Enhanced Chemotherapy Efficacy by Sequential Delivery of siRNA and Anticancer Drugs Using PEI-Grafted Graphene Oxide

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The RNA interference (RNAi) technique, an effective method to inhibit protein expression by targeted cleavage of messenger RNA (mRNA), has made substantial progress since the first demonstration of gene knockdown in mammalian cells.[1] Short interfering RNA (siRNA) induces specific silencing of targeted protein, thus offering significant potential in overcoming multiple drug resistance (MDR) of cancer cells.[2] For example, Bcl-2 protein, one of the main anti-apoptotic defense proteins, is closely related to the MDR of cancer cells.[3] Knockdown of the Bcl-2 protein expression level in cancer cells by Bcl-2-targeted siRNA would effectively overcome the MDR of cancer cells and sensitize cancer cells to anticancer drugs.[3d,4] Herein, we report sequential delivery of Bcl-2-targeted siRNA and the anticancer drug doxorubicin (DOX) using polyethylenimine (PEI)-functionalized graphene oxide (PEI-GO). We demonstrate that the PEI-GO is an excellent nanocarrier for effective delivery of siRNA and chemical drugs, and that sequential delivery of the siRNA and DOX by PEI-GO into cancer cells exhibits a synergistic effect, which leads to a significantly enhanced chemotherapy efficacy. To the best of our knowledge, this is the first report on applications of GO-based nanovectors for delivery of siRNA, and sequential delivery of siRNA and anticancer drugs into cancer cells.

Graphene, a newly discovered 2D nanomaterial, has been studied extensively due to its fundamental importance and potential applications,[5] while exploration of its biomedical applications has just started.[6] Noncovalent adsorption through π–π stacking, electrostatic, and other molecular interactions has proven to be effective for immobilizing chemical drugs, single-stranded DNA, and RNA onto GO sheets.[6a–e] However, it is hard to load double-stranded DNA (dsDNA) or double-stranded RNA (dsRNA) via the aforementioned physisorption method onto GO,[6d] thus limiting the widespread applications of GO as a gene or RNA vector. Cationic polymers such as PEI are widely used for gene and siRNA delivery.[7] We have recently demonstrated the potentiality of GO as an effective nanocarrier for delivery of multiple anticancer drugs.[6e] Therefore, integration of GO and cationic polymers is expected to facilitate loading of siRNA via electrostatic adsorption and aromatic anticancer drugs via π–π stacking onto GO sheets.

In our strategy, GO was first prepared according to our previous work,[6e] followed by covalent linking of PEI to GO through formation of an amide bond using EDC chemistry (EDC=*N*-ethyl-*N*'-[3-(dimethylamino)propyl]carbodiimide hydrochloride), as illustrated in **Figure 1**. The chemical conjugation of PEI to GO was confirmed by Fourier-transform infrared spectroscopy and elemental analysis. As shown in Figure S1 in the Supporting Information (SI), a strong IR absorption peak appearing at 1726 cm^{-1} is assignable to carbonyl stretching of the COOH groups of GO. After GO was reacted with PEI, the peak at 1726 cm^{-1} almost vanished and a new prominent peak at 1631 cm^{-1} appeared, which can be attributed to the stretching mode of amide groups.

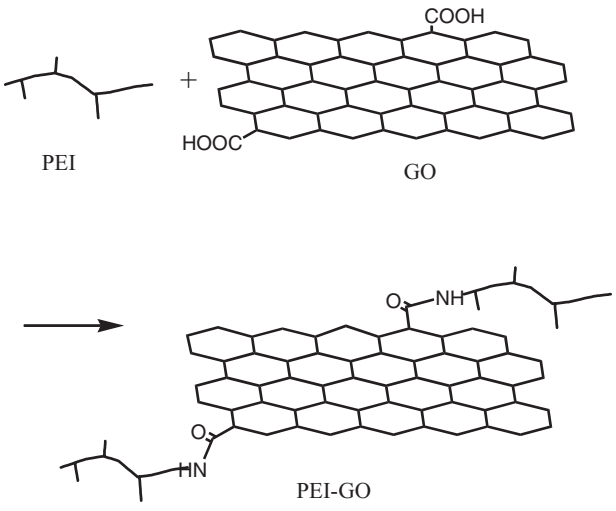

Figure 1. Schematic showing synthetic route of the PEI-GO nanocarrier.

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Furthermore, two strong peaks at 2850 and 2925 cm^{-1} are clearly seen in the IR spectrum of the resultant product. These two bands are due to the symmetric and asymmetric stretching modes of methylene groups of PEI molecules (Figure S1, SI). Elemental analysis shows that the PEI-GO consists of C 45.3, H 10.3, and N 18.4 wt%. The amount of conjugated PEI in PEI-GO was determined to be 42.9% based on elemental analysis.

