Polyethylene Glycol-Polyethylenimine-Tetrachloroplatinum (IV): A Novel Conjugate with Good Abilities of Antitumor and Gene Delivery

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ABSTRACT: It is much importance to develop novel multifunctional delivery systems for the combination therapy of drug and gene. In this work, a novel conjugate, polyethylene glycol-polyethylenimine-tetrachloroplatinum (IV) (PEG-PEI-Pt), with good abilities of antitumor and gene delivery was proposed by combining PEG (Mw 3400 Da), low molecular weight PEI (Mw 800 Da), and tetrachloroplatinum (IV). The antitumoral and gene transfection activities of PEG-PEI-Pt were analyzed in many tumor (A549, A375, HepG-2, HuH-7, and B16 cells) and normal (COS-7 cells) cell lines. Similar to cisplatin (one platinum anticancer drug), PEG-PEI-Pt showed much higher sensitivity in tumor cells than in normal cells. More importantly, PEG-PEI-Pt had a potential to treat drug-resistant tumors. Almost no transfection efficiency was observed for PEI (Mw 800 Da) and PEG-PEI. Very interestingly, PEG-PEI-Pt could condense plasmid DNA efficiently, and exhibited good transfection efficiency in B16, HepG-2, A375 and COS-7 cells, comparable to even higher than PEI 25 kDa. In addition, PEG-PEI-Pt could also effectively deliver siRNA into the cytoplasm of tumor cells. With the good antitumoral and gene delivery abilities, PEG-PEI-Pt may have a great potential for combination therapy of drug and gene. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 1509–1517, 2012

Key words: metal-polymer complexes; nanoparticles; polyelectrolytes; anticancer drug; gene delivery

INTRODUCTION

Since Rosenberg et al. first reported the antitumoral activity of cisplatin,[1,2] platinum based drugs have attracted much attention of many researchers. Pt(IV) complexes are substantially more kinetically inert than Pt(II) ones, making it possible to put the oral administration of Pt(IV) drugs into practice.[3–5] Polynuclear platinum anticancer drugs demonstrate high activity in a broad spectrum of human tumors commonly insensitive to chemotherapy, such as non-small-cell lung cancer, gastric cancer and glioma.[6–8] Polyethylenimine (PEI), one kind of the nonviral gene delivery vectors, has shown high transfection efficiency both *in vitro* and *in vivo*.[9,10] However, the transfection efficiency and cytotoxicity of PEI is dependent on its molecular weight. Low molecular weight PEI has been proved to be nontoxic, but displays poor transfer activity. In recent years, great attention has been paid to the low molecular weight PEIs. Polyethylene glycol (PEG)-modified PEI has improved solubility, minimized aggregation, reduced systemic toxicity and enhanced gene delivery activity.[11–13] Researchers involved in the field of polymer-platinum complexes foucsed on improving the delivery of platinum anticancer drugs to tumor cells,[14–19] but no one has yet undertaken the research on the potential gene delivery activity of certain polymer-platinum complexes.

It is much importance to develop novel multifunctional delivery systems for the combination therapy of drug and gene. To extend the function of platinum drugs, the concept of codelivery of platinum drugs and gene was introduced in this work. A novel conjugate PEG-PEI-Pt with good antitumoral

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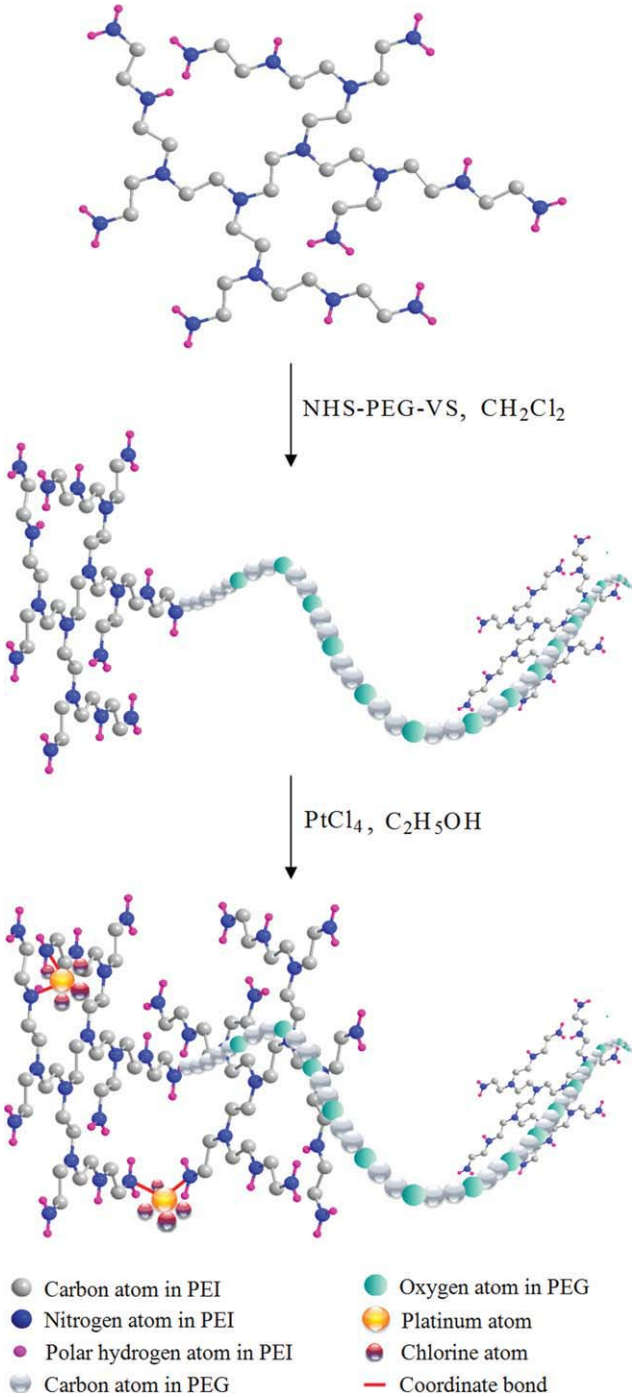
and gene transfection activities was designed by simply combining PEG, low molecular weight PEI (Mw 800 Da), and tetrachloroplatinum (IV). Similar to cisplatin, PEG-PEI-Pt showed higher sensitivity in tumor (A549, A375, HepG-2, HuH-7, and B16 cells) cells than in normal (COS-7) cells. PEG-PEI-Pt showed 3.3-fold higher sensitivity in A549 cells (drug-resistant human lung adenocarcinoma cells) than cisplatin. Very interestingly, PEG-PEI-Pt could also exhibit good transfection efficiency in different cell lines, comparable to even higher than PEI 25 kDa. In addition, it was also confirmed that PEG-PEI-Pt could effectively deliver siRNA into the cytoplasm of tumor cells. Such a conjugate with good antitumoral and gene delivery abilities may play an important role in the collaborative therapy of drug and gene for various malignant cancers in the future, especially for the ones that are drug resistant or not sensitive enough to the therapies in routine clinical use.

