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RESEARCH PAPER

Multi-functionalized single-walled carbon nanotubes as tumor cell targeting biological transporters

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Abstract Multi-functionalized single walled carbon nanotubes (SWNTs) were prepared and applied as tumor cell targeting biological transporters. A positive charge was introduced on SWNTs to get high loading efficiency of fluorescein (FAM) labeled short double strands DNA (20 base pairs). The SWNTs were encapsulated with the folic acid modified phospholipids for active targeting into tumor cell. The tumor cell-targeting properties of these multi-functionalized SWNTs were investigated by active targeting into mouse ovarian surface epithelial cells. The experimental results show that these multi-functionalized SWNTs have good tumor cell targeting property.

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Introduction

In the few past years carbon nanotubes (CNTs) are attracting increasing attention as new tools of carrier system for therapeutic agents. The biocompatibility of CNTs has been greatly improved based on the development of covalent and noncovalent functionalization methods, which makes their manipulation easier for applications as intracellular transporters. They have been shown to shuttle various molecular cargos inside living cells including proteins (Shi Kam and Dai 2005), peptides (Pantarotto et al. 2004), DNA (Shi Kam et al. 2006; Singh et al. 2005) and drugs (Zhu et al. 2005; Wu et al. 2005). The internalized nanotubes were found to be biocompatible and nontoxic at the cellular level (Singh et al. 2006). Therefore SWNTs can lead to new classes of novel nanomaterials for drug delivery and cancer therapy.

A series of receptors were expressed on the surface of tumor cells, which are able to mediate internalizing effect by specially connecting with corresponding ligands. These receptors are potential targets for drugs combined with conjugates. Therefore, the drug-conjugate compounds can be delivered to tumor cells. The folate receptors (FRs) is a promising target because of its overexpression in many malignant

tissues, and its ability to bind and internalize folic acid conjugates. Folic acid has been used widely as targeting molecule for the targeted delivery of therapeutic molecules (Ni et al. 2002; Zhou et al. 2002). For example, this strategy has been used in the receptor-mediated targeting therapy of some cancers such as leukemic cells (Ratnam et al. 2003) and ovarian tumor (Weiman et al. 1992).

However, the major limitation of non-viral mediated gene therapeutic approaches is the efficiency of delivery of therapeutic genes into the target cells. Since functionalized SWNTs can penetrate mammalian cell membranes effectively and they can also be multifunctionalized at the same time, we used –COOH–CO–NH–$(CH_2)_6$–$NH_3^+ \cdot Cl^-$ to mediate the conjugation of double strand DNA (dsDNA) chains to SWNTs and furthermore established targeted internalization of SWNTs into cancer cells by conjugation the functionalized SWNTs with folic acid.

Experimental

Apparatus and chemicals

Fourier transform infrared spectroscopy (FT-IR) (Tensor 27, BRUKER) and ultra–visable–near IR absorption spectrum (UV–vis–NIR) (JASCO, V-570) were used to characterized the functionalized SWNTs. Fluorescent inversion microscope (Leica, DFC300 FX), and confocal fluorescence microscopy (Olympus, FV1000) were used to detect the ability of the functionalized SWNTs uptaken by the tumor cells.

FAM-labeled DNA sequence: 5′-TGC-ATT-TTT-AAT-GGT-ATT-TA-3′-FAM and its complementary sequence: 5′-TAA-ATA-CCA-TTA-AAA-ATG-CA-3′ was purchased from Sangon Biological Engineering Technology & Services Co., Ltd, Shanghai, China. These two DNA sequence were mixed in boiling water and then cooled to 4 °C to permit annealing and the resulting hybridized FAM labeled double strand DNA (dsDNA-FAM) was prepared.

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)2000] (PL-PEG) was obtained from Avanti Polar Lipids, Inc, U.S.A. Folic acid (FA) was purchased from Boquan Technology Co., Ltd, Nanjing, China. Boc-anhydride (di-*tert*-butyl pyrocarbonate) were purchased from Beta Pharma Co., Ltd, Shanghai, China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1,6-hexamethylenediamine, thionyl chloride were purchased from Aldrich.