Atomic force microscopy measurements revealed that the lateral size of the GO sheet (about 200 nm) did not change much before and after PEI functionalization, while the thickness increased from 1–2 nm for GO to 3–4 nm for PEI-GO (**Figure 2**), mainly due to attachment of PEI on both planes of the GO sheet. The resultant PEI-GO was well dispersed in saline solution (10% NaCl) and did not precipitate even after centrifugation at 13 000 rpm for 2 h, which is very important for biological applications. The zeta-potential of PEI-GO was determined to be +55.5 mV, which facilitates adsorption of negatively charged DNA or RNA via electrostatic interaction and cellular uptake.[7b] Preparation of PEI-GO by noncovalent adsorption of PEI to GO by electrostatic and hydrogen-bonding interactions was also conducted.[6c] We found that the thus-obtained PEI-GO was unstable in saline solution. It precipitated completely in 10% NaCl solution in 24 h, most likely due to the gradual removal of adsorbed PEI in the saline solution (Figure S2, SI), thus limiting its applications in drug and RNA delivery.

Next, the biocompatibility of the covalently bonded PEI-GO and loading of siRNA by the PEI-GO carrier were investigated. Cell viability by the WST assay demonstrated that the PEI-GO conjugate shows much lower cytotoxicity than PEI 25 K (Figure 2b). Cellular uptake of free RNA molecules is usually difficult due to the strong negative charges that they bear.[7b] Loading of the negatively charged biomolecules by cationic polymers is widely adopted to solve the problem.[7b,8] In our experiment, loading of the siRNA on the PEI-GO vector was achieved by mixing of siRNA and GO-PEI in aqueous solution. An electrophoretic mobility experiment with the complex of siRNA and PEI-GO formed at different N/P ratios reveals complete adsorption of the siRNA on the PEI-GO conjugates when the N/P ratio was greater than 2.5 (**Figure 3**a). To study the uptake of siRNA loaded on PEI-GO in vitro, fluorescein isothiocyanate (FITC)-labeled siRNA was complexed with PEI-GO, and then incubated with HeLa cells for 2 h. Strong fluorescence from FITC was observed in the cells (Figure 3b). However, when FITC–siRNA was incubated with HeLa cells, no intracellular fluorescence was seen (Figure 3b). These facts indicate the important role of PEI-GO in transporting siRNA into cells.

Furthermore, we studied the knockdown efficiency of the Bcl-2 protein expression level by PEI-GO/Bcl-2-targeted siRNA. When the N/P ratio was 20, the protein expression level was dramatically downregulated (Figure 3c and SI, Figure S3). The siRNA with a concentration of 20 to 160 nM

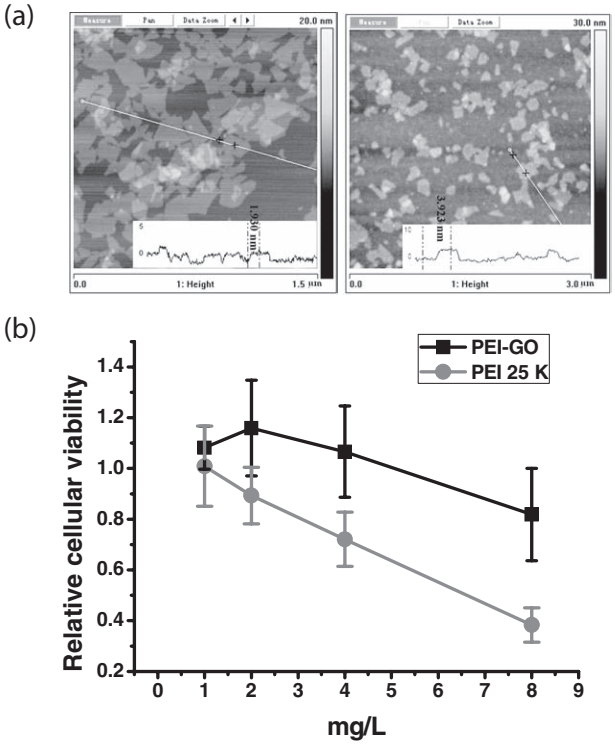

Figure 2. a) AFM images of GO (left) and PEI-GO (right); b) relative cellular viability of HeLa cells treated by PEI-GO and PEI 25 K, respectively.

