

# Polyethylenimine-Modified Multiwalled Carbon Nanotubes for Plasmid DNA Gene Delivery

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Carbon nanotubes (CNTs) are considered as unique materials with very promising biological and medical applications.<sup>1</sup> In particular, the use of CNTs as carriers of gene and drug represents a powerful approach.<sup>2,3</sup> Ammonium-functionalized CNTs (f-CNTs) are able to condense plasmid DNA (pDNA) and are taken up into the cells.<sup>3</sup> Polyethylenimine-grafted multiwalled carbon nanotubes (PEI-g-MWCNTs) can efficiently immobilize and transport pDNA into cells.<sup>4</sup> It was reported that acid-oxidized CNTs can also be used to afford noncovalent protein or DNA- nanotube conjugates.<sup>5</sup> Polyethylenimine (PEI) is one of the most effective nonviral gene delivery carriers. High - molecular - weight PEI has higher transfection efficiency but also higher toxicity than low - molecular-weight PEI.<sup>6,7</sup> Herein, we report an efficient molecular delivery technique based on the transporting high - molecular-weight PEI 600K-modified multiwalled carbon nanotubes (PEI-MWCNTs) into cell membranes. The PEI-MWCNTs exhibit low cytotoxicity and its associated pDNA is delivered to cells efficiently, and the green fluorescent protein (GFP) levels up to 18 times higher than that of naked DNA were observed.

Our strategy to design PEI - MWCNTs is depicted in Figure 1. Briefly, carboxylic acid groups were introduced onto MWCNTs by acid treatment as described elsewhere.<sup>8-10</sup> PEI 600K was attached covalently onto these cut MWNTs via 1 - (3 - dimethyl aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxyl succinimide (NHS) coupling to yield PEI-MWCNTs. The amount of immobilized PEI was determined by elemental analysis (Atlantic Micro Lab Inc., Norcross, GA) of the MWCNTs and the PEI-MWCNTs composites.<sup>11,12</sup> Analysis revealed that the sidewall coverage of MWCNTs by PEI was about 27 % of the available surface area. In the IR spectrum of PEI - MWCNTs, characteristic bands due to -NH<sub>2</sub> (1400 cm<sup>-1</sup>) and -CONH (1640 cm<sup>-1</sup>) were observed (see Supporting Information).<sup>13</sup> The migration of pDNA was totally inhibited in gel electrophoresis when the N / P ratio of PEI-MWCNTs to pDNA was about 5:1 (see Supporting Information). This result indicated that the PEI 600K anchored onto the surface of MWCNTs could condense pDNA.

Figure 2A and B show SEM images of mostly short MWCNTs and PEI-MWCNTs dropped on a silicon-oxide substrate. No significant amount of particles was observed on the substrate, suggesting good purity of MWCNTs in water solution. The MWCNTs contain large numbers of the carboxylic acid groups, which are the most active locations for chemical or physical functionalization. After PEI adhering preferably to MWCNTs, the diameters of MWCNTs seem to increase (Figure 2B). When a solution of PEI-MWCNTs (50 μg mL<sup>-1</sup>) in phosphate buffer solutions (PBS) was mixed with pDNA (10 μg mL<sup>-1</sup>), the electrostatic interactions of the positively charged PEI-MWCNTs with the phosphate groups of pDNA lead to condensation of pDNA

onto the sidewall of PEI-MWCNTs, globular structures were observed within regions where condensation of plasmids onto the CNTs, and the short PEI-MWCNTs were also condensed into individually dispersed particles (see in Figure 2C and insert d). The irregular spherical structures between 50 and 150 nm in diameters are typically obtained due to degrees of plasmid condensation depending on the charge density, the hydrophobic character of the interaction, and the number of pDNA molecules in the condensate.