Conjugation of amine-caped phospholipids with a polyethylene glycol (PL-PEG) and FA

FA (3.5 mM) and EDC (5 mM) were added to a solution of PL-PEG (0.35 mM) in 10 mM phosphate buffer at pH 7.5 according to the steps in the literature (Shi Kam et al. 2005). The reaction mixture after sonication was agitated for 24 h at room temperature. After reaction, the solution was dialyzed using a membrane (molecular weight cutoff 1,000) to remove unreacted FA and EDC. The dialysis was carried out for 3 days with frequent replacement of the buffer. After dialysis, the absorbance of the product PL-PEG–FA solution was recorded with UV absorption spectrum to ensure that excess free FA was removed from the solution.

The preparation of positively charged of SWNTs (SWNT-CONH–$(CH_2)_6NH_3^+$)

The SWNTs were prepared by a direct current arc-discharge method (Lv et al. 2005). The SWNTs were purified by use of nitric acid and cut using mixture acid as reported in literature (Liu et al. 1998).

To prepare the positively charged SWNTs ($SWNTs^+$) we first generated the *tert*-butyl *N*-(6-aminohexyl) carbamate (Boc-$NHC_6H_{12}NH_2$) using a procedure reported by M. Perree-Fauvet (Far et al. 2004). SWNT-COCl was prepared using a literature method (Chen et al. 1998) from the shortened SWNTs (s-SWNTs). SWNT-COCl (0.0691 g) and Boc-$NHC_6H_{12}NH_2$ (4.3 g) were then reacted in dimethylformamide (DMF) at 90 °C for 96 h under argon. The functionalized SWNTs with Boc protected amines from previous step, SWNT-CONH–C_6H_{12}NH-Boc, were collected with membrane filtration and washed with dioxane twice. Boc groups were removed using a similar procedure reported by Han et al (2001). A solution of HCl/dioxane (0.4 mL/9.6 mL) in a 25-mL round-bottom flask equipped with a magnetic stir-bar was cooled in an ice-water

bath under argon. SWNT-CONH–C_6H_{12}NH-Boc (30 mg) was then added with stirring. The ice-bath was removed and the mixture was kept stirred for 1 h. The reaction mixture was collected by centrifugation and then washed with dioxane twice. Finally, aqueous HCl solution (6 M) was added into the aqueous suspension of funtionalized SWNTs and the funtionalized SWNTs with positive charge ($SWNTs^+$) were collected using the standard centrifugation and membrane filtration. This functionalized procedure for SWNTs using above preparation method avoids reaction of amino groups of 1,6-diaminohexane with different SWNTs, which could cause much congregation of SWNTs. The SWNTs after functionalized using this method are dispersed relatively much better in water than the conventional method.

Conjugation of dsDNA-FAM with $SWNT^+$ (SWNTs-dsDNA-FAM)

To prepare the complex SWNTs-dsDNA-FAM, the generated SWNT-CONH–$(CH_2)_6NH_3^+$ with the final concentration of 0.06 mg/mL was incubated with dsDNA-FAM at the final concentration of 2.5 μM for 24 h at room temperature in dark.

The same preparation steps were taken to compare the coupling of dsDNA-FAM to SWNTs and $SWNTs^+$. The blank SWNTs and $SWNTs^+$ were prepared by the same steps as baseline of the analysis of UV–vis absorption spectrum. All samples were then ultracentrifuged for 150 min at 14,000 rpm. The dsDNA-FAM concentration in the upper layer was measured using a standard dsDNA-FAM concentration curve generated using the UV–vis spectra from a series of dsDNA-FAM solution with different concentrations. The dsDNA-FAM concentrations were measured at the wavelength of 260 nm. The loaded dsDNA-FAM amount of SWNTs or $SWNTs^+$ was determined using the Eq. 1:

$$\Phi = (M_{\mathrm{DNA}} - M'_{\mathrm{DNA}})/M_{\mathrm{SWNTs}} \quad (1)$$

where Φ is the loaded dsDNA-FAM amount of SWNTs or $SWNTs^+$, M_{DNA} is the initial dsDNA-FAM amount, M'_{DNA} is the dsDNA-FAM amount in the upper layer, and M_{SWNTs} is the added SWNTs or $SWNTs^+$ amount.