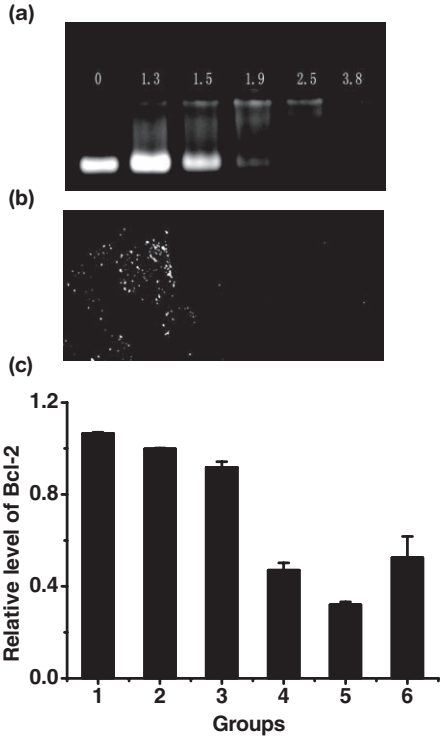

Figure 3. a) Electrophoretic mobility of siRNA complex with PEI-GO at different N/P ratios; b) fluorescent microscopy images of PEI-GO/FITC-siRNA complexes (left) and free FITC-siRNA (right) incubated with HeLa cells for 2 h; c) Relative Bcl-2 protein expression level of HeLa cells treated with different concentration of PEI-GO/Bcl-2 targeted siRNA at different N/P ratio (siRNA concentration 80 nM). N/P ratios of columns 1–5 are 2.5, 5, 10, 15, 20, respectively; column 6 is a control using PEI 25 K at its optimal condition with best down-regulated efficiency and low cytotoxicity.

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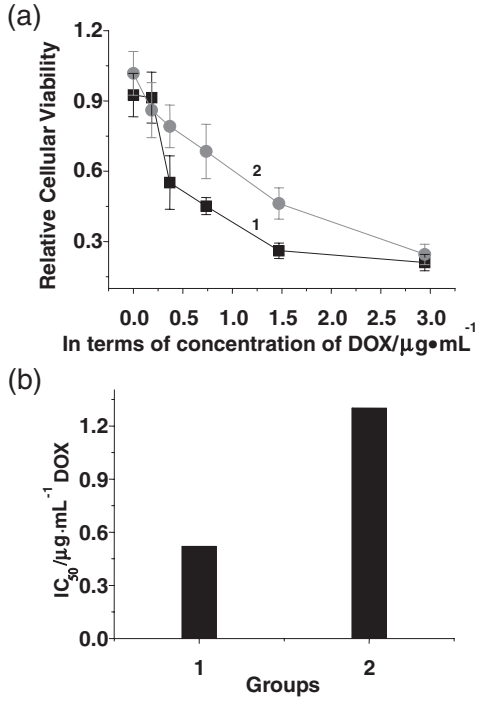

Figure 4. a) Relative viability of HeLa cells after being treated with 1) PEI-GO/Bcl-2 targeted siRNA and 2) PEI-GO/scrambled siRNA, for 48 h, followed by incubation with PEI-GO/DOX for 24 h; b) cytotoxicity, represented by IC50 in terms of DOX for the HeLa cells sequentially incubated with 1) PEI-GO/Bcl-2 targeted siRNA and PEI-GO/DOX, and 2) PEI-GO/scrambled siRNA and PEI-GO/DOX.

exhibits a substantial knockdown efficiency of 30 to 60% (SI, Figure S4). When the concentration of the siRNA was 80 nM and the N/P ratio was 20, the relative level of protein expression was only ~30% compared to that of the control cells (Figure 3c and SI, Figure S3,S4), while very low cytotoxicity of PEI-GO/siRNA was observed under these transfection conditions (the relative cellular viability was higher than 90%, see **Figure 4**a). When PEI was used to transfect siRNA into cells, the highest knockdown efficiency was 52.6%, under optimal conditions with equivalent cytotoxicity to PEI-GO (Figure 3c). Consequently, the siRNA loaded on PEI-GO gives a much better inhibition efficacy of the Bcl-2 expression level than that loaded on PEI 25 K.

