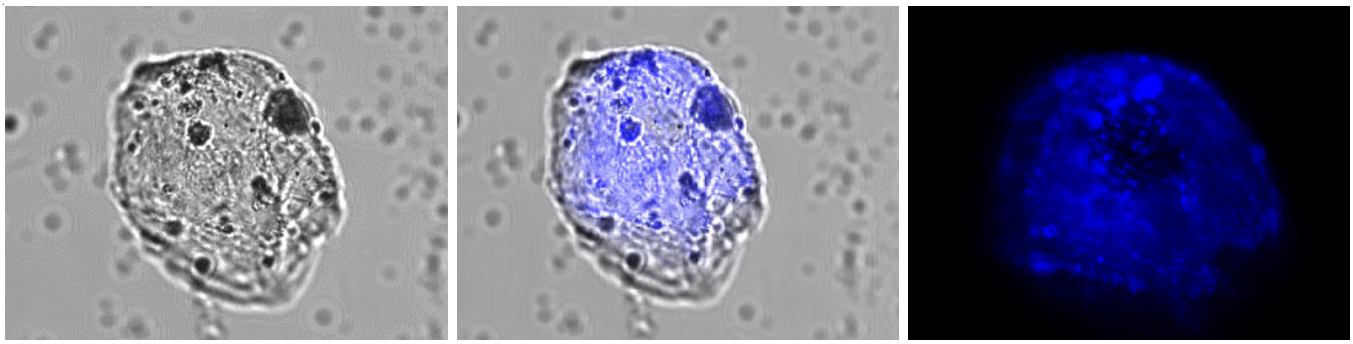


Fig. 4. NG-FA-PEI-PEG piece decorated with CdS QDs



(a) Bright filed (b) Merge (c) Dark filed

Fig. 5. CLSM images. Bright Filed (a); merge (b); Dark Filed (c) of CBRH-7919 liver cancer cells incubated with CdS-NG-FA-PEI-PEG for 3 h

Cytotoxicity of NG-FA-PEI-PEG: Cell lines and cell culture: CBRH-7919 liver cancer cells and HL7702 liver cells were maintained in DMEM medium supplemented with 10 % fetal bovine serum respectively. The cells were seeded in tissue culture flasks (about 2×10^4 cells) and incubated in a fully humidified atmosphere at 37 °C containing 5 % CO_2. For FCM (Flow Cytometer) assay[22], using 12-well culture plates, exponentially growing HL7702 liver cells were seeded (1×10^5 cell per well) and preincubated for 24 h, followed by co-incubation with different concentration NG-FA-PEI-PEG for 48 h. And then the medium was discarded and the cells were washed three times with PBS. Cells were then detached by trypsinization, centrifuged and dispersed again in PBS for the measurements. HL7702 liver cells treated by NG-FA-PEI-PEG were stained with Propidium Iodide (PI) and Annexin V-EGFP before FCM (COULTER EPICS XL, America) analysis.

RESULTS AND DISCUSSION

The TEM microscopy of nano graphene was shown in Fig. 6. Fig. 6a is the big piece reduced graphene oxide and there are very obvious ripple on the graphene sheets. Fig. 6b is the broken pieced of reduced graphene oxide sheets (the red dashed circle), which was caused by the sonication with 500 W sonicator for 24 h and the size of reduced graphene oxide sheets lied in 200 nm mostly.