EXPERIMENTAL

Materials

PEI (Mw 800 Da, the molar ratio of primary, secondary and tertiary amine groups is 4 : 2 : 3) and 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Tetrachloroplatinum(IV) was purchased from J and K Chemical Ltd. *N*-Hydroxysuccinimide polyethylene glycol vinylsulfone (NHS-PEG-VS, Mw 3400 Da) was obtained from Nektar (San Carlos, CA). Plasmid DNA pGL3 and Luciferase Kit Assay were purchased from Promega Corp. (Madison, WI). A549, A375, HepG-2, HuH-7, B16, and COS-7 cell lines were purchased from American Type Culture Collection (Rockville, MD). A375, HepG-2, and HuH-7 cells were cultured in DMEM (A549, B16 and COS-7 cells in 1640) containing 10% fetal bovine serum (GIBCO Invitrogen Corp. Greenland, NY) and 100 units/mL penicillin in a 5% CO_2 humidified atmosphere at 37°C. The plasmids were amplified in *Escherichia coli* and purified according the supplier's protocol (Qiagen, Hilden, Germany).



Scheme 1 Synthesis of PEG-PEI-Pt. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Synthesis of PEI-Pt and PEG-PEI-Pt

For PEI-Pt, $PtCl_4$ in ethanol was added dropwise into low molecular weight PEI (Mw 800 Da) in ethanol at a molar ratio of 1 : 1 in dark with the protection of nitrogen. A precipitate was formed immediately after the adding of the ethanol solution of $PtCl_4$. The mixture was stirred in dark for 4 h at room temperature and then vaporized at 37°C with a rotatory evaporator. The collected solid was washed in purified water for several times and then lyophilized, giving the final brown product PEI-Pt.

To synthesize PEG-PEI-Pt, the molar ratio of the reactants of PEI and $PtCl_4$ was kept at 1 : 1, and molar ratio of PEG and PEI in reactants was chosen to be 0.05 : 1, which was shown to be suitable for the conjugation of PEG and low molecular weight PEI.[20] NHS-PEG-VS (Mw 3400 Da) in CH_2Cl_2 was added into PEI (Mw 800 Da) in CH_2Cl_2 drop by

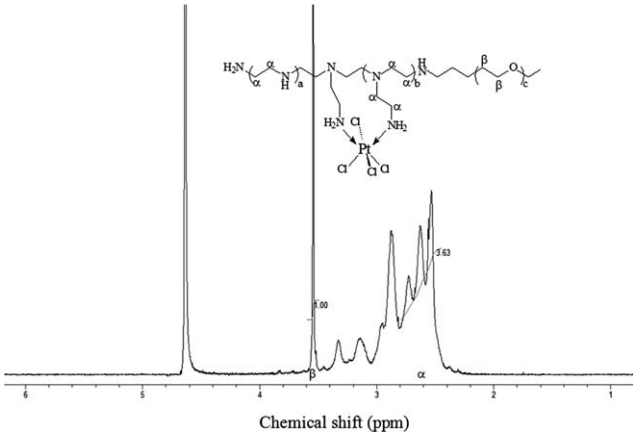


Figure 1 ^{1}H-NMR spectrum of PEG-PEI-Pt.

drop to form polyethylene glycol-polyethylenimine (PEG-PEI) via the reaction between the NHS group of PEG and the amino group of PEI. After that, $PtCl_4$ in ethanol was added dropwise into the above reaction system, stirred for 4 h in dark at room temperature. The mixture was vaporized at 37°C and the collected product was re-dissolved in water, purified and lyophilized, giving the final product PEG-PEI-Pt.

PEG-PEI-Pt characterization

^{1}H-NMR measurements were recorded on a Bruker 400 MHz NMR spectrometer with 32 scans in the Fourier transform mode. Chemical shifts were referred to the solvent peaks (δ = 4.70 ppm for D_2O). X-ray diffraction measurements were carried out using D/Max-2550 X-Ray Diffractometer, Ni-filtered Cu Kα radiation (40 Kv, 300mA). Powder samples were mounted on a sample holder and scanned in 0.02° steps from 3° to 50° (in 2θ) with 0.5 s per step. UV spectra were conducted on a UV spectrometer (UV757CRT), where wavelength scanning started at 500 nm and ended at 270 nm.

