Polyethylenimine-Grafted Multiwalled Carbon Nanotubes for Secure Noncovalent Immobilization and Efficient Delivery of DNA

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Gene Technology

Polyethylenimine-Grafted Multiwalled Carbon Nanotubes for Secure Noncovalent Immobilization and Efficient Delivery of DNA

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The functionalization of carbon nanotubes (CNTs) has been carried out in various ways for numerous applications in biotechnology,[1–15] including for the preparation of sensors,[2,3] as scaffolds for cell growth,[4] imaging reagents,[5] and transporters for drug delivery.[6–8,13,15] One way is to immobilize DNA onto the surface of CNTs through noncovalent interactions[8–13] or covalent bonds.[3,12–15] Covalent-bond approaches might compromise and even spoil the functions of DNA owing to chemical reactions and the difficulty in releasing DNA.[13,14b,15] Nevertheless, noncovalent approaches developed to date may only provide metastable immobilization of DNA onto the surface of CNTs. It was reported that the migration of DNA linked covalently to CNTs was retarded in gel electrophoresis but noncovalent interactions between DNA and CNTs did not completely prevent migration.[14b] Polyethylenimine (PEI) is a type of polymer with a high density of amines, thus DNA may be immobilized securely onto the surface of multiwalled carbon nanotubes (MWNTs) that have been functionalized with PEI through strong electrostatic interactions arising from these amines. Hence, we have adopted a grafting-from approach to prepare polyethylenimine-graft multiwalled carbon nanotubes (PEI-g-MWNTs). DNA has been immobilized securely onto the surface of PEI-g-MWNTs as demonstrated by the total inhibition of the migration of DNA in gel electrophoresis, and PEI-g-MWNTs showed transfection efficiency for delivery of DNA that was similar to or even several times higher than that of PEI (25 K) and several orders of magnitude higher than that of naked DNA.

PEI was grafted onto the surface of MWNTs by performing a cationic polymerization of aziridine in the presence of amine-functionalized MWNTs (NH_2–MWNTs). NH_2–MWNTs were obtained by introducing carboxylic acid groups onto the surface of MWNTs by heating at reflux in 3.0 M nitric acid. The carboxylic acid groups were transformed into acyl chloride groups by treatment with thionyl chloride[16] followed by treatment with ethylenediamine.[14] The grafting of PEI was realized through two mechanisms, the activated monomer mechanism (AMM) or the activated chain mechanism (ACM), by which protonated aziridine monomers or the terminal iminium ion groups of propagation chains, respectively, are transferred to amines on the surface of MWNTs.[17] (see Supporting Information.)

The relative amount of PEI grafted onto the surface of MWNTs was investigated by thermogravimetric analysis (TGA) performed under nitrogen. MWNTs were thermally stable up to 600 °C (Figure 1 A, curve a) whereas pure PEI degraded completely at about 500 °C (Figure 1 A, curve d). At 500 °C, pristine MWNTs, NH_2–MWNTs, and PEI-g-MWNTs showed negligible, about 2.3 %, and 10.5 % weight losses, respectively, thus PEI-g-MWNTs contained about 8.2 % PEI. Grafting with PEI made PEI-g-MWNTs easy to disperse in water, and the resulting suspension was still stable after six months. However, NH_2–MWNTs dispersed poorly in water and precipitation occurred within several hours (see Supporting Information). Transmission electron microscopy (TEM) provides direct evidence of grafting of PEI onto the surface of MWNTs. Figure 1 B shows TEM images of PEI-g-MWNTs on a holey carbon film: individually dispersed MWNTs are separated from others. High-resolution TEM (Figure 1 B, inset) indicates that PEI was grafted onto the surface of MWNTs as lumps with different sizes instead of as a uniform coating. This clumping results from the carboxylic acid groups, the ethylenediamine, and the PEI adhering preferably to the defects, which are the most active locations for chemical or physical functionalization;[18] these defects tend to cluster at the bends along the surface of MWNTs grown by chemical vapor deposition (CVD).[19] In Figure 1 C, the ^{1}H NMR spectrum of PEI-g-MWNTs in D_2O is compared with that of PEI in D_2O (pH 7.0). The signal at about $\delta =$ 3.1 ppm for PEI-g-MWNTs is attributed to grafted PEI, but the significantly decreased mobility of the PEI chains in PEI-g-MWNTs leads to broadening of the resonances. Some of the amine groups of PEI were protonated (pK_a of PEI is greater than 8.0); we found that protonation or partial protonation of PEI was necessary for the formation of a stable aqueous suspension of PEI-g-MWNTs and neutralizing PEI by adjusting the pH value to 9 or higher led to precipitation of dispersed PEI-graft-MWNTs within several hours.