In our previous work, GO was employed to load and deliver anticancer drugs such as DOX into cancer cells.[6c] In the current work, we studied loading and delivery of DOX by PEI-GO into HeLa cells. An absorption peak at ~490 nm in the UV–vis spectrum is indicative of successful formation of PEI-GO/DOX composites (SI, Figure S5).[6e] We then incubated the PEI-GO/DOX with HeLa cells for 2 h. After that the cellular uptake was checked with fluorescence microscopy. Adsorption of fluorescent molecules to PEI-GO usually results in quenching of the fluorescence, due to fluorescence energy transfer.[6a] As shown in the SI, Figure S6, loading of DOX onto PEI-GO leads to nearly complete quenching of DOX fluorescence. Release of DOX from the GO by addition of ethyl alcohol resulted in recovery of the fluorescence. Therefore, the fluorescence signal from DOX enables us to monitor the release of DOX from the PEI-GO carrier in the cells. For DOX loaded onto the PEI-GO, the fluorescence from DOX appears strong in cytoplasm after incubation with HeLa cells for 2 h (SI, Figure S7a). On the contrary, after free DOX was incubated with cells, DOX molecules were found mostly in the nucleus (SI, Figure S7c). The different intracellular distributions of DOX for the free DOX and that loaded on PEI-GO suggest that the PEI-GO/DOX complexes were stable in the cell medium, and little DOX was released before cellular uptake.

The strong fluorescence from DOX in the cytoplasm for the PEI-GO/DOX incubated with cells is indicative of intracellular release of DOX from the PEI-GO. It is necessary for DOX to be released from PEI-GO, enter the nuclei, and bind with DNA to show its anticancer activity, because DOX is an anticancer drug targeted towards the DNA. Unlike free DOX, which is a small molecule that can easily enter the cell nucleus by diffusion, DOX loaded onto PEI-GO is released from the carrier and then diffuses to the nucleus. Therefore, a longer incubation time is expected to allow observation of the drug in the nucleus. Just as we expected, strong fluorescence from DOX was observed in the nucleus when the HeLa cells were incubated with the PEI-GO/DOX complex for 6 h (SI, Figure S7b). The mechanism for uptake of the GO-PEI/DOX by cells and release of the drug from the nanocarrier is still not clear. It is very likely that endocytosis is responsible for the cellular uptake of the GO-PEI/DOX, and the lower pH in the endosome facilitates release of the drug from GO.[6b,e]

Finally, we investigated the synergistic effect of DOX and Bcl-2-targeted siRNA by codelivery and sequential delivery of DOX and the siRNA to HeLa cells by the PEI-GO nanocarrier. Simultaneous loading of siRNA and DOX was achieved by electrostatic and π–π interactions, respectively. We found that the codelivery of siRNA and DOX showed no significant synergistic effect on killing cancer cells, most likely due to slow downregulation of protein expression by Bcl-2-targeted siRNA compared with rapid DOX-DNA interactions, thereby generating anticancer efficacy. To address this issue, sequential delivery of siRNA and DOX into HeLa cells was studied. We first incubated HeLa cells with GO-PEI/siRNA containing 80 nM Bcl-2-targeted siRNA at N/P 20 for 5 h, and continued the incubation for another 43 h after the fresh medium was replaced. The cells were then treated with DOX-loaded PEI-GO complex for 24 h, and the WST assay was performed. For comparison purposes, scrambled siRNA was used as a control throughout the experiment. Under these transfection conditions, PEI-GO/Bcl-2-targeted siRNA complex would inhibit ~70% of the Bcl-2 expression level (Figure 3c), without generating much cytotoxicity. If no PEI-GO/DOX was added, the relative cellular viability was 92.5% (Figure 4a). However, the PEI-GO/Bcl-2-targeted siRNA complexes significantly enhanced the cytotoxicity of the PEI-GO/DOX, compared to the PEI-GO/scrambled siRNA complexes, as evidenced by a dramatic decrease in the IC_{50} (concentration of a drug that kills 50% of cells) of DOX. The IC_{50} value for DOX incubated with cells in the presence of PEI-GO/scrambled siRNA, and that in the presence of PEI-GO/Bcl-2-targeted siRNA, was determined to be 1.3 and 0.52 μg mL^{-1}, respectively (Figure 4b). The substantially lower cellular viability for the PEI-GO/DOX

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complexes in the presence of the Bcl-2-targeted siRNA suggests a strong synergistic anticancer effect of the PEI-GO/DOX and PEI-GO/Bcl-2-targeted siRNA.

In conclusion, we have developed a PEI-grafted GO nanocarrier for delivery of siRNA and chemical drugs. PEI-GO shows significantly lower cytotoxicity and substantially higher transfection efficacy of siRNA, at optimal N/P ratio, than PEI 25 K. Furthermore, we demonstrated that sequential delivery of siRNA and DOX by the PEI-GO nanocarrier leads to significantly enhanced anticancer efficacy, which, we believe, may provide insight into designing and constructing GO-based novel nanocarriers for efficient chemical drugs, siRNA, and gene delivery.