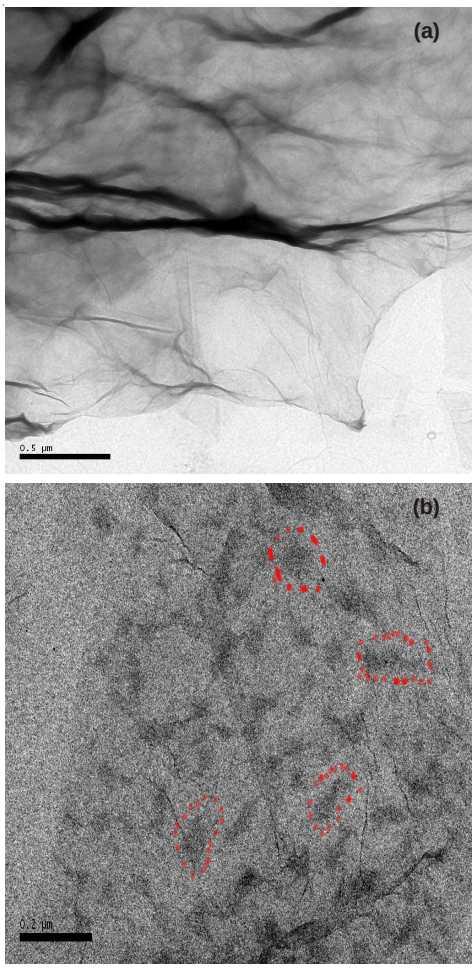


Fig. 6. TEM microscopy of big pieces reduced graphene oxide (a); the small pieces reduced graphene oxide which were obtained by sonication (b)

Fig. 7 showed the appearance of -OH and –C=O group (the middle picture of Fig. 7) suggested the successfully synthesis from PEG-OH (the above picture of Fig. 7) to PEG-COOH. The disappearance of the –OH and the appearance of NH-C=O (the down picture of Fig. 7) groups suggested the reaction of –COOH and $–NH_2$ conjugation successfully of PEG-COOH or FA-COOH and PEI-NH_2 and a very obviously characteristic peak came out at the 2256 cm^{-1} which is the NCO (O=C-N-) stretching vibration.

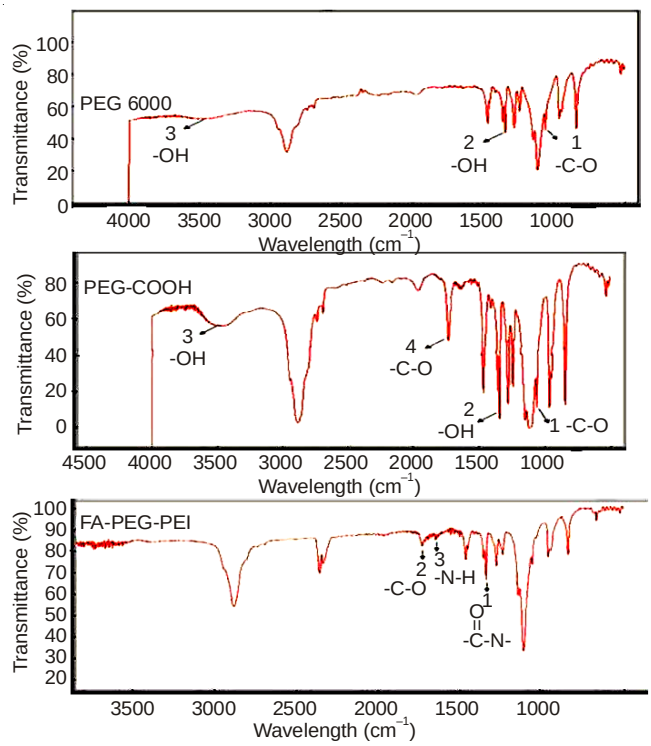


Fig. 7. FT-IR spectra of the PEG-6000, COOH-PEG-COOH and FA-PEI-PEG-PEI-FA

In the UV-visible spectra (Fig. 8), a peak at 253 nm of folic acid disappears while two new peaks at 318 nm and 350 nm appeared due to the conjugation of folic acid and PEI-PEG. The two peak at 318 nm and 350 nm disappears while a new peak at 270 nm arising due to the conjugation of FA-PEI-PEG and the nano graphene through the hydrophobic and π-π interaction.

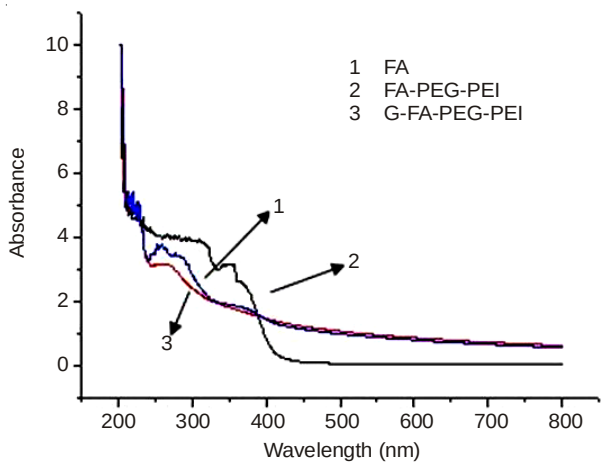


Fig. 8. UV-visible spectra of the folic acid, FA-PEG-PEI and G-FA-PEG-PEI in aqueous solution

Drug loading ratio: The drug loading ratio was determined according to the proposed method[5,11]. The doxorubicin (DOX) was chosen for the experiment[10,23]. The drug loading ratio on NG-FA-PEI-PEG (the weight ratio of drug loaded to NG-FA-PEI-PEG) is about 30.65 % (left tube of Figs. 2 and 9). The interactions of drug and carriers are π-π stacking and hydrophobic[5].