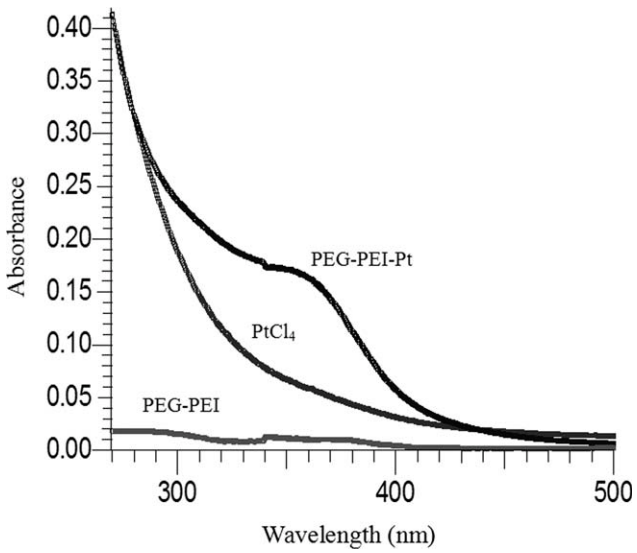


Figure 2 UV spectra of $PtCl_4$,PEG-PEI, and PEG-PEI-Pt.

TABLE I
IC_{50} (μM) for the 24 h of Action of Investigated Materials on A549, A375, HepG-2, HuH-7, B16, and COS-7 Cells Determined by MTT Assay

Cells	IC_{50} (μM)		
	$PtCl_4$	Cisplatin	PEG-PEI-Pt
A549	+	100	33
A375	78	16	43
HepG-2	+	20	63
HuH-7	+	17	65
B16	52	3.5	12
COS-7	+	+	+

"+": IC_{50} values higher than 100 μM.

Cell viability assay

Cell viability was evaluated using MTT assay as described previously.[13] Briefly, test cells were cultured in 96-well plates at a density of 8.0×10^3 cells/well and incubated for 16 h. Then, serial diluted test solutions prepared in complete culture media were used to replace the culture media. At the 24 h incubation, 20 µL of sterilized MTT (5 mg/mL) solution in PBS was added to each well reaching a final concentration of 0.5 mg/mL. After 4 h, unreacted dye was removed by aspiration, and the formazan crystals in each well were dissolved in 100 µL DMSO and measured spectrophotometrically in a microplate reader (Bio-Rad, Hercules, CA) at a wavelength of 570 nm. The relative cell growth (%) related to control cells cultured in media was calculated by $[A]_{test}/[A]_{control} \times 100\%$. All cytotoxicity experiments were performed in triplicate.

Wound healing assays

HepG-2 and A375 cells were kept in the four-well culture plate, respectively. After the cells reach confluence, a sterile pipette tip was drawn across the center of the well to produce a clear wound area. The cellular debris was removed by washing with PBS. The wounded monolayer cells were then incubated with 10% DMEM medium with or without PEG-PEI-Pt. Images were captured at the beginning and 24 h after cell migration to the wound.

Hematoxylin and eosin staining

HepG-2 and A375 cells were incubated on coverslips for 16 h, respectively. After incubated with different concentrations (0, 50 and 100 μM) of PEG-PEI-Pt solutions for 24 h, the cells were fixed in 4%