SWNTs-dsDNA-FAM encapsulated by PL-PEG–FA and PL-PEG (SWNTs-dsDNA-FAM–PL-PEG–FA, SWNTs-dsDNA-FAM–PL-PEG)

SWNTs-dsDNA-FAM was mixed with PL-PEG–FA and PL-PEG at the same concentration of 0.16 mM respectively, sonicated in ice-bath for 30 min, then agitated for 24 h in dark. The products of SWNTs-dsDNA-FAM–PL-PEG–FA and SWNTs-dsDNA-FAM–PL-PEG were obtained respectively by centrifugation and they were characterized by UV visible spectrum.

Uptake of the functionalized SWNTs by mouse ovarian surface epithelial cells (Mosec)

The Mosec first were cultured in 24 well plates in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, followed by exposure to SWNTs-dsDNA-FAM–PL-PEG–FA and SWNTs-dsDNA–FAM–PL-PEG with the final concentration of 0.006 mg/mL at 37 °C for 5 h respectively. Finally the incubated Mosec were washed with PBS buffer.

Uptake of the functionalized SWNTs by Hela

The Hela cells first were cultured in two different medium respectively: the FA-free RPMI-1640 medium and the normal RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin, followed by exposure to SWNTs-dsDNA-FAM–PL-PEG–FA with the final concentration of 0.006 mg/mL at 37 °C for 1 h respectively. Finally the incubated Hela cells were washed with PBS buffer.

Results and discussion

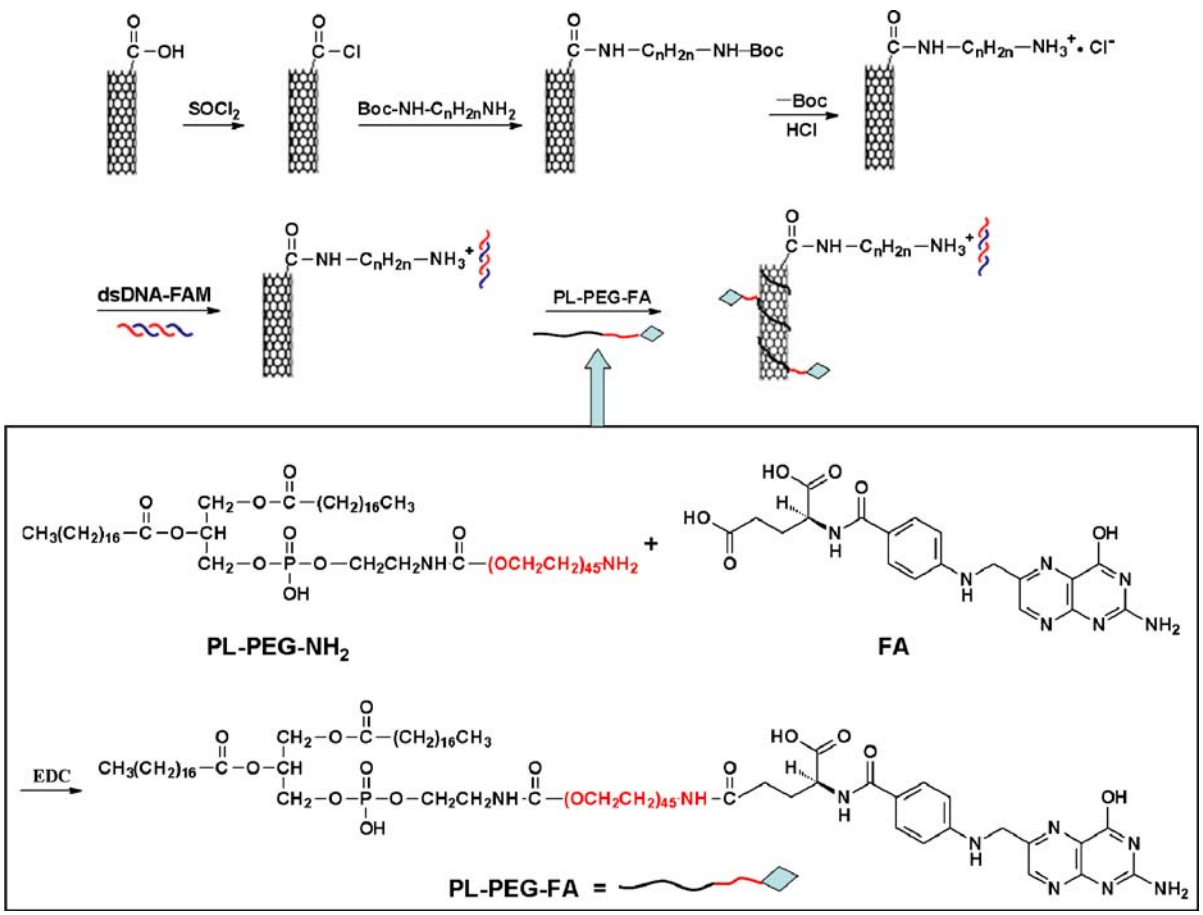
Characterization of the functionalized SWNTs

