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Article

Functionalized Multiwalled Carbon Nanotubes-anticancer Drug Carriers: Synthesis, Targeting Ability and Antitumor activity

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Abstract

A novel functionalized multiwalled carbon nanotubes(f-MWNTs)-anticancer drug carriers were prepared by modifying carbon nanotubes with polyethylenimine (PEI), folic acid and quantum dots for targeting and imaging cancer cells. The functionalized MWNTs exhibited good aqueous solubility, biocompatibility and high targeting ability. Water-insoluble anticancer drug Paclitaxel (PTX) were further loaded onto the f-MWNTs mainly through noncovalent π–π stacking interaction. In vitro cytotoxicity studies of f-MWNTs–PTX complexes using MTT assay exhibited a significant enhancement in the cytotoxic capability, suggesting these targeting drug carriers could facilitate intracellular delivery of anticancer drug and improve drug antitumor activity. Therefore, the highly versatile multifunctional carbon nanotubes could potentially be used for advanced drug delivery in biomedical fields .

Keywords: Multiwalled carbon nanotubes; Paclitaxel; Folic acid; Quantum dots; Drug delivery

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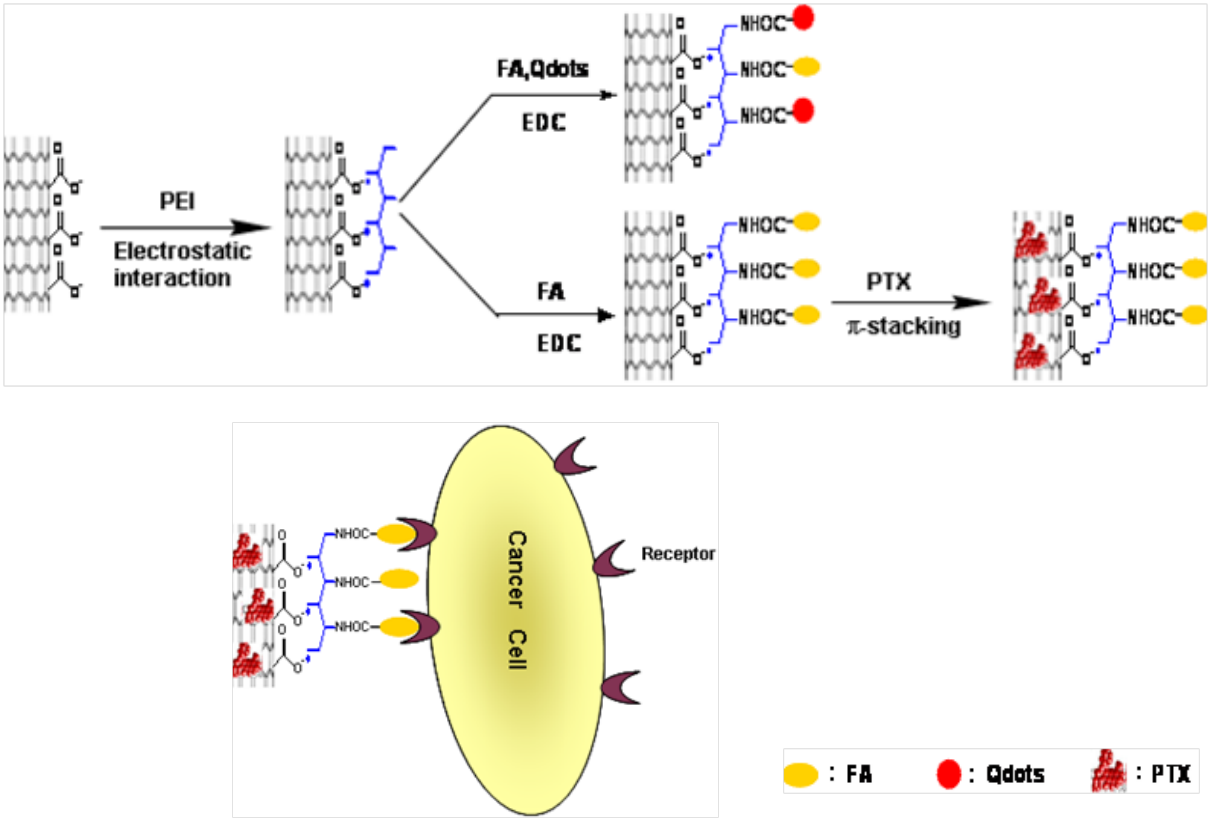
1. Introduction

