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PAPER

The preparation of functionalized graphene oxide for targeted intracellular delivery of siRNA†

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Functionalized graphene oxide (GO) for the targeted intracellular delivery of hTERT siRNA was prepared by conjugating GO with polyethylene glycol (PEG) and folic acid, followed by the loading of siRNA with the aid of 1-pyrenemethylamine hydrochloride *via* π–π stacking. It was found that it could target the HeLa *in vitro* and the transfected hTERT siRNA could knockdown the protein expression level and mRNA level efficiently.

1. Introduction

RNA interference (RNAi) is an effective method of investigating the functional consequences of target inactivation in mammalian cells and model organisms.[1] RNAi is mediated by small interfering RNA (siRNA), produced from long double stranded RNAs (dsRNA) of exogenous or endogenous origin, by an endonuclease called dicer. siRNA engages in sequence-specific interactions to inhibit gene expression by RNA degradation. Telomerase is the key enzyme for the stabilization of chromosomes by adding TTAGGG repeats to the telomere ends.[2] The activation of telomerase is critical for immortalization and is detected in the majority of malignant tumors, but not in most normal somatic cells. Knockdown of the telomerase protein expression level in cancer cells by telomerase targeted siRNA would effectively suppress the proliferation of cancer cells. However, the delivery of siRNA to target cells is also hampered by the siRNA stability, low transfection efficiency and properties of their biodistribution and pharmacokinetics. Thus, although siRNA has great promise as a tool to address basic biological questions and therapeutic strategies, better siRNA delivery is necessary to realize this potential.

Graphene is a fascinating new nanocarbon material with one-atom thickness and a large two-dimensional plane, which provides it with a large specific surface area. At the same time, the abundant surface chemistry of graphene after being oxidized offers an opportunity for loading large amounts of molecules, including small chemical molecules, biological macro-molecules and various targeting molecules. Its applications in biological and biomedical fields, including anticancer drug delivery,[3–8] photodynamic therapy[9,10] and imaging *in vivo*,[4,11] *etc.*, have been explored. Recently, GO based gene delivery systems have attracted increasing attention. Zhang *et al.*[12] reported the sequential delivery of siRNA and anticancer drugs using PEI-grafted graphene oxide. Liu *et al.*[13] prepared a complex of GO and PEI by noncovalent functionalization, followed by the binding of plasmid DNA *via* electrostatic interactions for the intracellular transfection of the enhanced green fluorescence protein gene in HeLa cells. In addition, Yang *et al.*[14] proved that functionalized nanoscale graphene oxide can protect oligonucleotides from enzymatic cleavage and efficiently deliver oligonucleotides into cells. Lin *et al.*[15] also reported that single-stranded DNA adsorbed on a graphene surface was effectively protected from enzymatic cleavage by DNase I, and by constraining a single-stranded DNA probe on graphene, improved the specificity of its response to a target sequence. These results indicate GO to be a promising candidate for gene therapy and gene interference.

The potential toxicity of GO is of more and more concern before it can be further used in biomedical fields such as drug delivery, photothermal therapy and imaging *in vivo*. There are numerous papers studying the behavior and toxicology of GO *in vitro* and *in vivo*.[9,16–22] The reports have shown that GO with a biocompatible coating (*e.g.*, protein, PEGylation) exhibited negligible *in vitro* toxicity to many cell lines.[23] In particular, it is of interest that Liu *et al.*[22] have studied the long-term *in vivo* biodistribution of 125I-labeled nanographene sheets (NGS) functionalized with polyethylene glycol (PEG) and systematically examined the potential toxicity of graphene. They found

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that PEGylated NGS mainly accumulated in the reticuloendothelial system, including the liver and spleen after intravenous administration and can be gradually cleared. This indicates that PEG modified NGS does not show appreciable toxicity at appropriate doses.