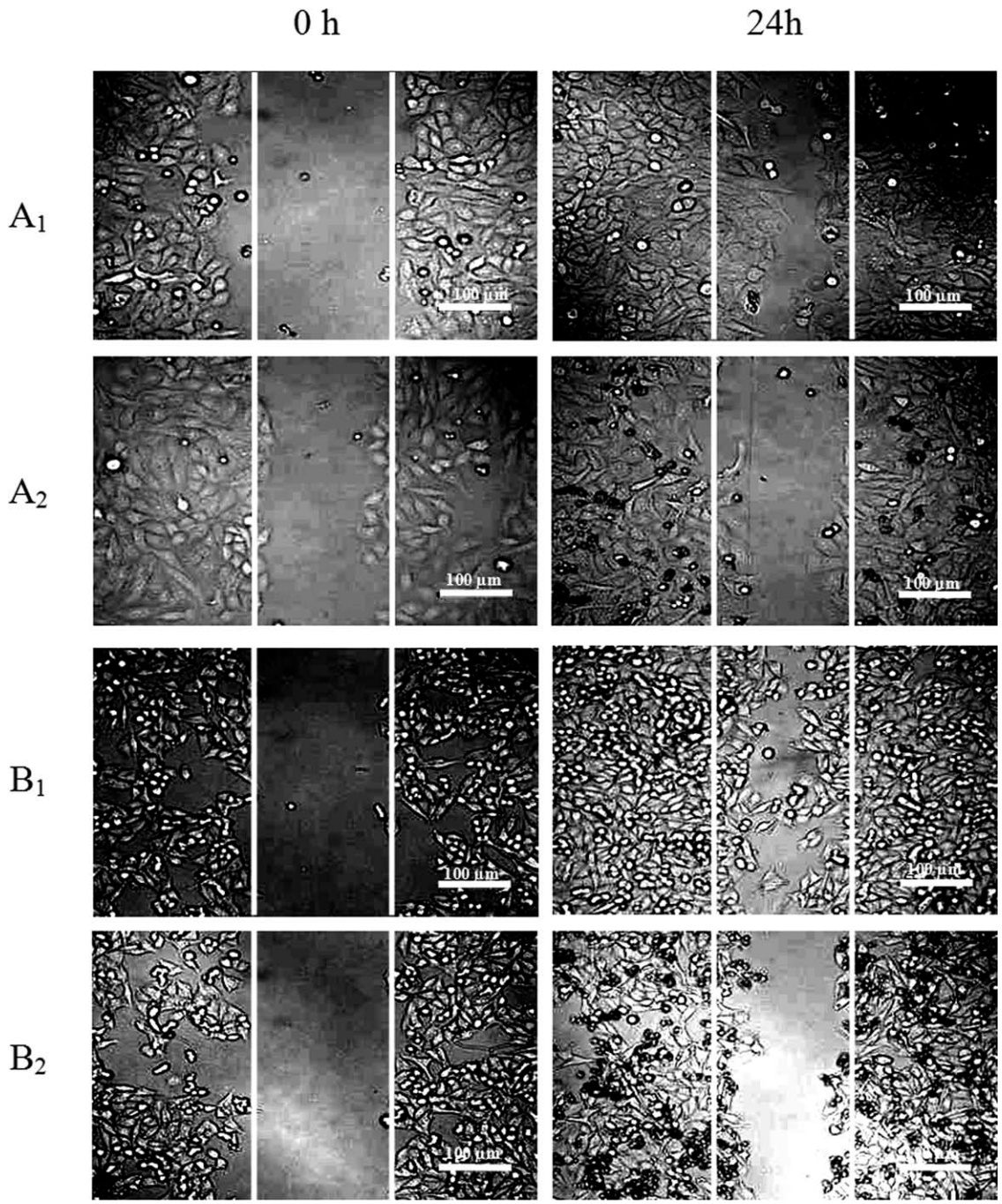


Figure 3 Wound healing assays at different incubation time: (A_1) HepG-2 cells without drug, (A_2) HepG-2 cells incubating with 63 μ*M* PEG-PEI-Pt, (B_1) A375 cells without drug, and (B_2) A375 cells incubating with 43 μ*M* PEG-PEI-Pt.

formaldehyde solution for 10 min. The fixed cells were then washed with PBS thrice, and permeabilized in hematoxylin solution for 15 min. Next, the hematoxylin solution was discarded. The cells were washed with PBS, treated with 1% acid alcohol for a few seconds, and placed in eosin solution for 5 min. After the cells were rinsed with PBS adequately, the images were visualized under fluorescence inverted microscopy (Nikon E400, Melville, NY).

Gene delivery

Transfection efficiency of PEG-PEI-Pt was evaluated in B16, HepG-2, A375, and COS-7 cells, respectively. For preparation of the polymer/DNA complexes, plasmid DNA (pDNA) stock solution was diluted to a chosen concentration (0.5 μg/μL) and was measured by optical density at 260 and 280 nm before use. PEG-PEI-Pt/DNA nanoparticles were prepared by mixing a series of PEG-PEI-Pt with 1.0 μg pGL-3

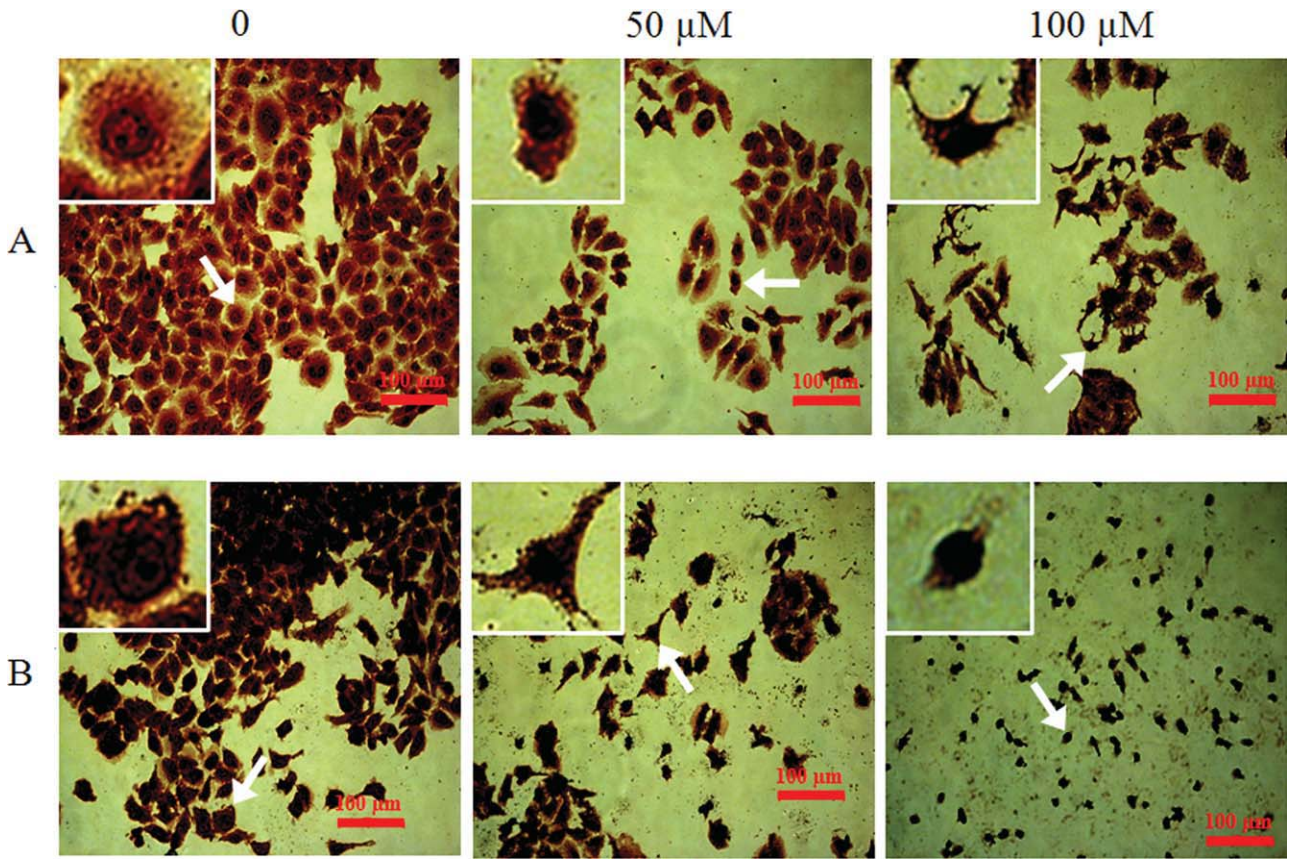


Figure 4 Hematoxylin and eosin stains of HepG-2 and A375 cells after incubating with different concentrations (0, 50 and 100 μ*M*) of PEG-PEI-Pt solutions for 24 h: (A) HepG-2 cells and (B) A375 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