f-CNTs are able to transport DNA into cells, but in vitro transfection efficiency of f-CNTs was less effective than that of lipids and only ten times higher than that of naked DNA.<sup>3</sup> Although the PEI anchored by polymerization of aziridine on surfaces of MWCNTs has no fine-tailoring molecular weight, transfection efficiency of PEI - grafting - MWCNTs was three times higher than that of PEI 25K, and four orders of magnitude higher than that of naked DNA.<sup>4</sup> In our study, in vitro gene delivery was demonstrated by using plasmid DNA expressing a green fluorescent protein, which was assayed as described previously.<sup>14,15</sup>

Transfection was carried out using PEI 600K and PEI-MWCNTs. The qualitative analysis of GFP expression upon transfection in human embryonic kidney cells (293T) was evaluated by fluorescence microscopy. It was shown by fluorescence imaging that within 4 h of transfection with 20 μg mL<sup>-1</sup> of PEI or PEI-MWCNTs / pDNA complexes, cells were able to express GFP. The fluorescent images of 293T cells confirmed that the GFP fluorescence was the highest with PEI-MWCNTs among all of transfected assays (Figure 3A). However, the transfection efficiency of PEI / pDNA complexes reduced, which should be attributed to the lower cell viability (Figure 3B). These complexes protect pDNA during its delivery which may be later taken up by the cells through the endosomal pathway and released into the cytosol.<sup>16</sup> Similar results were also observed in HeLa and 293 cells (see Supporting Information). As a control, naked (non - condensed) pDNA showed only neglectable fluorescence intensity (relative to background level) in the cytoplasm of 293T cells (Figure 3C), which demonstrated that pDNA without any carrier had very low transfection efficiency as reported in other work.<sup>15,16</sup>

Furthermore, quantitative analysis of transfection efficiency was performed by gene expression analysis, as previously described.<sup>14,15</sup> Naked DNA, and complexes of PEI/DNA and PEI - MWCNTs/DNA were prepared at PBS solution (pH 7.2). As shown in Figure 4, compared transfection efficiency of PEI-MWCNTs for pDNA delivery with that of naked DNA and PEI 600K in 293T cells, the gene expression of PEI-MWCNTs was more than 1.5 times higher than that of PEI 600K, and 18 times higher than that of naked DNA. The optimal weight ratio for PEI-MWCNTs to DNA was about 8:1 with N/P ratio of about 10:1 (4:1 for PEI 600 K). PEI-MWCNTs show good transfection efficiency of pDNA in other cells

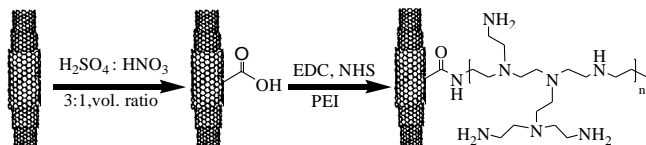
as well. The transfection efficiencies of PEI-MWCNTs in 293 and HeLa cells were around 1.4 and 1 times, respectively, of those of PEI 600K, and much higher than those of naked DNA under an optimal N/P ratio of 10:1 (see Supporting Information). The lower transfection efficiency at PEI/DNA ratio of 4:1 could be ascribed to high cytotoxicity of cell-surface binding and then lead to less GFP expression.

Pristine and some functionalized carbon nanotubes have been demonstrated to be of low cytotoxicity.<sup>11,17,18</sup> PEI-g-MWCNTs show a higher cytotoxicity when its concentration was about 5  $\mu\text{g mL}^{-1}$ , and the increased concentration of PEI-g-MWCNTs showed less effect on the cell viability as compared with PEI 25 K.<sup>4</sup> The cytotoxicity of PEI-MWCNTs and PEI 600K at various concentrations against 293 T cell was showed in Figure 5. The cell viability of PEI-MWCNTs was around 93 % when the concentration of PEI-MWCNTs was about 10  $\mu\text{g mL}^{-1}$ , but the increased concentration of PEI-MWCNTs to 100  $\mu\text{g mL}^{-1}$  showed less effect on the 60% of cell viability. In addition, the tendency that cell viability decreased with increasing concentration of PEI-MWCNTs could be relative to the enhanced positive charge of PEI 600 K. While the cell viabilities of PEI 600K was around 29 % at the same concentration. Compared with PEI 600K, the lower cytotoxicity of PEI-MWCNTs could be due to MWCNTs moiety introduced. The toxicity of PEI-MWCNTs on HeLa and 293 cell lines was similar as on 293T cell lines (see Supporting Information). In general, the cytotoxicity of PEI is related to the molecular weight: a higher molecular weight results in a higher cytotoxicity,<sup>6, 7</sup> but PEI-MWCNTs may not behave as high-molecular-weight PEI 600K.