Good biocompatibility is essential for materials that are used for various biological deliveries, such as gene and drug delivery and intracellular tracking. PEG is biocompatible with a high aqueous solubility and stability in physiological solutions, including serum, and is non-toxic. Surfaces covered with PEG have been shown to prevent the absorption of plasma proteins, thereby increasing the nanoparticles' blood circulation time and allowing them to reach their target tissues and decreasing their uptake by macrophage cells.[24] Therefore, GO modified with PEG can not only decrease the toxicity of GO, as proved by many groups,[3,4,22] but can also enhance both its solubility and biocompatibility.

The ability for efficient delivery and the enhanced special cellular uptake into cancer cells is one major focus area for research into gene carriers. A well-known strategy for achieving efficient tumor targeting is to conjugate carriers with specific ligands that can recognize molecular signatures on the cancer cell surface. It is reported that the surface of many tumor cells, such as ovarian, colorectal, breast and cervical cancer cells, overexpress the folate receptor, while the surfaces of normal tissues and cells do not express the folate receptor.[25] Therefore, folic acid (FA) functionalized GO can be specifically absorbed onto the tumor cells through the specific interaction between the FA on the surface of the functionalized GO and the folate receptor on the surface of tumor cells. Zhang *et al.*[26] reported the targeted delivery of two anticancer drugs by the covalent binding of FA molecules to nanoscale GO and found it can specifically target MCF-7 cells. Based on the above reports, the present work focuses on the exploration of functionalized GO as a carrier for the targeted intracellular delivery of siRNA. We combined the biocompatibility, solubility and the decrease of toxicity by PEG modification, the tumor targeting function of FA molecules and the siRNA-bearing ability, to construct a GO based targeted siRNA vehicle. The conjugation of PEG and FA were used to modify GO to give a tumor targeting GO, which was then loaded with hTERT siRNA with the aid of 1-pyrenemethylamine hydrochloride *via* π–π stacking, to act in the tumor targeting intracellular delivery of siRNA.

2. Experimental

Materials

Graphite was purchased from Qingdao Tianhe Graphite Co. Ltd., Qingdao, China with an average particle diameter of 4 μm (99.95% purity). 1-Pyrenemethylamine hydrochloride ($PyNH_2$), poly(ethylene glycol)bis(3-aminopropyl) terminated (NH_2-PEG-NH_2) (M_n = 2100), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid monohydrate (MES) were purchased from Aldrich. FA was bought from Nanjing Boquan Technology Co., Nanjing, China and used as received. Sephadex G-15 was bought from Shanghai Seebio Biotechnology Inc., Shanghai, China. The FAM-labeled DNA sequence, 5′-TGC-ATT-TTTAAT-GGT-ATT-TA-3′-FAM, was purchased from Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China. Rabbit monoclonal to telomerase reverse transcriptase and β-actin antibody were all purchased from Abcam Co. Goat anti-rabbit IgG-HRP was purchased from Bioworld Technology Inc. siRNA targeting human telomerase reverse transcriptase was designed and synthesized by Santa Cruz Biotechnology Inc. RPMI 1640 culture medium was purchased from HyClone Co. FA-free RPMI-1640 medium and fetal bovine serum (FBS) were purchased from GIBCO Co.

Conjugation of amine-capped polyethylene glycol (NH_2-PEG-NH_2) and FA

FA (0.35 mmol), EDC (0.5 mmol) and NHS (0.5 mmol) were placed into a 50 mL mixture solvent of water and DMSO (1 : 4) in the presence of 10 mM MES. After sonication, the reaction mixture was agitated overnight. Then, 200 μL triethylamine and 0.7 mmol NH_2-PEG-NH_2 were added into the reaction mixture and it went on to be stirred for 24 h at room temperature. After reaction, the solution was condensed by rotary evaporation. The small molecular compounds were then removed from the condensate by a Sephadex G-15 column. The first yellow fraction was condensed and lyophilized. The product NH_2-PEG–FA was obtained.