To prepare SWNTs-based biomolecular transporters with high loading efficiency and the ability of targeting tumor cells, positively charged SWNTs were first prepared from shortened SWNTs by forming amide linkages with 1,6-hexamethylenediamine

of which one end was protected with Boc-anhydride (Scheme 1). Then Boc-anhydride was removed by the addition of hydrogen choloride to get the acidificated ammonium salt positive ion, namely $SWNTs^+$. $SWNTs^+$ were then used as carrier of FAM-labled dsDNA chains by the electrostatic interactions between the positive charged SWNTs and the negative charged dsDNA. Finally the complexes of SWNTs and dsDNA-FAM were encapsulated by PL-PEG–FA via the wrap of polymer on the exterior surface of SWNTs.

Since the surface of many tumor cells overexpressed the folate receptor while the surface of the normal tissues and cells don't express the folate receptor, the folic acid functionalized SWNTs can absorbed onto the tumor cells though the specific interaction of the folic acid on the wall of the functionalized SWNTs and the folate receptor on the surface of tumor cells. The functionalized SWNTs which can target tumors cells were delivered targeting to tumor cells and then uptaken by tumor cells by both endocytosis and phagocytosis (Shi Kam et al. 2005, 2006).

This functionalization method has two noticeable advantages: (1) the long hydrophobic chains of fatty acid moiety of phospholipid can be absorbed and wrapped onto the wall of SWNTs by van der Waals and hydrophobic interaction. Moreover, the solubility of phospholipids has been increased after modified by amino (polyethylene glycol) 2000 due to the addition of long hydrophilic chains. At the same time, phospholipid can be utilized by organism and have no toxicity. Therefore SWNTs encapsulated by PL-PEG have enhanced both its solubility and compatibility. (2) By using the positively charged SWNTs to conjugate with dsDNA, the efficiency of dsDNA chains loading onto SWNTs can be improved and the



Scheme 1 Preparation of functionalized SWNTs which has targeting function for tumor cells

dsDNA don't need to be modified, so the integrity of dsDNA can be kept. As the structure of small interfering RNAs (siRNAs) is similar to that of dsDNA, this method can be used for carriering siRNAs which degrade easily and penetrating mammalian cell membranes effectively.

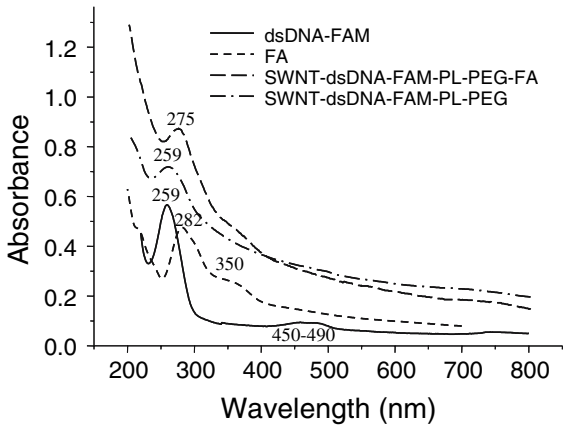


Fig. 1 UV visible spectra of dsDNA-FAM, FA, SWNT-dsDNA-FAM–PL-PEG–FA and SWNT-dsDNA-FAM–PL-PEG in aqueous solution

The positively charged SWNTs were first characterized by FT-IR spectrum. The functionalization of SWNTs with $-CONH-(CH_2)_6-NH_3^+ \cdot Cl^-$ was demonstrated by new peaks at $\sim$2,923 cm^{-1} (C–H) and $\sim$1,401 cm^{-1} (C–C) corresponding to the saturated hydrocarbons belonging to 1,6-diaminohexane and the bands at 1,713 cm^{-1} and 1,632 cm^{-1} assigned to amide band (C=O).