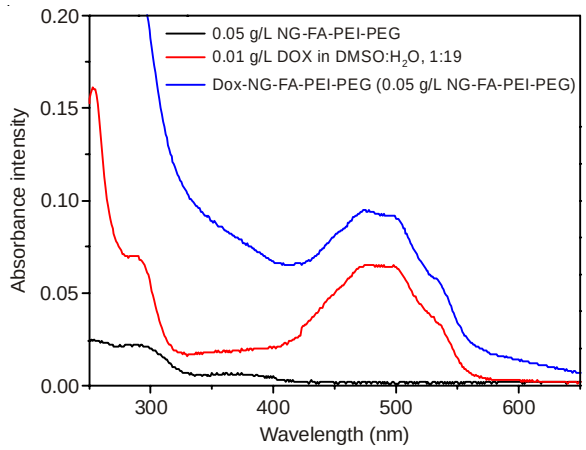


Fig. 9. UV-visible spectra of DOX, NG-FA-PEI-PEG and Dox-NG-FA-PEI-PEG

pH dependent releasing of doxorubicin from NG-FA-PEI-PEG (Fig. 10): Doxorubicin was choice as the model drug to find out the releasing behaviour on NG-FA-PEI-PEG sheets according to the method proposed[5,11,14]. The NG-FA-PEI-PEG was incubated in both PBS (pH = 7.4) and ABS solution (acetate buffered saline solution, pH = 5.5). After 12, 24, 36, 48, 60 and 72 h incubation, the released doxorubicin was removed by filtration through an Anodisc membrane filter (47 mm in diameter, 0.22 μm pore size; Whatman) and the super-natant DOX-NG-FA-PEI-PEG was re-determined for UV-visible spectroscopy measurement. The released percentage of doxorubicin was calculated based on the dropping of EA absorption peak at 485 nm. Fig. 10 showed the release of doxorubicin from the NG-FA-PEI-PEG sheets in pH = 5.5 ABS solution is more obvious than in pH = 7.4 PBS solution with time increasing, so we think the release of doxorubicin from the NG-FA-PEI-PEG sheets is pH dependent. The pH dependence of drug release from NG-FA-PEI-PEG sheets could be exploited for drug delivery applications since the micro-environments of extracellular tissues of tumors, intracellular lysosomes and endosomes are acidic.

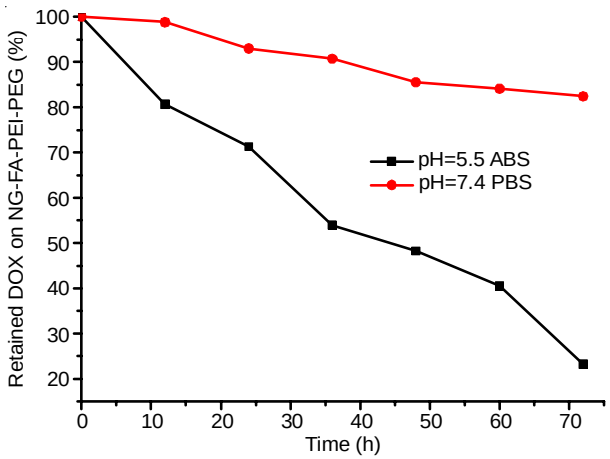


Fig. 10. Correlation plot of retained doxorubicin on reduced graphene oxide sheets with time increasing in different pH solution

Fig. 5 depicted the different concentration of NG-FA-PEI-PEG could induce the cell apoptosis in different degree. When the concentration is less than 30 mg/L, the cytotoxicity effect of NG-FA-PEI-PEG to HL7702 liver cells is slightly decreased. 90 mg/L is a very important point which can affect the cells obviously [Fig. 11(d) and (e)]. In the present experiment, the concentration of 10 mg/L was chosen as the drug delivery system.