plasmid DNA, and PEI 25 kDa/DNA was acted as a control. After 30-min mixing, the nanoparticles were incubated with the four cells in serum-free medium for 4 h and then in 10% serum medium for 48 h at 37°C. At the end of transfection, the cells were washed with PBS solution twice and lysed in the cell culture lysis reagent (Promega Co., Cergy Pontoise, France). Luciferase gene expression was quantified using procedures similar to those described earlier.[13] The ability of PEG-PEI-Pt to deliver fluorescein isothiocyanate-labeled small interfering RNA (FITC-siRNA) was also investigated in A549 cells at the weight ratio of 35/1 using similar procedures.

RESULTS AND DISCUSSION

Synthesis and characterization of PEG-PEI-Pt

PEI-Pt was first prepared as the control at a molar ratio of 1 : 1 by adding $PtCl_4$ dropwise into PEI (Mw 800 Da) solution. The resultant PEI-Pt is insoluble in water. Even after modified by PEG afterward, PEI-Pt remained insoluble, probably because it is very difficult to introduce PEG chain into PEI-Pt powders. But the PEG-PEI-Pt prepared according to Scheme 1 is water-soluble. To synthesize PEG-PEI-Pt, NHS-PEG-VS (Mw 3400 Da) in CH_2Cl_2 was added into PEI (Mw 800 Da) in CH_2Cl_2 drop by drop via the reaction between the NHS group of PEG and the amino group of PEI. After that, $PtCl_4$ was added dropwise into the above reaction system. After purification, the final water-soluble PEG-PEI-Pt was obtained. The representative ^{1}H-NMR spectrum of PEG-PEI-Pt in D_2O was shown in Figure 1. The weight ratio of PEG and PEI in PEG-PEI-Pt was calculated based on the proton integral values of ^{1}H-NMR spectrum (δ 3.5–3.6 for CH_2 of PEG and δ 2.4–3.0 for CH_2 of PEI), and was shown to be 0.28 : 1, a suitable value for gene transfer indicated by earlier reports.[13] Platinum content in the chosen PEG-PEI-Pt was 5.1% (wt/wt), measured by inductively coupled plasma mass spectrometry (ICP-MS). Thus, the molar ratio of PEG, PEI, and Pt in PEG-PEI-Pt was 1 : 15 : 4.

PEG-PEI-Pt was also characterized by X-ray diffraction (XRD) measurements and ultraviolet-visible (UV) spectra. Sharp peaks in the XRD spectrum of $PtCl_4$ appeared at $2\theta = 12.7°$,13.8°, 14.7°, 15.9°, 16.6°, and 17.4°, obviously different from those of PEI-Pt (a broad peak at $2\theta = 11.9°$) and PEG-PEI-Pt (three peaks at $2\theta = 12.6°$, 19.0°, and 23.0°). The difference among the three XRD spectra illustrated the validity