PEI obtained by cationic polymerization of aziridine has a dendritic structure that contains primary, secondary, and tertiary amines with a molar ratio of about 1:2:1.[20] Grafting PEI onto the surface of MWNTs should have a negligible effect on the chemistry. The migration of DNA was totally inhibited in gel electrophoresis when the weight ratio of PEI-

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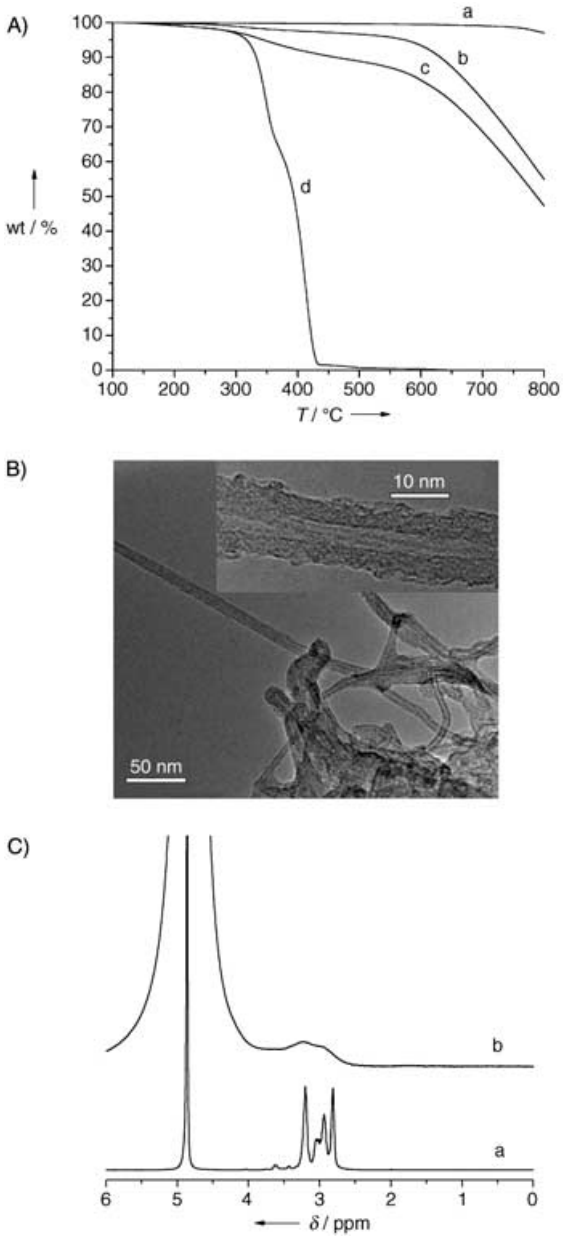

Figure 1. A) TGA curves of a) pristine MWNTs, b) NH_2–MWNTs, c) PEI-g-MWNTs, and d) PEI. B) TEM images of PEI-g-MWNTs; inset, high-resolution TEM image of PEI-g-MWNTs . C) ^1H NMR spectra of a) PEI in D_2O (pH 7) and b) PEI-g-MWNTs in D_2O.

g-MWNTs to DNA was about 4:1 (see Supporting Information). This result indicated that the dendritic grafted PEI with a high content of primary, secondary, and tertiary amines could function as anchor points for the secure immobilization of DNA onto the surface of MWNTs. In contrast, the presence of pristine MWNTs and NH_2–MWNTs showed little effect on the migration of DNA, even at a high weight ratio of 100:1 (see Supporting Information). Furthermore, a monolayer of PEI (25 K) was adsorbed onto the surface of CNTs directly after thorough rinsing,[21] but this monolayer of PEI did not prevent the migration of DNA (see Supporting Information). Thus, DNA could not be securely immobilized onto the surface of pristine MWNTs, NH_2–MWNTs, and MWNTs that were covered by a physically absorbed monolayer of PEI (25 K) because the interaction was too weak.