Conjugation of GO with NH_2-PEG–FA

Graphene oxide (GO) was prepared from purified natural graphite according to a modified Hummers method.[27] The mole percentage of the carboxylic acid groups on GO was about 8%, determined according to our previous method. 5 mg GO (the concentration of carboxylic acid groups on GO is about 0.033 mmol) was dissolved in 5 mL aqueous solution by sonication, then MES (0.25 mmol), EDC (0.5 mmol) and NHS (0.5 mmol) were added into the above aqueous solution and it was sonicated for 1 h followed by stirring overnight. The reaction solution was adjusted to pH ~ 9 by dropping in 100 μL triethylamine, then 0.05 mmol NH_2-PEG-FA was added into the reaction mixture and it was stirred for 4 days at room temperature. After the conjugation reaction, the suspension was ultracentrifuged and the precipitates were washed with distilled water, with several ultracentrifugation and redispersion cycles over several days. The absorbance of the supernatant was recorded on a UV absorption spectrometer to ensure that any excess free NH_2-PEG-FA was removed from the solution. The product GO-PEG-FA was dispersed in 10 mL distilled water. GO was reacted with NH_2-PEG-NH_2 to obtain GO-PEG as a control by the same steps.

Conjugation of GO-PEG-FA with 1-pyrenemethylamine hydrochloride ($PyNH_2$)

The above 5 mL GO-PEG-FA was mixed with 0.5 mL (1 mg mL^{-1}) 1-pyrenemethylamine hydrochloride and alternately agitated and immersed in a laboratory sonication bath at room temperature for 24 h. The resulting GO-PEG-FA-$PyNH_2$ was washed with distilled water, followed by several ultracentrifugation and redispersion cycles. The absorbance of the

supernatant was recorded on a UV absorption spectrometer to ensure that any excess free $PyNH_2$ was removed from the solution. All of the supernatant was collected after washing and detected using UV-vis absorption spectroscopy at a wavelength of 341 nm to measure the concentration of $PyNH_2$ in the supernatant. This concentration was used to calculate the loading amount of $PyNH_2$ on GO-PEG-FA according to the following formula:

$$PyNH_2 \text{ loading capacity} = (W_{\text{administered dose}} - W_{\text{residual dose in solution}})/W_{\text{GO-PEG-FA}}$$

where $W_{\text{administered dose}}$ is the weight of initial $PyNH_2$ for loading, $W_{\text{residual dose in solution}}$ is the weight of residual $PyNH_2$ in solution after loading onto GO-PEG-FA, and $W_{\text{GO-PEG-FA}}$ is the weight of GO-PEG-FA for loading.

GO-PEG was reacted with $PyNH_2$ to obtain GO-PEG-$PyNH_2$ as a control by the same steps.

Uptake of GO-PEG-FA-$PyNH_2$ by human cervical carcinoma cell line (HeLa)

Cell uptake studies were performed using HeLa. To investigate the targeted uptake of GO-PEG-FA-$PyNH_2$ by HeLa, cellular uptake of the GO-PEG-FA-$PyNH_2$ was measured by confocal fluorescence microscopy. FAM-labeled DNA was loaded on GO-PEG-FA-$PyNH_2$ by the incubation of FAM-labeled DNA (20 μmol L^{-1}, 3 μL) with an aqueous suspension of GO-PEG-FA-$PyNH_2$ (0.06 mg mL^{-1}, 500 μL) for 2 h to mix them together. As a control, GO-PEG-$PyNH_2$ was treated with FAM-labeled DNA by the same steps.

The HeLa were first cultured in an FA-free RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a fully humidified atmosphere at 37 °C containing 5% CO_2, followed by exposure to GO-PEG-FA-$PyNH_2$ and GO-PEG-$PyNH_2$ loaded with FAM-labeled DNA, with a final concentration of 0.02 mg mL^{-1} at 37 °C for 1 h and 5 h respectively. Finally the incubated HeLa were washed with PBS buffer.