In cancer therapy, a major challenge is to deliver anticancer drug molecules precisely to tumor sites for maximum treatment efficacy while minimizing side effects to normal organs[1-2]. In recent years, advanced drug delivery systems have been considered to hold great promise for improving cancer therapy outcomes[3-7]. Active targeting drug delivery systems can be developed using specific interactions between receptors on the cell surface and targeting moieties conjugated to surface of drug carriers. In this way, therapeutic drugs conjugated to targeting carriers can be effectively transported to tumor cells. At present, selectively targeting drug delivery systems still faced challenges including improving specificity and stability, regulating bioavailability, and developing targeting lower toxicity carriers [8-12]. Therefore, it is of high value to develop novel effective tumor-targeted drug delivery systems.

Carbon nanotubes(CNTs) are unique, one-dimensional molecular-scale tubes of graphitic carbon with outstanding properties including high aspect ratio, high surface area, high mechanical strength, unique electronic properties, excellent chemical and thermal stability,etc [13-14]. With these unique structures and properties, CNTs have been developed as promising nanoplatforms to immobilize biological or therapeutic drug molecules, such as proteins, antibodies, siRNA or drugs on their surface or in the hollow cavity, and especially these functionalized CNTs are capable of crossing biological barriers independently of the cell type, which makes them ideal candidates for drug delivery systems [15-21].

Paclitaxel (PTX) is a powerful antimitotic agent that against a wide range of solid tumors, especially drug-resistant ovarian cancer, metastatic breast cancer, and non-small-cell lung cancer [22-24]. Improving the applications of PTX in clinical therapy is limited by poor aqueous solubility, inefficient distribution, and the lack of selectivity. In this work, we explored an efficient targeting carbon nanotube-based drug delivery system, in which folic acid as targeting ligand and quantum dots(Qdots) as fluorescence labeling probes were covalently conjugated onto to the PEI-modified MWNTs by 1-(3-(dimethylamino)propyl)-3- ethylcarbodiimide



Scheme. 1 Schematic illustration of preparing functionalized multiwalled carbon nanotubes-anticancer drug carriers. Polyethylenimine (PEI), folic acid (FA), quantum dots (Qdots) and Paclitaxel (PTX) were attacheded onto the carboxylated MWNTs.

hydrochloride (EDC) crosslinker (Scheme 1). Herein, quantum dots were used as fluorescent labeling probes for clearly tracking the intracellular transporting and targeting delivery of functionalized multiwalled carbon nanotubes(f-MWNTs). Furthermore PTX binded strongly to f-MWNTs mainly through noncovalent π–π stacking interaction. The results demonstrated that improved aqueous solubility and targeting anticancer activity in vitro of the f-MWNTs–PTX complexes were obtained.

2. Materials and Methods

2.1 Materials

MWCNTs (95% purity, diameter 10-20nm, length1-2μm) were obtained from Shenzhen Nanotech Port Co. Quantum dots (mercaptoacetic acid-capped CdTe, emission wavelength of 615 nm) were obtained from Wuhan JiaYuan Quantum Dots Technological Development Co. Polyethylenimine(PEI) (25K),1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich. Human cervical carcinoma HeLa cells and Human Umbilical Vein Endothelial Cells cells(HUVEC) were obtained from Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS, China). All other reagents used were available commercially and were of the high purity grade.

2.2 Preparation of MWNTs-PEI-FA and MWNTs-PEI-FA-Qdots

MWNTs-PEI were synthesized according to our previous reported method[21]. Briefly, pristine MWNTs (95% purity, diameter 10–30 nm, length 1–2 μm) were dispersed in a concentrated sulfuric acid/nitric acid mixture (3:1 v/v) and sonicated for 16 h. After this treatment, the oxidized and shorten MWNTs were obtained after centrifugalized and washed thoroughly by deionized water. Then the cationic polymer polyethylenimine(PEI) was used to modify the MWNTs to obtain functionalized MWNTs (MWNTs-PEI).