SWNTs-dsDNA-FAM–PL-PEG–FA and SWNTs-dsDNA-FAM–PL-PEG were then investigated by UV absorption spectra, as shown in Fig. 1. The dsDNA-FAM shows a characterized peak at 259 nm, and after SWNTs-dsDNA-FAM encapsulated by PL-PEG, this peak didn't change. FA shows a strong absorbance at 282 nm and a shoulder peak at about 350 nm. While SWNTs-dsDNA-FAM–PL-PEG–FA shows a peak at 275 nm and a weak shoulder at about 350 nm. The peak shift to 275 nm may result from the interaction of SWNTs-dsDNA-FAM and

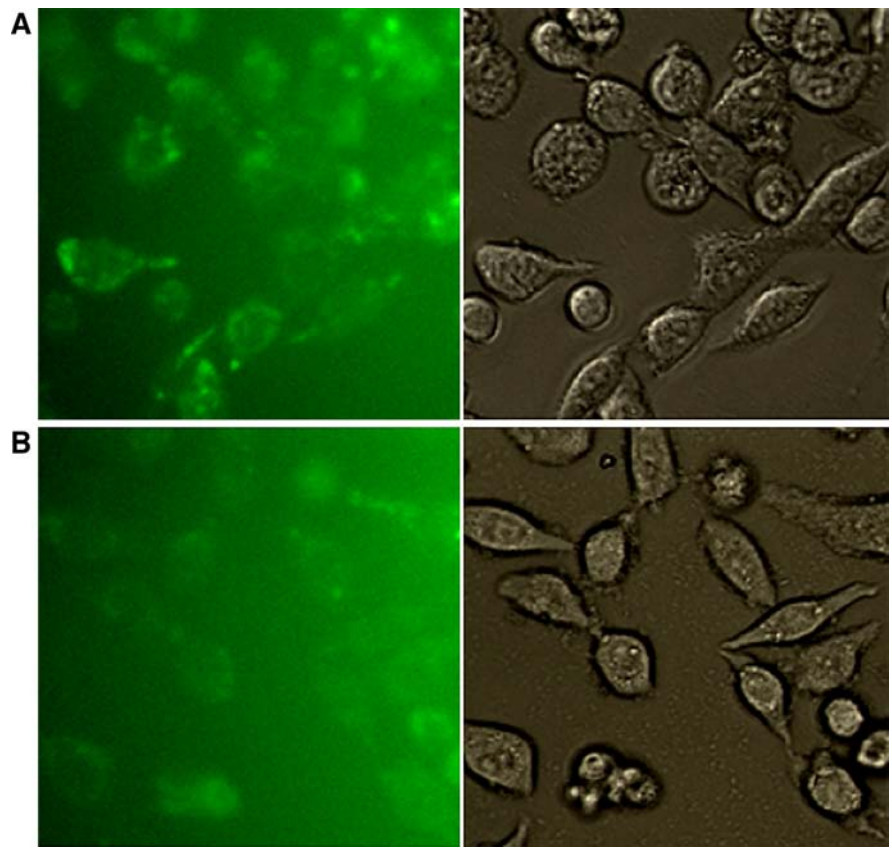


Fig. 2 The fluorescence microscope photos of SWNTs-dsDNA-FAM–PL-PEG–FA (**a**) and SWNTs-dsDNA-FAM–PL-PEG (**b**) with the final concentration of 0.006 mg/mL after incubated with Mosec at 37 °C for 5 h and washed with PBS buffer