Recently it was demonstrated that amine-terminal oligoethylene glycol functionalized MWNTs (*f*-MWNTs) are promising for the delivery of DNA into cells, but the in vitro transfection efficiency of *f*-MWNTs was much less effective than that of lipids and only ten times higher than that of naked DNA.[8] In comparison, PEI-g-MWNTs that we have developed showed good transfection efficiency for DNA delivery. Figure 2 compares the transfection efficiency of PEI-g-MWNTs for DNA delivery in 293 cells with that of naked DNA and PEI (25 K), one of the most efficient polymers for the delivery of DNA.[22] The optimal weight ratio for PEI-g-MWNTs to DNA was about 45:1, with a N/P ratio of about 28.5:1 (10:1 for PEI at 25 K).[22] Under these conditions, the transfection efficiency of PEI-g-MWNTs was more than three times higher than that of PEI (25 K), and four orders of magnitude higher than that of naked DNA. PEI-g-MWNTs showed good transfection efficiency of DNA in other cells as well. The transfection efficiencies of PEI-g-MWNTs in COS7 and HepG2 cells were around twice and half, respectively, of those of PEI (25 K) and much higher than those of naked DNA under an optimal N/P ratio of between 10 to 16:1 (see Supporting Information).

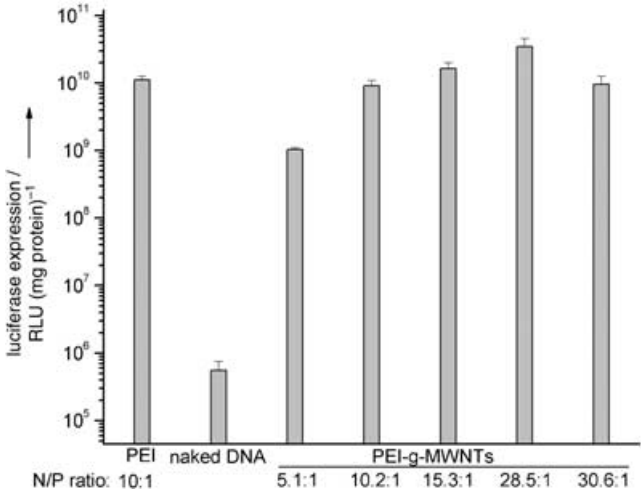

Figure 2. Transfection efficiency of PEI-g-MWNTs for DNA delivery relative to that of PEI (25 K) and naked DNA in 293 cells. The level of gene (pCMV-Luc) expression is given in relative light units (RLU) per mg of protein for quadruplicate runs (mean ± standard deviation ($n = 4$)).

The uptake of CNTs or their conjugates by the cells was suggested to occur by phagocytosis[5] or endocytosis,[6] which is in contrast to an insertion and diffusion mechanism in which MWNTs function as nanoneedles that inject DNA through the cell membranes.[7,8] We labeled PEI-g-MWNTs with fluorescein isothiocyanate (FITC); confocal microscope imaging demonstrated that the complexes of DNA with fluorescently labeled PEI-g-MWNTs entered cells after incubation for 1 h at 37 °C, but only very weak green fluorescence could be detected after incubation for 1 h at 4 °C (see Supporting Information). Therefore, the uptake of

the complexes of PEI-g-MWNTs and DNA should occur by endocytosis as reported.[5,6] The high transfection efficiency of PEI-g-MWNTs is attributed to several factors. The first factor is the secure immobilization of DNA onto the surface of MWNTs which leads to the formation of stable complexes that protect DNA well from degradation. The second factor is that the proton-sponge effect of the grafted PEI would allow the PEI-g-MWNTs/DNA complexes to escape easily from endosomes or other vesicles in cells, as has been well documented.[22] Furthermore, the larger complexes of PEI-g-MWNTs and DNA would improve the proton-sponge effects of PEI and facilitate a more effective sedimentation onto the cells.[23]