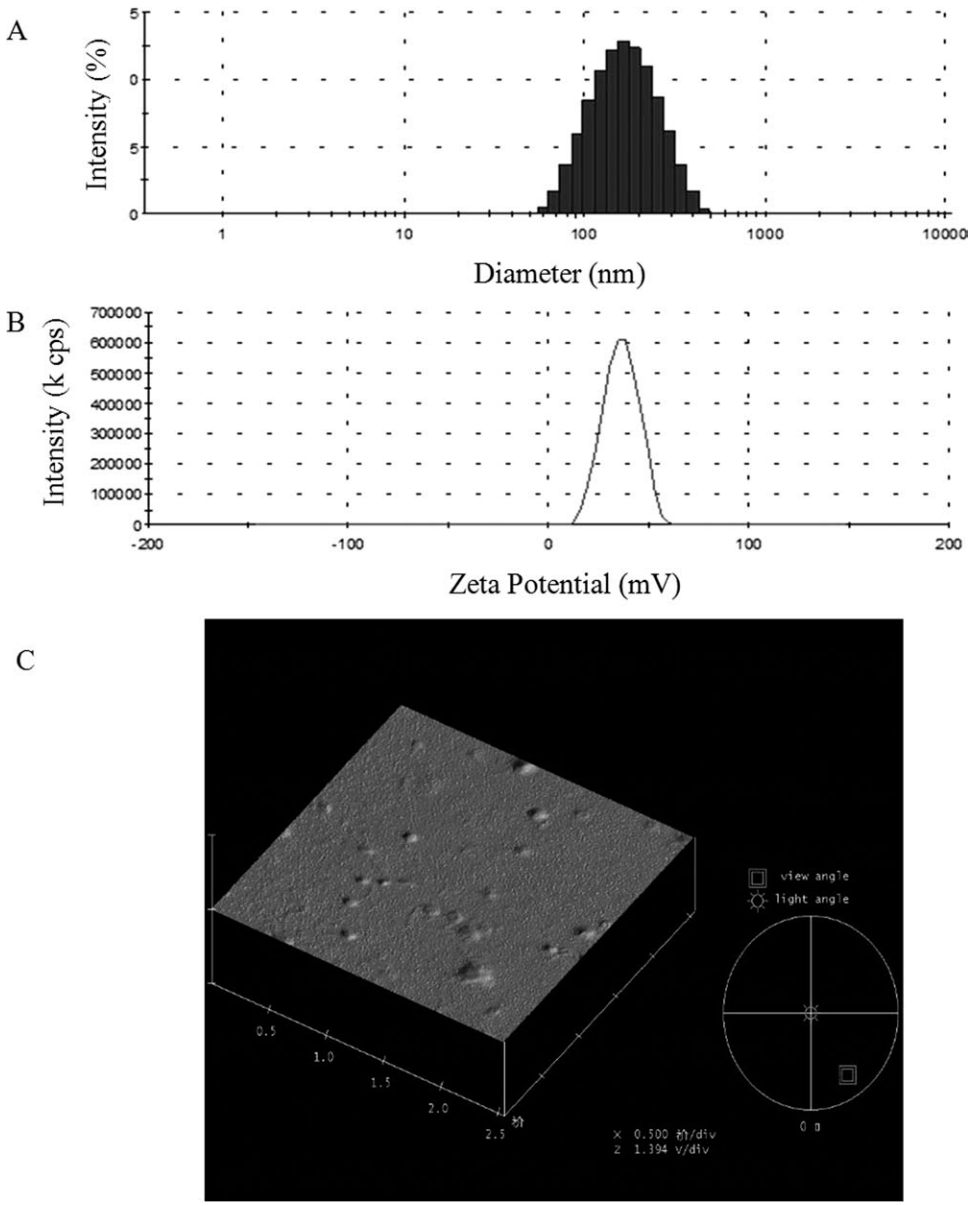


Figure 5 Measurements of the PEG-PEI-Pt/DNA complex: (A) particle size measurement, (B) zeta potential measurement, and (C) atom force microscopy.

of PEG modification and platinum (IV) complexation. The UV spectra of $PtCl_4$, PEG-PEI, and PEG-PEI-Pt were shown in Figure 2. No band was observed for PEG-PEI, while a new band at 350 nm appeared when the complex PEG-PEI-Pt was formed, which was in accordance with the research results of Duff[21] and Liang.[22]

Antitumor activity of PEG-PEI-Pt

The antitumor activity of PEG-PEI-Pt was tested by MTT assay in tumor cells (A549, A375, HepG-2, HuH-7, and B16 cells) and normal COS-7 cells in comparison with those of the control $PtCl_4$ and cisplatin. IC_{50} was used to evaluate the antitumor effect. IC_{50} is defined as the concentration of a drug or compound at which the relative cell viability decreases to 50%. Table I showed the IC_{50} (μM) for the 24 h of action of investigated materials on the above six cell lines. Among all the materials evaluated, PEG-PEI-Pt was the better potent inhibitor against all the five test tumor cell lines than $PtCl_4$. It should be noted that the IC_{50} (33 μM) of PEG-PEI-Pt in A549 cells was observed much lower than that (100 μM) of cisplatin. A549 cells were the cisplatin-resistant human lung adenocarcinoma cells, indicating that PEG-PEI-Pt had potential to treat certain drug-resistant tumors. In addition, PEG-PEI-Pt showed higher sensitivity in the five test tumor cells than in normal COS-7 cells. PEG and low molecular weight PEI are almost nontoxic.[23] PEG-PEI-Pt probably contains certain structures that show resemblance with cisplatin,