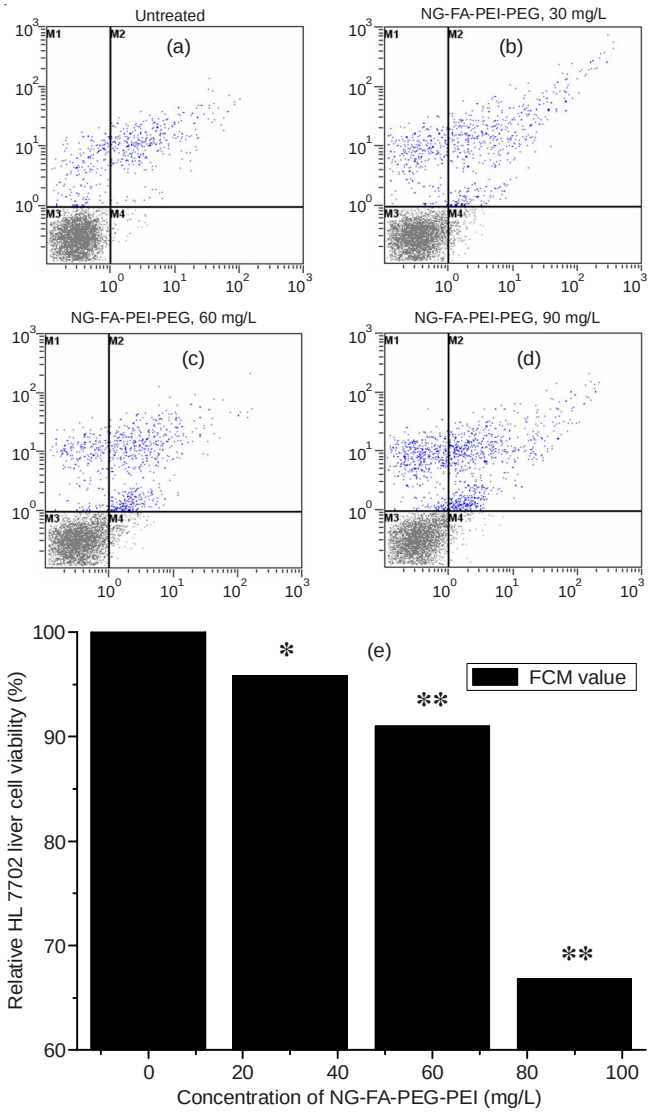


Fig. 11. FCM assay pictures for apoptosis assay of HL7702 liver cells after NG-FA-PEG-PEI treatment. HL7702 liver cells were incubated with NG-FA-PEG-PEI at 0 mg/L (a), 30 mg/L (b), 60 mg/L (c) and 90 mg/L (d) for 48 h. (e) is the column plot which was corresponding to the (a)-(d). M1 represent the regions of necrosis, M2 represent the regions of terminal apoptosis cells, M3 represent the regions of normal cells and M4 represent the regions of early apoptosis cells respectively.The asterisks (*) indicated $P < 0.05$ *versus* the control group (0 mg/L) and double asterisks (**) indicated $P < 0.01$ *versus* the control group. Comparing the all NG-FA-PEG-PEI groups with control group, very obviously statistically significant was obtained. When the P value was less than 0.05, differences were considered statistically significant

NG-FA-PEG-PEI/DOX induce CBRH7919 mouse liver cancer cell to apoptosis: FCM was used to analyze the CBRH7919 mouse liver cancer cell apoptosis, for FCM assay, the caner cell cocultured with the doxorubicin, NG-FA-PEG-PEI and NG-FA-PEG-PEI/DOX. The FCM data of control group (Fig. 12(a)) and the group treated with NG-FA-PEG-PEI, DOX and NG-FA-PEG-PEI/DOX [Fig. 12(a)-(d) were shown in Fig. 12]. Comparing the treated group to control group, the concentration of 10 mg/L NG-FA-PEG-PEI could affect little to the apoptosis of cancer cell [Fig. 12 (b and e)], but the doxorubicin (1 mg/L) can induce the cancer cell to apoptosis obviously as shown in Fig. 12 (c and e). An obvious apoptosis was observed when the cell was treated with the NG-FA-PEG-PEI/DOX (1 mg/L DOX, 10 mg/L NG-FA-PEG-PEI), the relative cellular viability decreased from 82 to 60 % (NG-FA-PEG-PEI/DOX *versus* DOX) [Fig. 12 (c, d and e)]. It suggested that the conjugation of doxorubicin and NG-FA-PEG-PEI could induce the cancer cell apoptosis effectively.