Pristine and some functionalized carbon nanotubes have been demonstrated to be of low cytotoxicity.[5–8] In 293 cells, the complexes of PEI-g-MWNTs/DNA with a weight ratio of 10:1 showed no significant effects on cellular metabolism but higher ratios led to a decreased cell number. Pure PEI-g-MWNTs showed a higher cytotoxicity. Figure 3A shows that

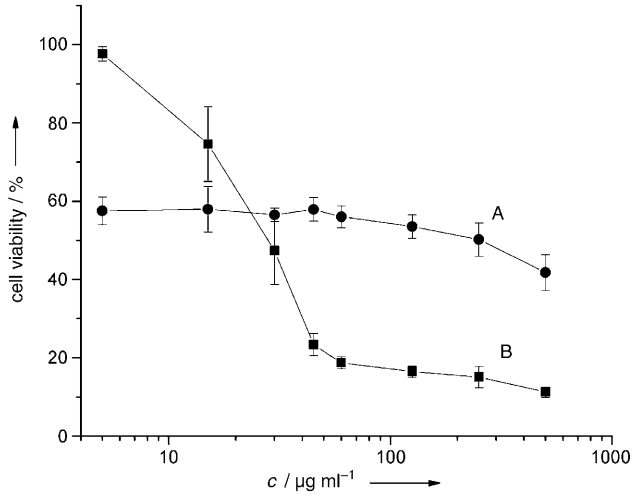

Figure 3. Cytotoxicity of A) PEI-g-MWNTs and B) PEI (25 K) in 293 cells.

about 60% of the cells were viable when the concentration of PEI-g-MWNTs was about 5 $\mu g\,mL^{-1}$, but the increased concentration of PEI-g-MWNTs showed less effect on the cell viability as compared with PEI (25 K; Figure 3B). Similar results were observed in COS7 and HepG2 cells (see Supporting Information). The cytotoxicity of PEI is related to the molecular weight: a higher molecular weight results in a higher cytotoxicity.[23] One PEI-g-MWNT may behave as high-molecular-weight PEI and thus it would have a certain degree of cytotoxicity.

In conclusion, we have prepared PEI-g-MWNTs. The grafted PEI chains function as efficient anchors to securely immobilize DNA onto the surface of MWNTs. This approach might be exploited to prepare highly sensitive CNT-based DNA sensors or probes and novel safe and efficient gene-delivery systems by fine-tailoring the PEI or by adopting other low toxic and biocompatible substitutes.

Experimental Section

PEI-graft-MWNTs: MWNTs were prepared by catalytic CVD of methane on Co–Mo/MgO catalysts. NH_2–MWNTs[14] and aziridine[25] were prepared by procedures similar to those reported. In a typical process for preparing PEI-g-MWNTs, NH_2–MWNTs (0.1 g) were dispersed in 1,2-dichloroethylene (20 mL) by ultrasonication over 20 min; aziridine (0.45 mL) was then added into the mixture under stirring followed by HCl (10 M; 10 μL). The reaction mixture was left at 80 °C for 24 h, after which time PEI-g-MWNTs were collected by filtration through a 0.2-μm pore polycarbonate membrane then washed with dichloromethylene ten times. PEI-g-MWNTs were purified by dispersing in deionized water, filtering through a 0.2-μm pore PVDF membrane, and washing with deionized water followed by drying under vacuum at 60 °C.

TGA was performed by scanning from 100 to 820 °C under nitrogen at a heating rate of 20 °C min^{-1} by using a Perkin Elmer TGA7. TEM was performed on a Philips CM300 FEGTEM instrument at 300 kV, and the samples were prepared by dropping one droplet of an aqueous suspension of PEI-g-MWNTs onto a holey copper mesh covered with carbon. NMR spectra were obtained on a Bruker DRX-400 spectrometer. The procedures for gel electrophoresis, evaluation of transfection efficiency for delivery of DNA, and cytotoxicity, were similar to those reported previously,[26] and these procedures are described in the Supporting Information together with the preparation of FTIC-labeled PEI-g-MWNTs, their incubation with cells, and confocal microscopy experiments.

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