MWNTs-PEI-FA (or MWNTs-PEI-FA-Qdots) were prepared as follows: folic acid (1 mg mL^{-1} in DMSO) and Qdots (8 μM) was firstly reacted with 2 mg mL^{-1} EDC for 10 min at room temperature, respectively. Then 0.05 mg mL^{-1} MWNTs-PEI solution was added and reacted for 3 h. The resulting MWNTs-PEI-FA or MWNTs-PEI-FA-Qdots were obtained by centrifuging at 12000 rpm for 10 min several times and were resuspended in aqueous solution and used immediately.

2.3 Supramolecular Assembly for PTX Loading onto f-MWNTs

PTX loaded onto f-MWNTs (MWNTs-PEI-FA) were prepared by mixing 1 mL of 0.05 mg mL^{-1} MWNTs-

PEI-FA solution with 50 µl of 1 mg mL^{-1} PTX ethanol solution and stirring overnight at room temperature. The mixture solution was then centrifuged three times at 12000 rpm for 10 min. After removing the supernantant, the PTX-MWNTs-PEI-FA complexes were obtained and resuspended in aqueous solution with gentle sonication. Likewise, PTX-MWNTs-PEI complexes were prepared in similar way. These supramolecular assembly of the complexes were systematically characterized by transmission electron microscopy (JEOL 2100, TEM), zeta potential analyzer (Malvern Instruments, nano ZS90), UV–vis spectrophotometry (Varian, Cary-100), and fluorescence spectrophotometry (Varian, Cary-Eclipse 500).

2.4 Confocal Microscopy assay

Confocal microscopic analysis was performed using a Carl Zeiss LSM 5 PASCAL laser scanning confocal microscope. HeLa cells and HUVEC cells were seeded at 3×10^4cells in a 35-mm Petri dish and were cultured in MEM and DMEM containing 10% fetal bovine serum at 37°C with 5% CO_2, respectively. After cell attachment overnight, both HeLa cells and HUVEC cells were treated with MWNTs-PEI-FA-Qdots bioconjugates (5µg ml^{-1}), and incubated for an additional 4h in fresh media and washed by PBS (pH=7.4) three times before confocal imaging.The targeting cellular uptake of MWNTs-PEI-FA-Qdots complex were examined by confocal laser microscopy .

2.5 In Vitro Cell Toxicity Assay

HeLa cells were seeded in 96-well tissue culture plates and maintained overnight in MEM medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2, respectively. Cells were then treated with various concentrations of MWNTs, MWNTs-PEI at different concentrations. Cells were also incubated with PTX (0.5µg ml^{-1}) , MWNTs-PEI (1.5µg ml^{-1}), PTX-MWNTs-PEI (1.5µg ml^{-1}), PTX-MWNTs-PEI-FA (1.5µg ml^{-1}). Relative cell viability was measured by standard MTT assay. All the cells were allowed to grow in regular growth medium for 48 h followed by incubation with fresh serum-free medium containing 20µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg mL^{-1}) for 4 h at 37 °C for the proliferation assay. The medium was removed, the cells were lysed by adding 150 µL of DMSO, and the absorbance of the purple formazan was recorded at 540 nm using a Thermo Multiskan spectrum reader. Cell numbers were determined from a standard plot of known cell numbers versus the corresponding optical density.

3. Results and discussion

The shorten and oxidized MWNTs were firstly obtained by a combined treatment of strong acids and sonication.Then the MWNTs were functionalized by PEI polymer molecules to make their well dispersion and stable in aqueous suspensions. As can be seen from the TEM image (Fig. 1a), surface of the MWNTs was wrapped with PEI (black arrow). Furthermore, the zeta potential of oxidized MWNTs and after MWNTs functionalized by PEI are measured. The zeta potential of the oxidized MWNTs was -25.7 mV, which are mainly attributed to anionic carboxylate group of the MWNTs by treatment of strong acids and sonication. After PEI modified MWNTs , the superfluous amine group of PEI lead that the zeta potential value was changed to 50.5 mV. This result suggested that the negatively charged surface of the MWNTs was fully covered by PEI polymer via electrostatic interaction. The amine groups on the surface of MWNTs-PEI could then be beneficial for further bioconjugates. Moreover, MTT assay showed(Fig. 1b) that MWNTs-PEI had high reduction in the cytotoxic