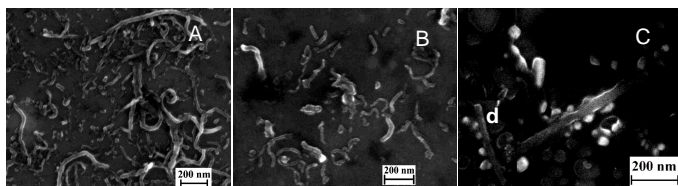
In conclusion, the PEI600K-MWCNTs showed enough ability to condense DNA and obviously enhance gene transfer activity. Due to lower cytotoxicity than that of PEI600K, this approach might be exploited to prepare novel safe and efficient gene delivery systems by adopting other biocompatible substitutes.

**Acknowledgment.** Financial support from the 973 program of MOST (2006CB 705600), and National Natural Science Foundation is greatly acknowledged.

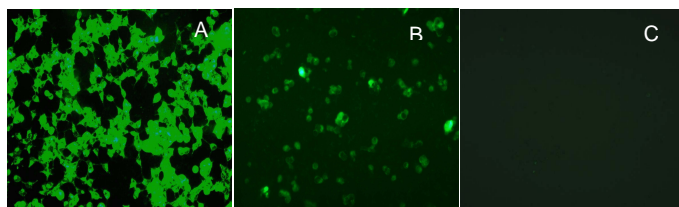
**Supporting Information Available:** FT IR spectrum of PEI 600K-MWCNTs, Complexes of PEI600K-MWCNTs with plasmid DNA, salt dissociation, cell transfection assay, and cytotoxicity evaluation (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.



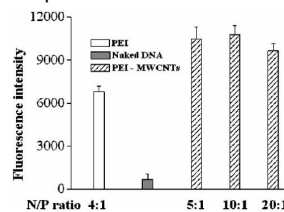
**Figure 1.** Scheme of the procedure for immobilizing PEI onto MWCNTs.



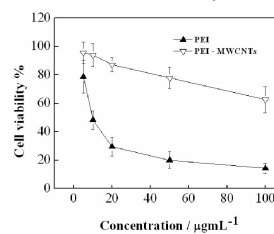
**Figure 2.** SEM images of MWCNTs (A), PEI-MWCNTs(B) and PEI-MWCNTs / pDNA complexes (C and insert d).



**Figure 3.** Expression of green fluorescence protein in HEK 293T cells after 4 h transfection with PEI-MWCNTs/pDNA (A) and PEI/pDNA (B) nanoparticles compared with control assays of naked DNA (C). Pictures were taken after 24 h post transfection under fluorescence microscope.



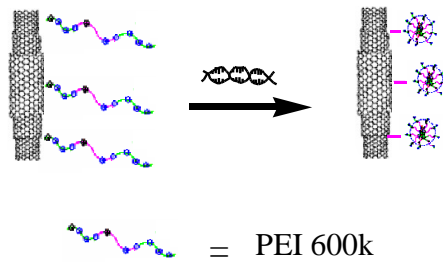
**Figure 4.** Expression of green fluorescence protein in 293T cells for nanoparticles of PEI / pDNA, naked pDNA and PEI-MWCNTs/pDNA. GFP quantification was done 24 h after transfection. Indicated values are means of four experiments  $\pm$  SD.