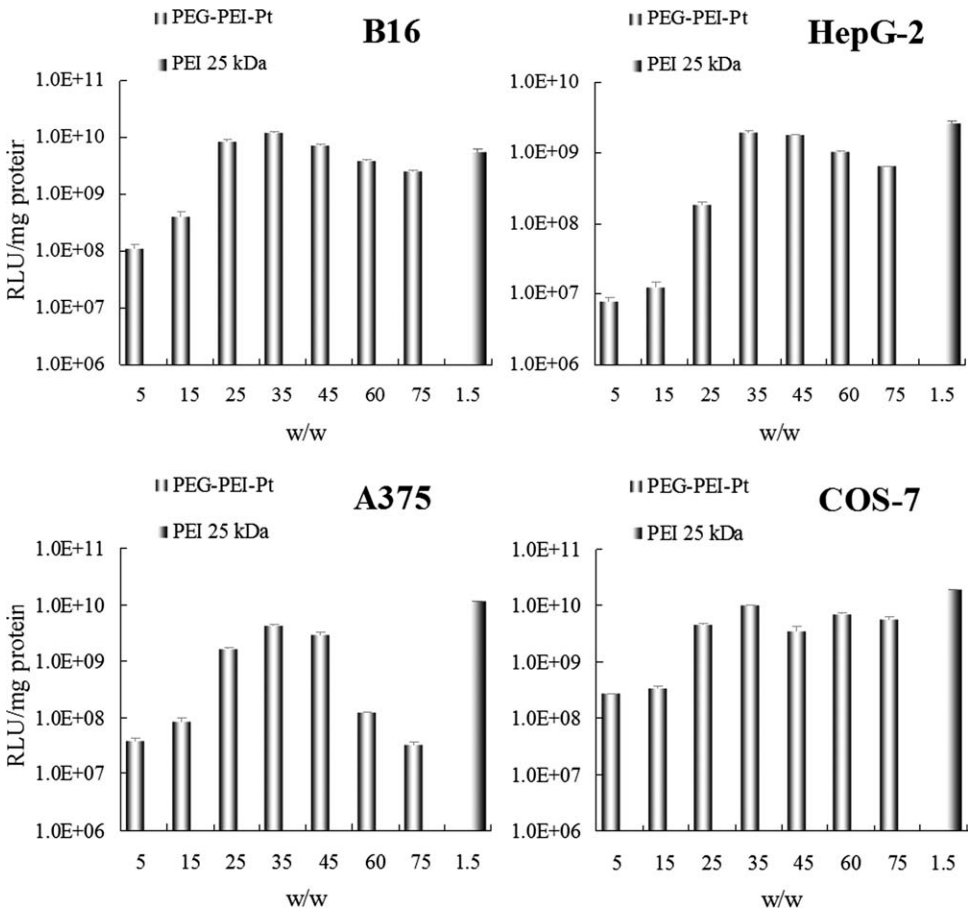


Figure 6 *In vitro* luciferase transfection of PEG-PEI-Pt and PEI 25 kDa in B16, HepG-2, A375, and COS-7 cells.

polynuclear platinum anticancer drugs or other platinum drugs. Such unique structures may contribute to the activity to kill tumor cells and even the cisplatin-resistant ones.

To futher investigate the antitumoral activity of PEG-PEI-Pt, the wound healing assays in HepG-2 and A375 cells for cell migration (Fig. 3) and hematoxylin and eosin stains of HepG-2 and A375 cells after

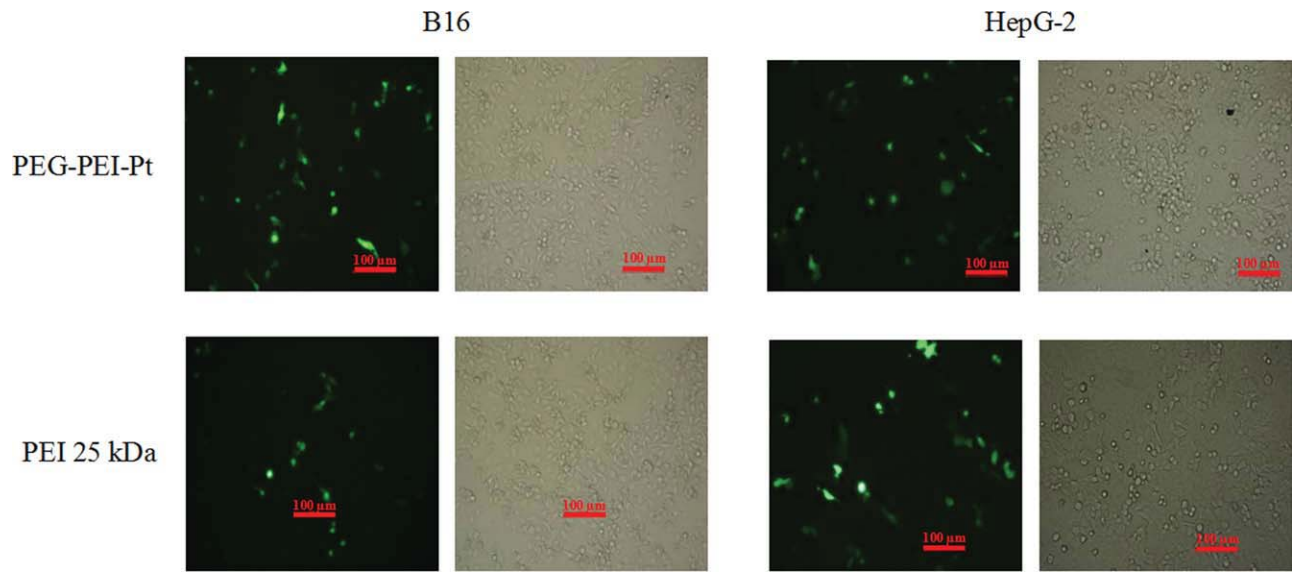


Figure 7 Transfection efficiency of PEG-PEI-Pt and PEI 25 kDa in B16 and HepG-2 cells reported by green fluorescent proteins. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

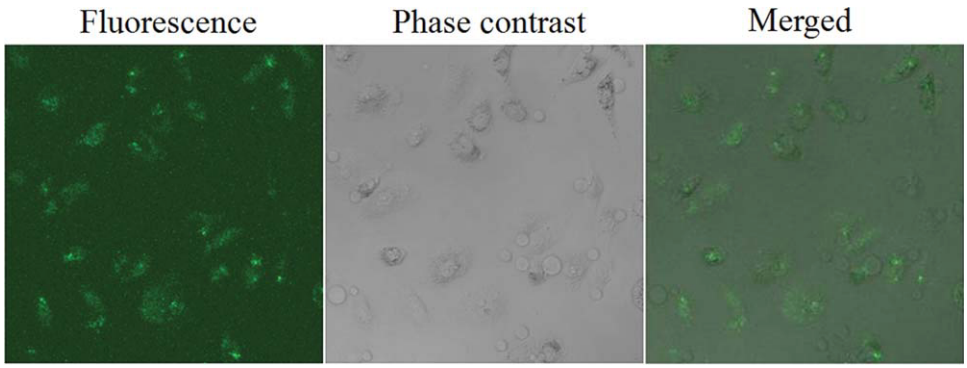


Figure 8 Laser confocal scanning microscope detection of the distribution of FITC-siRNA in A549 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

incubating with different concentrations of PEG-PEI-Pt solutions for 24 h (Fig. 4) were performed. The ability of tumor cells to migrate is closely associated with their metastatic potentiality. Metastasis, the process whereby cancer cells leave the primary tumor and form secondary tumor at a distant site, is one of the major causes of mortality in cancer patients. The development of drugs that specifically inhibit tumor metastasis of cancer cells is therefore of great significance in cancer treatment. The wound healing assays showed that PEG-PEI-Pt could prevent the migration of cells efficiently and led to the change of cellular morphology, indicating PEG-PEI-Pt possess antimetastasis ability. The morphology changes were also proved by the results of hematoxylin and eosin stains (Fig. 4). The cells treated with PBS were perfectly maintained integrated, while substantial changes in cell morphologies were observed after exposure with PEG-PEI-Pt. Notably, cells were significantly damaged and shrunk with increase in the incubation concentration of PEG-PEI-Pt. These results suggest that PEG-PEI-Pt possesses the good ability to inhibit tumor cell proliferation, consistent with those of MTT assay (Table I).