GO-PEG-FA-$PyNH_2$ loading with siRNA

GO-PEG-FA-$PyNH_2$ was redispersed in Milli-Q with a concentration of 0.5 mg mL^{-1}. GO-PEG-FA-$PyNH_2$ with a final concentration of 0.05 mg mL^{-1} or 0.2 mg mL^{-1} was incubated with 60 nmol L^{-1} hTERTsiRNA in 2 mL RPMI-1640 medium at room temperature for 1 h. The coupling of siRNA with GO-PEG-FA-$PyNH_2$ with a final concentration of 0.05 mg mL^{-1} or 0.2 mg mL^{-1} was incubated with HeLa cells in 6-well plates. Liposome and GO-PEG-$PyNH_2$ with a final concentration of 0.05 mg mL^{-1} or 0.2 mg mL^{-1} coupled siRNA and GO-PEG-FA-$PyNH_2$ without siRNA were used as controls.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The level of hTERT mRNA was analyzed by reverse transcription-PCR after 24 h. Briefly, the total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol, and cDNA was synthesized from 1 μg of RNA using the RNA PCR kit version 2 (TaKaRa, Otsu, Japan) with the primers. The primers used include the following: hTERT, 5′-CGGAAGAGTGTCTGGAGCAA-3′ and 5′-GGATGAAGCGGAGTCTGGA-3′. Reaction parameters were: 94 °C, 30 s; 60 °C, 30 s and 72 °C, 30 s for 35 cycles. The efficiency of cDNA synthesis from each sample was estimated by PCR with glyceraldehyde-3-phosphate dehydrogenase, sense strand siRNA: GTTGCCATCAATGACCCCTTC, antisense strand siRNA: CTTCTCCATGGTGGTGAAGAC.

Western blot analysis

The expression of hTERT protein was analyzed by Western blotting after 48 h. For Western blotting of hTERT protein, nuclear extract was prepared and 30 μg of nuclear extract were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% non-fat dried milk, the membrane was incubated with rabbit anti-TERT monoclonal antibody (1 : 2000) overnight at 4 °C. The membranes were then washed and incubated with goat anti-rabbit IgG-HRP in TBST for 2 h. As a loading control, actin was also assessed using anti-actin antibody.

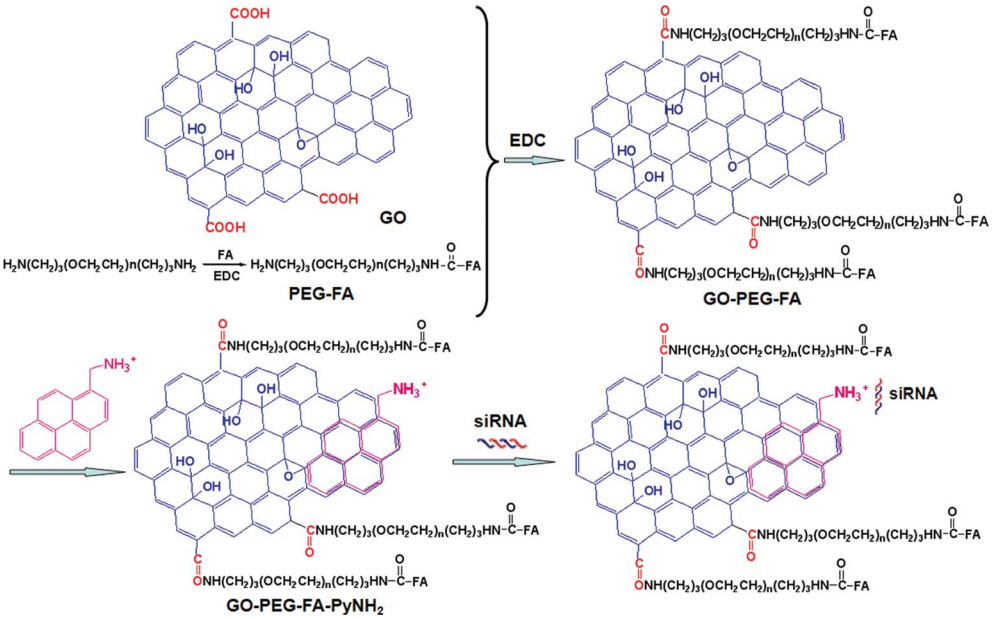
The cell toxicity of GO-PEG-FA-$PyNH_2$ at different concentrations

To investigate the cytotoxicity of the GO-PEG-FA-$PyNH_2$, WST assays were performed and HeLa cells were employed. HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells per well in normal RPMI-1640 medium supplemented with 10% FBS and maintained for 24 h. The cells were then incubated with GO-PEG-FA-$PyNH_2$ (with a final concentration of 0.1, 1, 10, 100, 500 mg L^{-1}) for 24 h. HeLa was treated with GO by the same steps as a control. The relative cell viability was checked by the WST assay.