**Figure 5.** Cytotoxicity of PEI-MWCNTs, and PEI600K for 293T cells.

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An efficient molecular delivery technique based on the transporting high-molecular-weight PEI 600K-modified multiwalled carbon nanotubes (PEI 600K-MWCNTs) into cell membranes is reported. The PEI 600K-MWCNTs exhibit low cytotoxicity and its associated plasmid DNA (pDNA) is delivered to cells efficiently, and the green fluorescent protein (GFP) levels up to 18 times higher than that of naked DNA were observed.

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## Supporting Information

For PEI – MWCNTs modified MWCNTs with PEI (KBr pellet), the characteristic peaks at 3285  $\text{cm}^{-1}$  were assigned to N-H of PEI. 2922, and 2854  $\text{cm}^{-1}$  was attributed to

vibration bands of methylene. 1650  $\text{cm}^{-1}$  band ( $\nu_{\text{C=O}}$  amide) in the amide linkages.

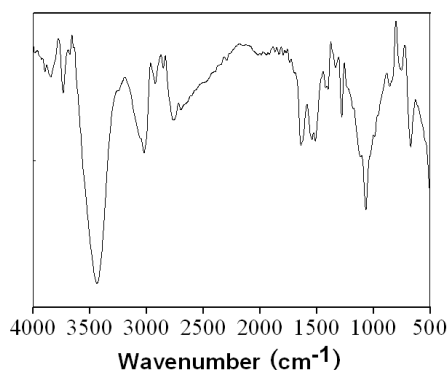


Figure S1. FT - IR spectrum of MWCNTs modified with PEI 600K

### Complexes of PEI - MWCNTs with pDNA (plasmid DNA)

To prepare PEI – MWCNTs / pDNA complexes, the appropriate concentration of PEI – MWCNTs was diluted to a total volume of 200  $\mu\text{L}$  with phosphate buffer solution (PBS, pH 7.2) and then split into four 50  $\mu\text{L}$  aliquots for each concentration of PEI – MWCNTs. Depending on ratio needed, PEI – MWCNTs concentrations ranged from 50 to 500  $\mu\text{g}/\text{mL}$ . An equal volume of a 20  $\mu\text{g}/\text{mL}$  DNA( the enhanced green fluorescence protein, EGFP) solution was then added to three of the PEI – MWCNTs aliquots and then mixed by rapidly pipetting 10 times, yielding a final DNA concentration of 10  $\mu\text{g}/\text{mL}$ . A 50  $\mu\text{L}$  of deionized water was added to the fourth PEI – MWCNTs aliquot of each group as alone MWCNTs control. Complexes were allowed to form for 15 min at room temperature prior to use. This process was repeated for each ratio tested, yielding three samples per condition plus a CNTs-only sample at the corresponding concentration.

### Salt dissociation of Complexes

The complexes were formulated as described above yielding PEI – MWCNTs final concentrations of 50  $\mu\text{g}/\text{mL}$ , and mixed with NaCl at a range of concentrations from 0 to 2 M. The samples were allowed to incubate for 5min at room temperature before being loaded into 1% agarose gel in TBE buffer containing ethidium bromide. The gel was run for 1.5 h at 80 V and then photographed under ultra - visible light using Quantity One analysis software.

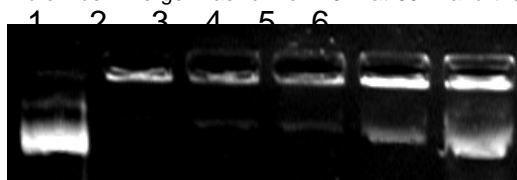


Figure S2. Gel electrophoresis of DNA complexes: lane 1 = naked DNA; lane 2, PEI – MWCNTs / DNA complexes in deionized water; lane 3, complexes in 0.2 M NaCl; lane 4, complexes in 0.5 M NaCl; lane 5, complexes in 1 M NaCl; lane 6, complexes in 2 M NaCl.