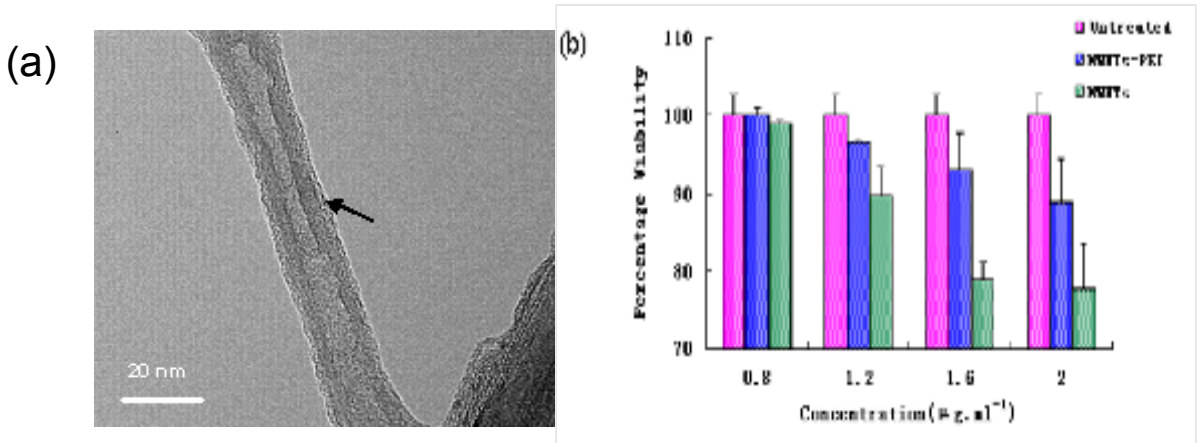


Fig. 1 (a) TEM image of MWNTs-PEI . (b) The percentage cell viability of Hela cells after 48h incubation with MWNTs, MWNT-PEI at various concentrations.

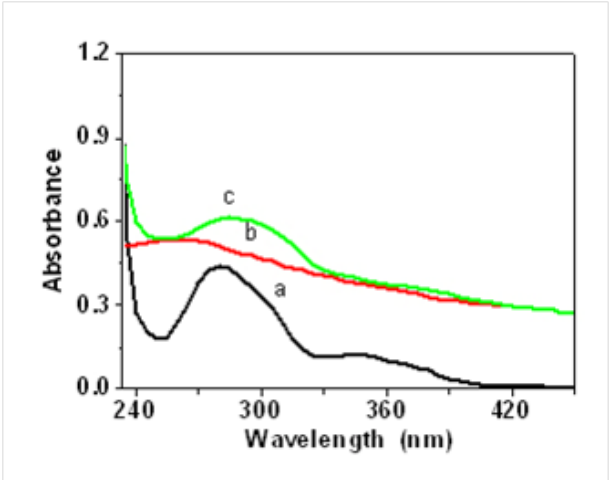


Fig. 2 UV–vis absorbance spectra of solutions of free FA (line a, black), MWNTs-PEI (line b, red), and MWNTs-PEI- FA (line c, green).

effect compared to the oxidized MWNTs, indicating the biocompatibility of PEI modified carbon nanotubes was greatly enhanced.

UV-vis absorption spectra were used to observe the targeting molecules (FA) successfully conjugating onto the MWNTs-PEI. Fig. 2 shows the UV–vis absorbance spectra of free FA, MWNTs-PEI and MWNTs-PEI complexed with FA. It can be clearly seen that the obvious absorption peak of MWNTs-PEI-FA at 280 nm (line c) can be attributed to the FA characteristic absorption peak (line a). It suggested that the cancer-targeting ligand FA molecules had successfully anchored to the MWNTs-PEI via amide reaction between the carboxyl group of the FA and the amine group of PEI segment to obtain the MWNTs-PEI -FA conjugates.

In order to clearly track the intracellular transport and targeting delivery of functionalized MWNTs, the mercaptoacetic acid-capped Qdots as fluorescent labeling were then covalently bound to the MWNTs-PEI-FA conjugates, which could be verified by TEM and fluorescence spectra. It can be investigated from TEM image (Fig. 3a) that Qdots appearing as black dots had tightly decorated onto the functionalization MWNTs. Furthermore, as shown in Fig. 3b, the PL spectrum of Qdots bound to MWNTs–PEI–FA displayed similar emission peaks to the characteristic luminescence peak for red Qdots. The results suggested that the Qdots hadsucceefully assemblied on the nanotubes.