3. Results and discussion

A representation of the functionalized graphene oxide loaded with siRNA for targeted intracellular delivery was shown in Scheme 1. We employed amino ended PEG for conjugation with FA and GO. GO was prepared from purified natural graphite according to a modified Hummers method.[14] Amino terminated PEG was first conjugated with FA *via* an amide linkage to form a PEG-FA conjugate. PEG-FA was then connected to GO *via* the amide linkage between the amino groups on the other side of PEG-FA and the carboxylic groups on GO, to afford GO-PEG-FA. GO-PEG-FA was then reacted with an anchor molecule, 1-pyrenemethylamine hydrochloride, *via* π–π stacking. Thus, the positive ammonium ion was introduced on the surface of the functionalized GO after acidification, to give GO-PEG-FA-$PyNH_2$. GO-PEG-FA-$PyNH_2$ was then loaded with siRNA by the electrostatic interactions between the positively charged GO-PEG-FA-$PyNH_2$ and the negatively charged siRNA. In our strategy, we used GO-PEG-FA-$PyNH_2$ to transfect human telomerase reverse transcriptase (hTERT) siRNA targeted into the human cervical carcinoma cell line HeLa, and examined the ability of the delivered hTERT siRNA to silence the expression of hTERT.

The morphology of GO-PEG-FA-$PyNH_2$ was characterized with AFM (Fig. 1). GO, before functionalization, shows a height of 0.8–1.0 nm, with a smooth surface, suggesting a single layer



Scheme 1 The preparation of functionalized graphene oxide for targeted intracellular delivery of siRNA.

graphene sheet, as shown in Fig. 1A. Most of the GO has a size below 200 nanometers. After GO was modified, many protuberances were observed on the surface of GO-PEG-FA-$PyNH_2$ (Fig. 1B), suggesting that a large amount of decorations are immobilized onto the GO sheets. The height of the GO-PEG-FA-$PyNH_2$ ranges from several nanometers to around 20 nanometers, since PEG-FA may be bound on the surface of GO as a coil. The GO-PEG-FA-$PyNH_2$ can be stably dispersed in blood (insert of Fig. 1), which may offer an advantage for its long duration circulation in blood.

UV-Vis absorption spectra were used to confirm that PEG-FA and $PyNH_2$ were conjugated to GO, as shown in Fig. 2A. The peak at 282 nm in the UV-vis spectrum of PEG-FA in Fig. 2A(b) is attributed to the characteristic absorbance peak of FA, which has a strong peak at 278 nm for GO-PEG-FA in Fig. 2A(c). The shift from 282 to 278 nm for the FA species before and after the PEG-FA is conjugated with GO may be due to the interaction of GO and the conjugated PEG-FA components. The peaks at around 241, 276 and 342 nm, observed for GO-PEG-FA-$PyNH_2$ in Fig.2A(d), were attributed to the characteristic absorption of the $PyNH_2$ molecules.

The conjugation of PEG with FA and PEG-FA on GO was further characterized by FTIR. Fig. 2B displayed the FTIR spectra of FA (a), PEG-FA (b), GO-PEG-FA (c) and GO (d). The FTIR absorbance peaks for FA-PEG were observed at 1642 cm^{-1} (C–O) and 1536 cm^{-1} (N–H). At the same time, the peaks at around 2872 and 1107 cm^{-1}, corresponding to the C–H of PEG and the peaks at 1690 and 1607 cm^{-1}, attributed to the characteristic peaks of FA, both appeared. All of the peaks for the C–H of PEG and the characteristic peaks of FA in Fig. 2B were slightly shifted after PEG-FA was conjugated with GO. This suggests that PEG-FA is successfully conjugated with GO.