### Cell culture conditions

HEK 293T cells were obtained from the database of Shanghai Cell Bank, Chinese Academy of Sciences and maintained at 37°C under 5% CO<sub>2</sub> and 90% relative humidity in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat - inactivated (30 min at 56°C) fetal calf serum, 1.5 g /L NaHCO<sub>3</sub>, 200 mM L - glutamine, penicillin (100 U / mL) and streptomycin (100 mg / mL). HEK 293T cells were selected as a model cell lines for in vitro transfection, and studied to simulate the conditions of in vivo transfection.

### In vitro transfection assay

HEK 293T cells were seeded on a 24 well plate at an initial concentration of  $5 \times 10^4$  cells per well and incubated for 24 h before addition of the complexes solution. The condition of incubation set up was the same as described above. The duration of incubation time on intracellular uptake was defined at 4 h. Culture cells were harvested for the enhanced green fluorescent protein (EGFP) assay after 24 h of incubation. The qualitative analysis of EGFP expression upon transfection in HEK 293T, HeLa and 293 cells was evaluated by fluorescence microscopy. These cells were able to express GFP with 20  $\mu\text{g mL}^{-1}$  of PEI or PEI - MWCNTs / pDNA complexes. The fluorescent images showed that the PEI – MWCNTs / pDNA complexes have high EGFP fluorescence with among all of transfected assays. In contrast, the PEI / pDNA complexes have low transfection efficiency due to lower cell viability than that of PEI – MWCNTs / pDNA complexes. As a control, naked (non - condensed) pDNA showed only neglectable fluorescence intensity (relative to background level) in the cytoplasm of these cells, which demonstrated that pDNA without any carrier had very low transfection efficiency as reported in other work.

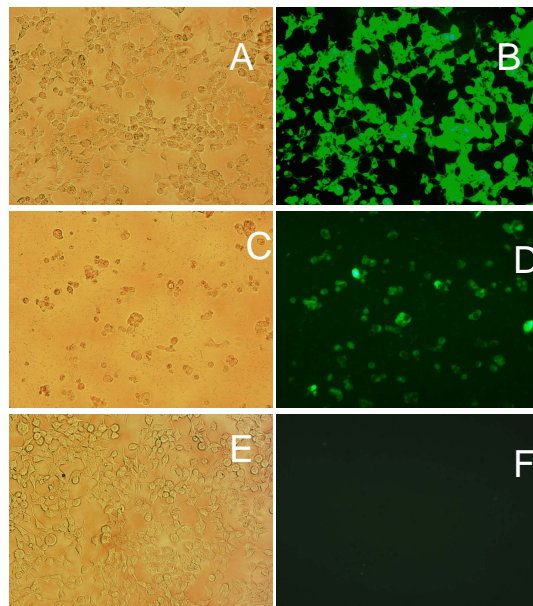




Figure S3. Expression of green fluorescence protein in HEK 293T cells after 4 h transfection with PEI - MWCNTs/ pDNA (A, B) and PEI /pDNA (C, D) nanoparticles compared with control assays of naked DNA (E, F). Pictures were taken after 24 h post transfection under fluorescence microscope.