Gene delivery

For cellular transfection, DNA has to be condensed by cationic polymers into polymer/plasmid nanoparticles suitable for cellular uptake. The ability to condense DNA is a prerequisite for polymeric gene vectors. The ability of PEG-PEI-Pt to condense plasmid DNA (pDNA) into particulate structures was confirmed by agarose gel electrophoresis, particle size [Fig. 5(A)] and zeta potential [Fig. 5(B)] measurements and atom force microscopy (AFM) [Fig. 5(C)]. As expected, almost no condensation ability was observed for PEI (Mw 800 Da) and PEG-PEI. Very interestingly, PEG-PEI-Pt could condense plasmid DNA efficiently. Agarose gel electrophoresis assay indicated that the migration of DNA was completely retarded when the weight ratio of PEG-PEI-Pt/DNA was 0.4/1. The particle size measurement and AFM image of the PEG-PEI-Pt/DNA complex at the weight ratio of 35/1 showed the average sizes were around 200 nm. Zeta potential is an indicator of surface charges on the polymer/pDNA naoparticles. A positively charged surface allows electrostatic interaction with anionic cell surfaces and facilitates cellular uptake. The corresponding zeta potential of PEG-PEI-Pt/DNA complex at the weight ratio of 35/1 is about +30 mV.

Transfection efficiency of PEG-PEI-Pt was evaluated in B16, HepG-2, A375, and COS-7 cells. PEG-PEI-Pt/DNA nanoparticles were prepared by mixing a series of PEG-PEI-Pt with 1.0 μg pGL-3 plasmid DNA, where PEI 25 kDa/DNA was acted as the control at the weight ratio of 1.5 (around its optimal N/P ratio of 10).[13] After 30-min mixing, the nanoparticles were incubated for gene transfection. As shown in Figure 6, PEG-PEI-Pt was able to transfect all the experiment cells at different weight ratios from 5/1 to 75/1, and its optimal weight ratio is about 35/1. The optimal transfection efficiency of PEG-PEI-Pt was 1.5-fold higher than that PEI 25 kDa in B16 cells. In general, low molecular weight PEI has almost no transfection efficiency.[23] In this work, no obvious transfection in the above four cells were also observed for PEI (Mw 800 Da) and PEG-PEI. It is very surprising that PEG-PEI-Pt could exhibit the good transfection efficiency, comparable to or even higher than high molecular weight PEI (25 kDa) in different cell lines. There are several probable reasons for such interesting results. As mentioned above, the molar ratio of PEG, PEI and Pt in PEG-PEI-Pt was 1 : 15 : 4, indicating it is possible for several PEI chains to complex one Pt(IV). Pt(IV) complexation may lead to the crosslinkage of PEIs, increasing the molecular weight of PEI relatively. Such complexation may have certain sturctures similar to star-shaped PEG-PEI copolymers which exhibited a DNA condensation potential as high as high molecular weight PEI.[20] In addition, Pt(IV) complexation may help the polycation to penetrate the cell membrane, and then increase the transfection efficiency.

In an attempt to confirm the gene delivery capability of PEG-PEI-Pt, direct visualization of gene expression of GFP in B16 and HepG-2 cells at the weight ratio of 35/1 was also observed under fluorescence microscopy (Fig. 7). Plasmid pEGFP encoding GFP was also efficiently delivered by PEG-PEI-Pt in the tested cell lines, which was consistent with the luciferase transfection assay (Fig. 6).

RNA interference (RNAi) is becoming the popular choice for gene function analysis and RNAi-based gene therapy.[24] The RNAi delivery remains to be challenging. The ability of PEG-PEI-Pt to deliver fluorescein isothiocyanate-labeled small interfering RNA (FITC-siRNA) was preliminarily investigated in A549 cells at the weight ratio (35/1) of PEG-PEI-Pt/FITC-siRNA. Confocal microscopic images (Fig. 8) indicated that PEG-PEI-Pt could act as a good vector to take siRNA into the tumor cells, and the taking-efficiency was over 90%.

CONCLUSIONS

The novel conjugate of PEG-PEI-Pt was successfully synthesized by combining PEG, low molecular weight PEI (Mw 800 Da), and tetrachloroplatinum (IV). The antitumoral activity and transfection efficiency of PEG-PEI-Pt in different cell lines were evaluated. Similar to cisplatin, PEG-PEI-Pt showed higher sensitivity in tumor (A549, A375, HepG-2, HuH-7, and B16 cells) cells than in normal (COS-7) cells. PEG-PEI-Pt was also a potential drug for treating drug-resistant tumors. Furthermore, PEG-PEI-Pt could condense plasmid DNA efficiently, and showed a sastisfactory extent of transfection efficiency in different cell lines, comparable to or even higher than PEI 25 kDa. In addition, it was also confirmed that PEG-PEI-Pt could effectively deliver siRNA into the cytoplasm of tumor cells. Such a conjugate with good antitumoral and gene delivery abilities may play an important role in the collaborative therapy of drug and gene for various malignant cancers in the future.

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