In addition, thermogravimetric analysis (TGA) and the corresponding derivative, thermogravimetry (DTG), were used to characterize the thermal properties of GO-PEG-FA (Fig. S1†). For GO, a weight loss of 18 wt% at about 70 °C and 25 wt% at about 230 °C can be observed, which can be assigned to the loss of adsorbed water on GO and groups on GO such as hydroxyl and carboxylic groups, respectively. In the case of GO-PEG-FA, the initial degradation of 16% mass loss at about 185 °C may be due to the residual oxygen functional groups on GO, the formed amide groups between GO and PEG-FA and the decomposition

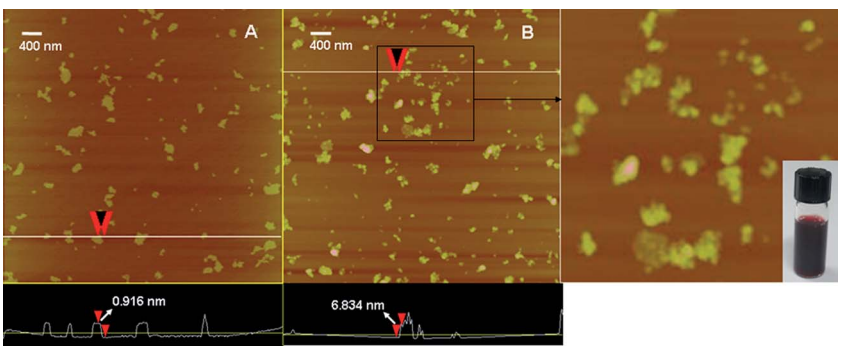


Fig. 1 Typical AFM images of GO (A) and GO-PEG-FA-$PyNH_2$ (B). The inset is a photo of GO-PEG-FA-$PyNH_2$ stably dispersed in blood.

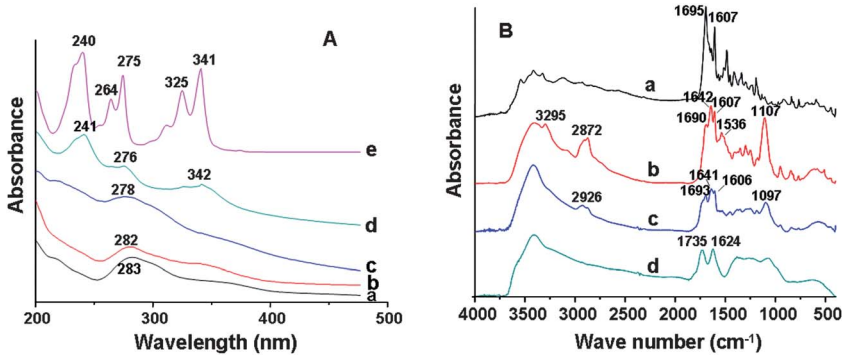


Fig. 2 (A) UV spectra of FA (a), PEG-FA (b), GO-PEG-FA (c), GO-PEG-FA-PyNH$_2$ (d) and PyNH$_2$ (e). (B) FTIR spectra of FA (a), PEG-FA (b), GO-PEG-FA (c) and GO (d).