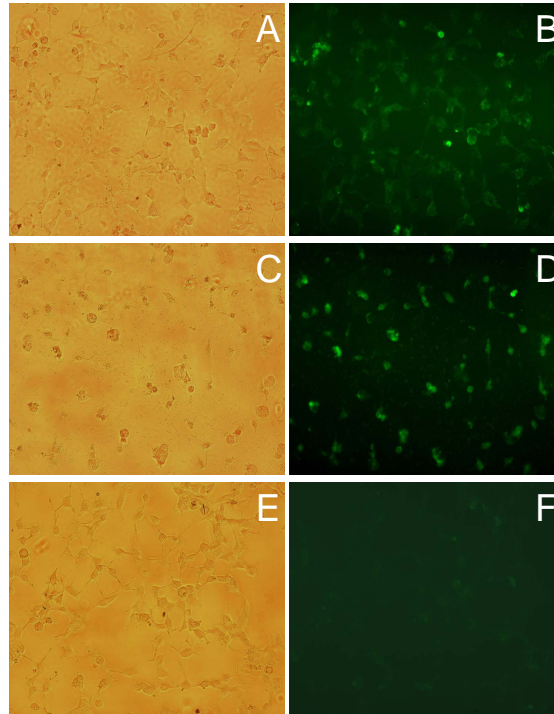
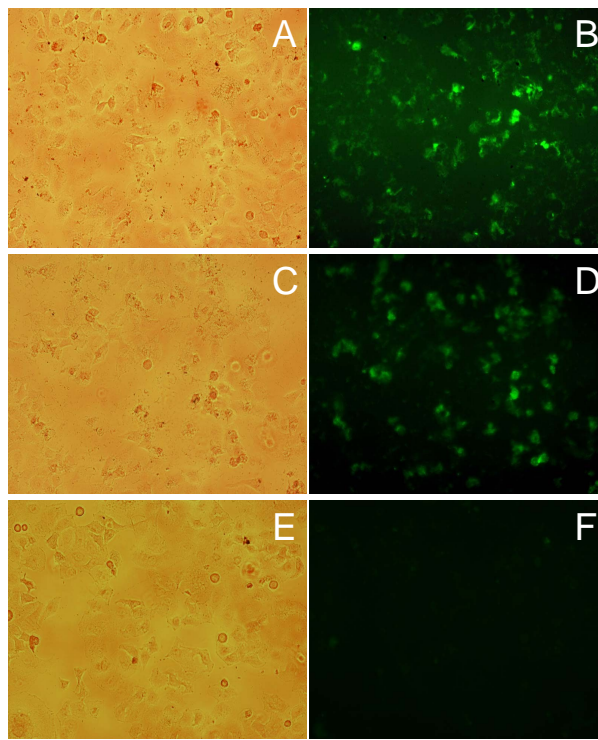


Figure S4. Expression of cells after 4 h transfection with PEI /pDNA (C, D) nanoparticles compared with control assays of naked DNA (E, F). Pictures were taken after 24 h post transfection under fluorescence microscope.

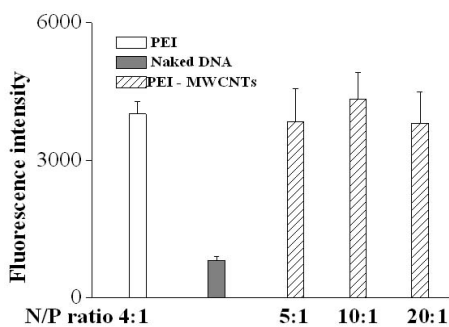


green fluorescence protein in 293 - MWCNTs / pDNA (A, B) and PEI compared with control assays of taken after 24 h post transfection

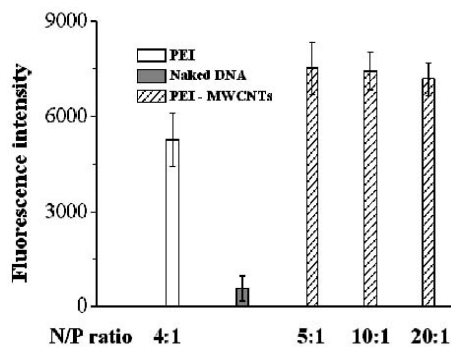
Figure S5. Expression of green fluorescence protein in HeLa cells after 4 h transfection with PEI – MWCNTs/pDNA (A, B) and PEI /pDNA (C, D) nanoparticles compared with control assays of naked DNA (E, F). Pictures were taken after 24 h post transfection under fluorescence microscope.

A quantitative analysis of transfection efficiency was performed by gene expression analysis. Naked DNA, and complexes of PEI / DNA and PEI - MWCNTs / DNA were prepared at PBS solution (pH 7.2). As shown in Figure 4, compared with transfection efficiency of PEI - MWNTs for pDNA delivery in 293T cells, the gene expression of PEI - MWCNTs was more than 1.5 times higher than that of PEI 600K, and 18 times higher than that of naked DNA. The optimal weight ratio for PEI - MWCNTs to DNA was about 8:1, with N / P ratio of about 10:1 (4:1 for PEI 600 K).