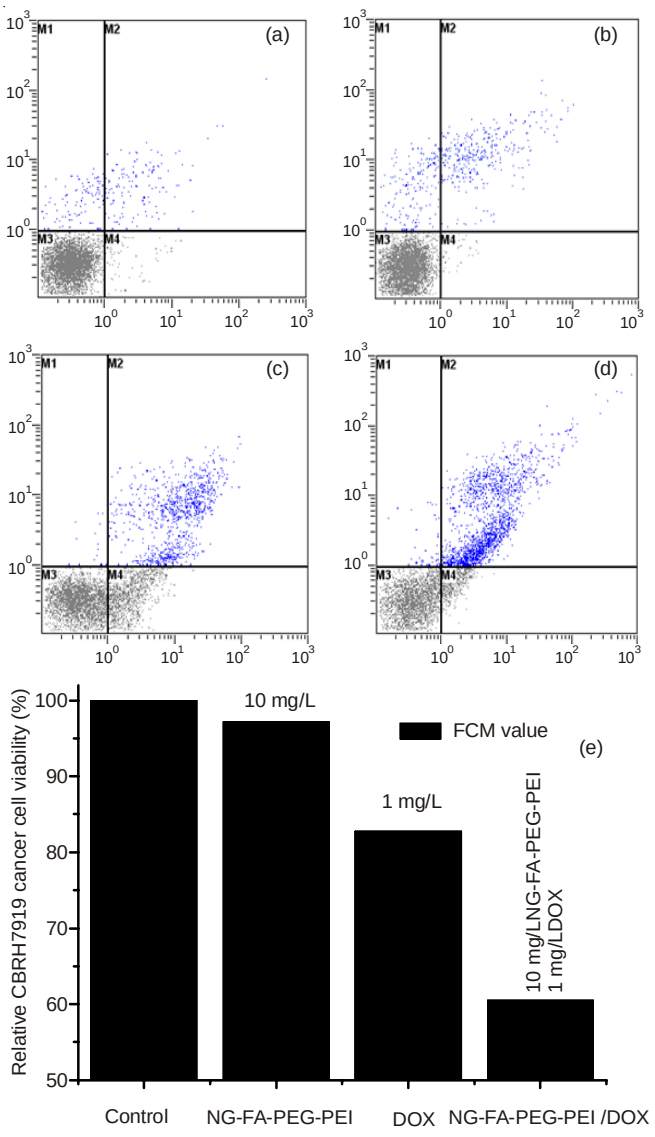


Fig. 12. FCM pictures and the column plot of relative cell viability to the concentration of NG-FA-PEG-PEI and DOX. (a) is the control group data of FCM; (b–d) are FCM date of the group treated with the NG-FA-PEG-PEI (10 mg/L), DOX (1 mg/L) and NG-FA-PEG-PEI/DOX (1 mg/L DOX, 10 mg/L NG-FA-PEG-PEI). (e) is the column plot of relative cell viability to the concentration of NG-FA-PEG-PEI, DOX and NG-FA-PEG-PEI/DOX, respectively

Conclusion

In conclusion, the synthesis of a functional nano graphene with good biocompatibility, physiological stability and its application to the controlled loading and targeted delivery of drugs have been reported. Anticancer drugs were loaded onto nano graphene with high capacity and selectively transported into specific cancer cells by receptor-mediated endocytosis. This work demonstrates that the novel graphene nanostructures is feasible for the controlled loading and targeted delivery of insoluble drugs, which may suggest promising applications of graphene materials in biological and medical areas.

ACKNOWLEDGEMENTS

This work was financially supported by NSFC (Grant No. 21201020) and Shandong Provincial Natural Science Foundation, China (Grant No. ZR2011BL026). Thanks are also due to Ms. Lixia Zhang (Researchers of Binzhou Medical College) for help with cell culture and useful discussion.

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