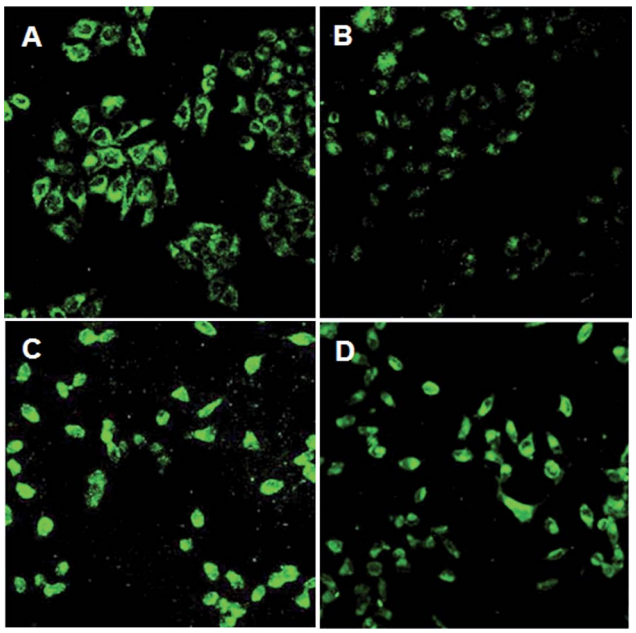


Fig. 3 Confocal fluorescence images of GO-PEG-FA-PyNH$_2$ loaded with FAM-labeled DNA after incubation with HeLa at 37 °C for 1 h (A) and 5 h (C) and GO-PEG-PyNH$_2$ loaded with FAM-labeled DNA after incubation with HeLa at 37 °C for 1 h (B) and 5 h (D).

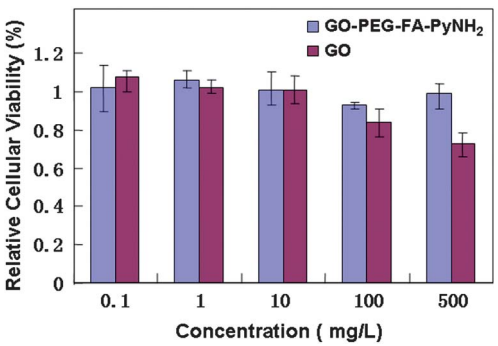


Fig. 5 *In vitro* cell toxicity of GO-PEG-FA-PyNH$_2$ and GO at different concentrations.

of the FA. The weight loss of 17% GO-PEG-FA at around 400 °C is presumably assigned to the decomposition of the PEG in GO-PEG-FA. This also indicates that the PEG-FA is bound onto GO. The loading amount of PyNH$_2$ on GO-PEG-FA was determined as about 0.245 mg mg^{-1} by a UV-vis spectrum at 341 nm, which was calculated by the difference of PyNH$_2$ concentrations between the original PyNH$_2$ solution and the supernatant solution after loading.

First, the ability of functionalized GO tumor targeting cells was evaluated by investigating their uptake by HeLa *in vitro*. In order to evaluate the ability of GO-PEG-FA-PyNH$_2$ to enter into HeLa, we observed the fluorescence levels after

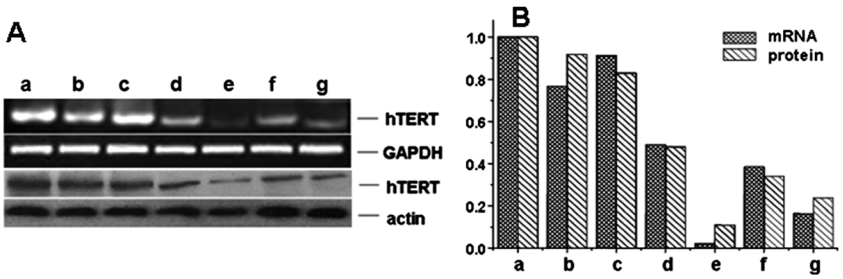


Fig. 4 Specific gene silencing in HeLa cells. A: Upper, level of hTERT transcripts after treatment of HeLa for 24 h with liposome, (b), GO-PEG-FA-PyNH$_2$ (c), GO-PEG-FA-PyNH$_2$ with 0.05 mg mL^{-1} (d), GO-PEG-FA-PyNH$_2$ with 0.2 mg mL^{-1} (e), GO-PEG-PyNH$_2$ with 0.05 mg mL^{-1} (f) and GO-PEG-PyNH$_2$ with 0.2 mg mL^{-1} (g) transfected hTERT siRNA (60 nmol L^{-1}) *versus* untreated control cells (a). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Lower, protein level of hTERT and actin in HeLa treated for 48 h as in (upper). B: The quantitative results for the level of hTERT transcripts and protein levels of hTERT.