PEI - MWCNTs show also good transfection efficiency of pDNA in 293 and HeLa cells as well. The transfection efficiencies of PEI-MWCNTs in 293 and HeLa cells were around 1.4 and 1 times, respectively, of those of PEI 600K, and much higher than those of naked DNA under an optimal N/P ratio of 10:1. The lower transfection efficiency at PEI /DNA ratio of 4 :1 could be ascribed to high cytotoxicity of cell - surface binding and then lead to less GFP expression.



HeLa cells



293 cells



Figure S6. Expression of green fluorescence protein in 293T cells for nanoparticles of PEI / pDNA, naked pDNA and PEI – MWCNTs / pDNA. GFP quantification was done 24 h after transfection. Indicated values are means of four experiments  $\pm$  SD.

### Cytotoxicity Evaluation

293T cells were plated at  $1 \times 10^4$  cells in  $100 \mu\text{l}$  of medium per triplicate well in flat bottom 96 - well microwell plates (Corning) 1 days before the experiment were incubated with increasing. Thereafter, the medium was removed and equivalent volumes of medium containing known dose levels of PEI - MWCNTs or PEI were added. The cells were incubation for 24 h. 10 ml of MTT (Sigma) dissolved in medium at 5 mg/ml were added to the cultures and MTT formazan production was allowed to occur for 4 h.  $100 \mu\text{l}$  of dimethyle sulfoxide were added for 6 h. The plate was centrifuged at 4,500 g for 15 min to remove most nanotubes that aggregated as the sediment, and  $100 \mu\text{l}$  of the supernatant was removed to a new plate. Solubilized MTT formazan was determined by measurement of optical density at 570 nm in a microplate reader. Survival for treated cells was determined as a percentage of the optical density of MTT formazan in control cultures. The means  $\pm$  S.D. from replicate experiments with triplicate cultures are reported.

The toxicity of PEI - MWCNTs and PEI 600K was similar as on 293T cell lines at various concentrations against 293 cell lines and HeLa cell lines, shown in Fig. S5. For 293 cell lines, the cell viability of PEI - MWCNTs was around 93 % when the concentration of PEI - MWCNTs was about  $10 \mu\text{g mL}^{-1}$ , but the increased concentration of PEI - MWCNTs to  $100 \mu\text{g mL}^{-1}$  showed less effect on the 70 % of cell viability, while the cell viabilities of PEI 600K was around 23 % at the same concentration. As compared with 293 cell lines, the viability of HeLa cells was around 64 % when the increased concentration of PEI - MWCNTs was about  $100 \mu\text{g mL}^{-1}$ , while the cell viabilities of PEI 600K was around 20 % at the same concentration.

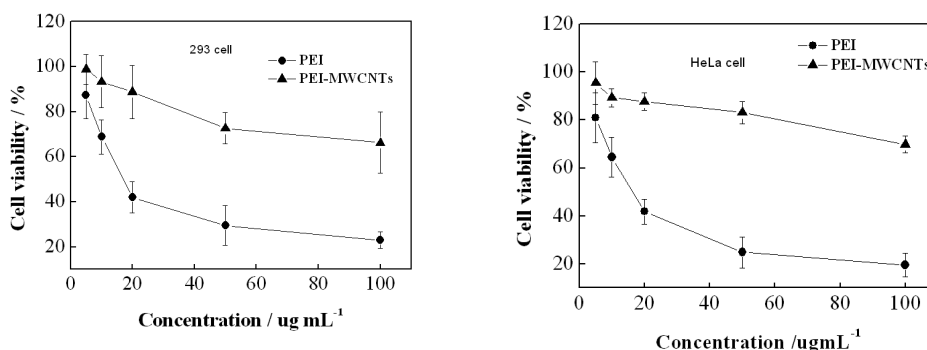


Figure S 7. Cytotoxicity of PEI - MWCNTs, and PEI 600K for 293 and HeLa cells.