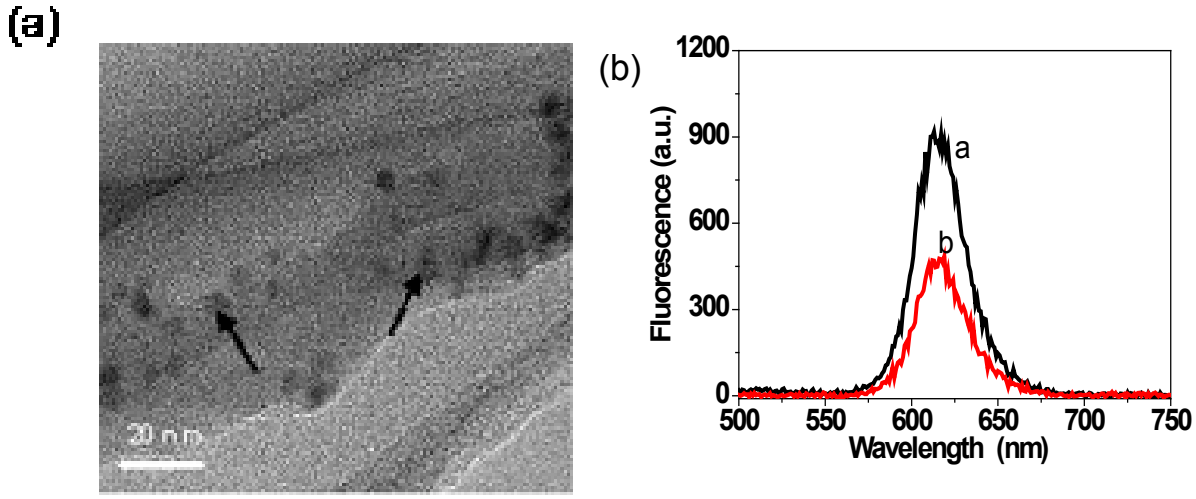


Fig. 3 (a) Transmission electron micrograph of MWNTs-PEI-FA-Qdots, (b) Fluorescence spectra of Qdots (line a, black) and Qdots bound to MWNTs-PEI-FA (line b, red)(excitation at 260 nm).

To examine the cellular targeting ability of MWNTs-PEI-FA, HeLa cells and HUVEC cells were respectively incubated with Qdots-labled MWNTs-PEI-FA complexes for 4 h and visualized using confocal fluorescence microscopy (Fig. 4). As it is known that human cervical cancer HeLa cells line exhibited a high level of folate receptor (FR) over-expression[25-26], the cell membrane of Hela cells has high expressing levels of folate receptor. Obviously, much brighter Qdots fluorescence signals were observed in HeLa cells incubated with MWNTs-PEI-FA-Qdots, compared to those observed in HUVEC cells treated by the same amount of MWNTs-PEI-FA-Qdots complexes, due to the folate-mediated endocytosis. HeLa cells surface with highly over-express folate receptor (FR) led to enhance the celluar uptake of MWNTs-PEI-FA-Qdots (Fig. 4a). In contrast, for low FR expressing HUVEC cells, there is no obvious fluorescence inside the cells (Fig. 4b). Therefore, It indicated the MWNTs-PEI-FA supramolecules have active targeting delivery efficiency.

We next investigated the binding of PTX to f-MWNTs, which could be confirmed by UV–vis spectra. As shown in Fig. 5, the characteristic UV–vis absorbance peak for PTX at ca. 240 nm superimposed on the characteristic MWNTs-PEI-FA absorption spectrum. It suggested PTX had successfully loaded onto the f-MWNTs, which

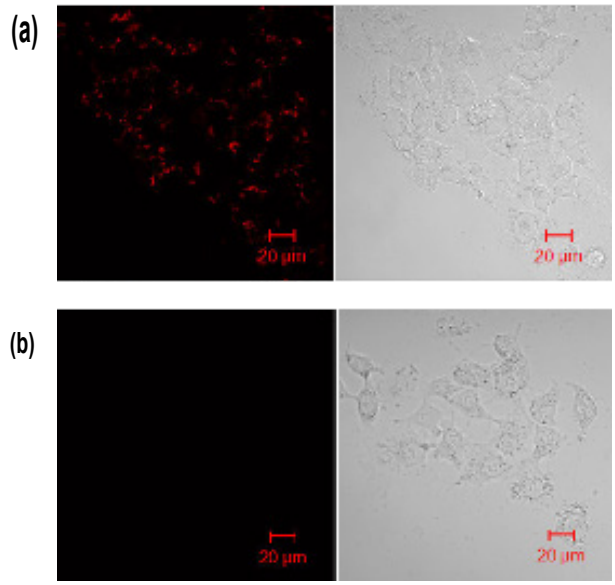


Fig. 4 Confocal fluorescence images (left) and light images(right) of (a) Hela cells and (b) HUVEC cells after incubation with MWNTs-PEI-FA-Qdots complexes for 4h.