GO-PEG-FA-$PyNH_2$ or GO-PEG-$PyNH_2$ (FA free) loaded with FAM-labeled DNA was incubated with HeLa at 37 °C for 1 and 5 h respectively and the cells were observed by confocal fluorescence microscopy. The confocal fluorescence images show that the fluorescence level of FAM-labeled DNA loaded GO-PEG-FA-$PyNH_2$, transfected into HeLa for 1 h, is much higher than that of GO-PEG-$PyNH_2$ (Fig. 3A and 3B) and most of the loaded DNAs were adsorbed on the surface of HeLa. However, after they were incubated with HeLa for 5 h, their fluorescence level is approximately the same and they were both uptaken by HeLa cells. This indicates that GO-PEG-FA-$PyNH_2$ can selectively target the surface of HeLa quickly and both GO-PEG-FA-$PyNH_2$ and GO-PEG-$PyNH_2$ can deliver FAM-labeled DNA into HeLa effectively.

Furthermore, we investigated the knockdown efficiency of the hTERT protein expression level and the hTERT transcript level using GO-PEG-FA-$PyNH_2$ transfected hTERT siRNA. Compared with liposome transfected hTERT siRNA, both GO-PEG-FA-$PyNH_2$ and GO-PEG-$PyNH_2$ transfected hTERT siRNA clearly downregulated the protein expression level and mRNA level as shown in Fig. 4. The knockdown efficiency of the hTERT protein expression level and hTERT transcript level are enhanced with increasing amount of GO-PEG-FA-$PyNH_2$ and GO-PEG-$PyNH_2$. The folate-targeted delivery of hTERT siRNA resulted in more significant gene suppression compared to the non-specific delivery for both the hTERT mRNA and protein expression levels. GO-PEG-FA-$PyNH_2$ without hTERT siRNA did not affect hTERT mRNA expression.

The cell toxicity of GO-PEG-FA-$PyNH_2$ in the absence of hTERT siRNA and GO was detected by WST assay as shown in Fig. 5. After HeLa was incubated with GO for 24 h, the relative cellular viabilities were about 100% below the concentration of 10 mg L^{-1} and they decreased with increasing concentrations of GO, when the concentration of GO is higher than 100 mg L^{-1}. However, the relative cellular viabilities showed a high level after HeLa was incubated with GO-PEG-FA-$PyNH_2$ at relatively higher concentrations (even as high as 500 mg mL^{-1}). No obvious toxicity was observed for various concentrations of GO-PEG-FA-$PyNH_2$ without siRNA loading. These results suggested that the PEG modified GO were not cytotoxic after loading $PyNH_2$.

4. Conclusions

In summary, we prepared a functionalized GO with tumor targeting functions, which can be used for the targeted delivery of hTERT siRNA. Cell uptake studies indicated that GO-PEG-FA-$PyNH_2$ specifically transfected the FAM-labeled DNA to the HeLa cells. RT-PCR and Western blot assays showed that hTERT siRNA delivered *via* GO-PEG-FA-$PyNH_2$ silenced the mTERT expression and suppressed the expression of mTERT protein in HeLa. WST assays indicated that the PEG modified GO was not cytotoxic after loading $PyNH_2$. These results demonstrate that multi-functionalized GO may represent a new class of molecular transporters applicable for siRNA therapeutics.

Acknowledgements

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Supporting Information

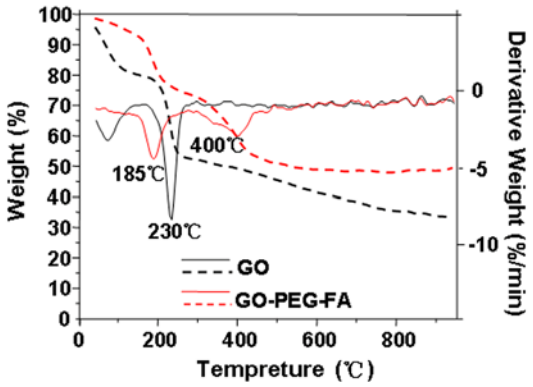

Fig S1. TGA and DTG curves of GO and GO-PEG-FA