Experimental Section

Synthesis of PEI-GO: GO was prepared according to our previous work.[6e] The GO was conjugated with PEI by formation of an amide bond between PEI and GO in the presence of EDC. Briefly, GO (10 mg) was dispersed in distilled water (10 mL), to which an aqueous solution of branched PEI 25 K (100 mg, dissolved in 10 mL distilled water) and EDC solution (10 mg mL^{-1}, 5 mL) were added. After the mixture was stirred for 30 min, additional EDC (10 mg mL^{-1}, 15 mL) was added and stirring was continued overnight. The product was purified according to the following protocol: NaCl (1.6 g) was added to the mixture and dissolved. The supernatant was collected after centrifugation at 13 000 rpm for 2 h, and filtered via a 100 K ultrafilter. The retainer in the ultrafilter was repeatedly washed with aqueous solution containing 10% NaCl and 10% urea to remove any unreacted PEI. Free PEI in the ultrafiltrate was detected by ninhydrin, to confirm complete removal of unreacted PEI. The product was repeatedly washed and then ultrafiltered to remove NaCl and urea, and was finally dispersed in water to form a stable solution (~1.57 mg mL^{-1} PEI-GO).

Cell Culture: HeLa, HepG 2, and HEP-2 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were seeded in tissue culture flasks (about 3×10^5 cells) and incubated in a fully humidified atmosphere at 37 °C with 5% CO_2. For WST assays, the cells were seeded in 96-well plates at a density of 3×10^3 cells per well in culture medium (200 μL) and maintained for 24 h. For siRNA transfection assays, cells were seeded in six-well plates at a density of 5×10^5 cells per well in culture medium (2 mL) for 16 h.

Uptake of PEI-GO/FITC-siRNA Complexes: PEI-GO/FITC-siRNA complexes were prepared by mixing of PEI-GO (4.8 μg) and FITC-siRNA (10 μg) in cell medium (500 μL) for 30 min. The resultant PEI-GO/FITC-siRNA complexes were incubated with HeLa cells in 24-well plates for 2 h at 37 °C. Finally, fluorescence microscopy images were captured. The same concentration of free FITC-siRNA was also incubated with HeLa cells for comparison purposes.

siRNA Transfection: PEI-GO/siRNA complexes were prepared at different N/P ratios by mixing of PEI-GO and siRNA. After incubating the cells (seeded in six-well plates) with the complexes for 5 h, the medium in the wells was changed to fresh medium containing FBS. The cells were further incubated for another 43 h. The relative Bcl-2 protein expression level was checked by Western blot assay. Bcl-2-targeted siRNA sequences were outlined according to the literature[4c] as follows: sense strand: 5'-GUACAUCCAUUAUAAGCUGdtdt-3'; and antisense strand: 5'-CAGCUUAUAAUGGAUCdtdt-3'.

Western Blot Assay: To probe the change in Bcl-2 protein level, total proteins were extracted from PEI-GO/siRNA-transfected cells. The proteins were resolved electrophoretically on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel under denaturing conditions and transferred onto poly(vinylidene fluoride) (PVDF) membranes. The membranes were blocked overnight at 4 °C and probed with rabbit primary polyclonal anti-Bcl-2 (1:900 dilution) and anti-tubulin β (1:900 dilution) antibodies at 25 °C for 2 h. Following incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies for 50 min, the proteins were detected using ECL reagent. Band intensities were quantified using FUJIFILM Multi Gauge Version 3.11. Values were normalized to tubulin β level, and then expressed relative to the nontransfected control.

Codelivery and Sequential Delivery of siRNA and DOX: Loading of DOX onto PEI-GO was carried out according to our previous work,[6e] and was monitored by UV–vis spectroscopy and quantified by the absorbance at 490 nm. PEI-GO/DOX/siRNA complexes were prepared by mixing of PEI-GO/DOX with siRNA for 30 min. For codelivery of siRNA and DOX, HeLa cells were treated with PEI-GO/DOX/siRNA complexes containing 80 nM siRNA at N/P ratio 20 and different concentrations of DOX for 5 h, and then the cells were incubated with fresh cell medium. For sequential delivery of siRNA and DOX, HeLa cells were treated with PEI-GO/siRNA complexes containing 80 nM siRNA at N/P ratio 20 for 5 h, and then the cells were incubated with fresh cell medium for another 43 h, followed by incubation with PEI-GO/DOX for 24 h. For evaluation of cytotoxicity, WST assays were conducted and IC_{50} doses of DOX loaded were then calculated.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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