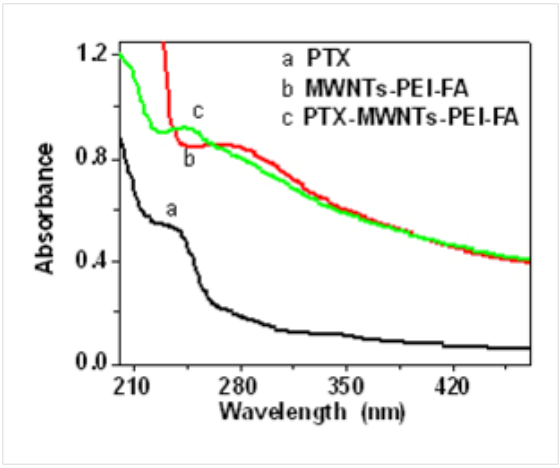


Fig. 5 UV–vis absorbance spectra of free PTX (line a, black), MWNTs-PEI-FA (line b, red), and PTX-MWNTs-PEI-FA complex (line c , green).

may be mainly driven by π–π stacking and hydrophobic interactions. Furthermore, PTX-MWNTs-PEI-FA complexes were observed to be good stablity in aqueous solution. Thus, the structure of the CNT backbone could act as a suitable platform for the formation of supramolecular complexes with insoluble, aromatic drug molecules.

Further, in order to evaluate their antitumor activity of these PTX-MWNTs-PEI-FA supramolecular complexes, *in vitro* cytotoxicity studies were performed by MTT assay, as shown in Fig. 6. The Hela cells were incubated with MWNT-PEI(b), PTX-MWNTs-PEI(c) PTX-MWNTs-PEI-FA(d) and free PTX(e) for 48 h, respectively. Untreated cells were used as control(a). It can be seen from Fig. 6 that due to their folate receptor-mediated targeting, f-MWNTs–PTX-FA complexes displayed significant enhancement in the cytotoxic

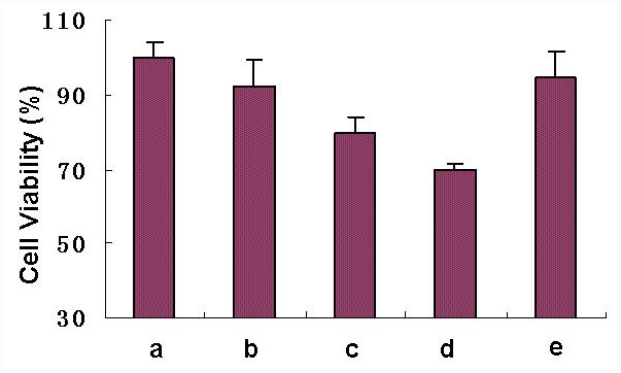


Fig. 6 The percentage cell viability of untreated Hela cells (a) and Hela cells after 48h incubation with MWNT-PEI(b), PTX-MWNTs-PEI(c), PTX- MWNTs-PEI-FA(d) and free PTX (e). Untreated cells were used as control.

capability compared to that of the PTX alone and the equivalent PTX-MWNTs-PEI. The enhanced antitumor activity obtained with f-MWNTs-PTX complexes suggested that f-MWNTs could act as suitable carriers for effectively improving intracellular delivery of the anticancer drug.

4. Conclusions

In summary, we explored a novel targeting carbon nanotube-based drug delivery system, in which folic acid was used as targeting ligand and quantum dots as fluorescence labeling probes. The f-MWNTs-PTX complexes exhibited efficient targeting intracellular delivery and enhanced antitumor activity. Therefore, these functionalized carbon nanotubes-based complexes could hold great promising in drug delivery applications for cancer therapy.

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