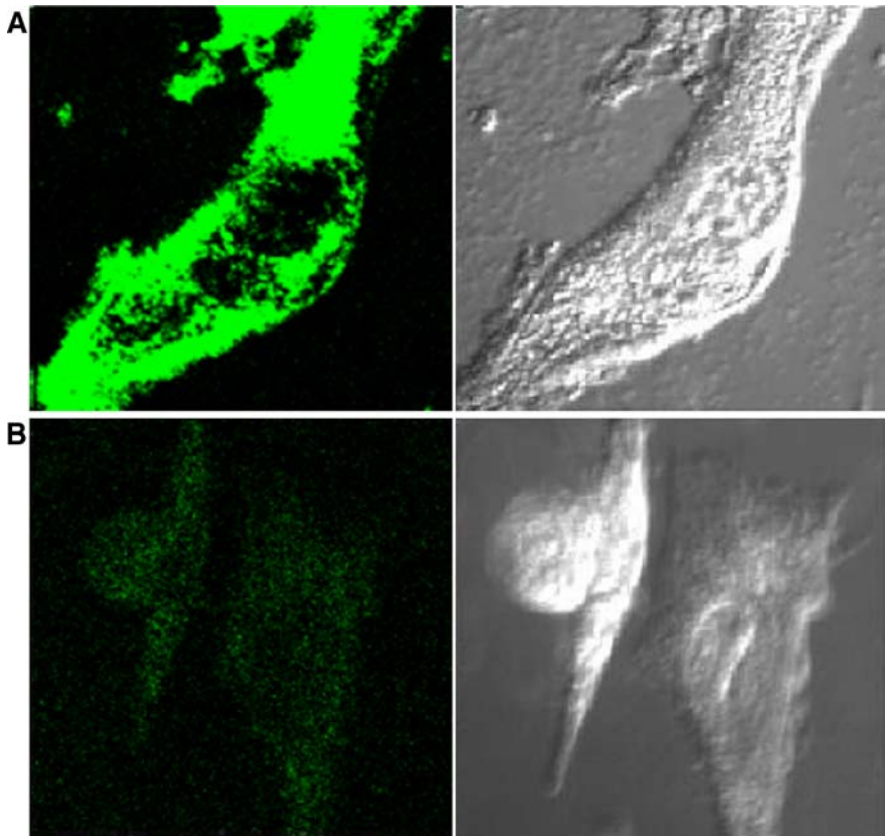


Fig. 3 Confocal fluorescence images of Mosec after incubated with SWNTs-dsDNA-FAM–PL-PEG–FA (**a**) and dsDNA-FAM (**b**) with the final dsDNA-FAM concentration of 0.25 μM for 1 h

PL-PEG–FA. This indicates FA and ds-DNA-FAM were both bound onto the SWNTs.

Using Eq. 1, the analysis of UV absorption spectra at the wavelength of 260 nm was performed to compare the coupling of dsDNA-FAM to SWNTs and to $SWNTs^{+}$. The loading of dsDNA-FAM on SWNTs was found to be 53.1 μg/mg and that of dsDNA-FAM on $SWNTs^{+}$ was 222.8 μg/mg. The significant enhancement of loading suggests that SWNTs conjugating with $–CONH–(CH_2)_6–NH_3^+ \cdot Cl^-$ promoted the coupling of dsDNA-FAM to SWNTs.

Targeted delivery of the functionalized SWNTs by Mosec

First, the ability of functionalized SWNTs targeting tumor cells was evaluated by investigating their uptake by Mosec in vitro. FRs is overexpressed on many epithelial cancers. It is reported that the folate-linked liposomes in a FR(+) mouse model of ovarian cancer is mainly targeted in ovarian cancer cells (Turk et al. 2004). Therefore, we used folic acid to modify the functionalized SWNTs and made them bind to the folate receptor on the surface of Mosec. In order to compare the ability of entering into Mosec for the functionalized SWNTs modified with folic acid and without folic acid, we observed the fluorescence levels after the two different functionalized SWNTs were incubated with Mosec for 5 h, respectively. The fluorescence microscope photos show the fluorescence level of the functionalized SWNTs modified with folic acid is much higher than that of the functionalized SWNTs without folic acid. (Fig. 2) This clearly indicates that the folic acid modified functionalized SWNTs can selectively target the surface of Mosec and penetrate into the cells.

Figure 3 is the confocal fluorescence images of Mosec after incubated with SWNTs-dsDNA-FAM–PL-PEG–FA and dsDNA-FAM, respectively. The images show the fluorescence level of SWNTs-dsDNA-FAM–PL-PEG–FA in Mosec is very high, while the fluorescence level of dsDNA-FAM in Mosec is very low and its fluorescence is dispersed.

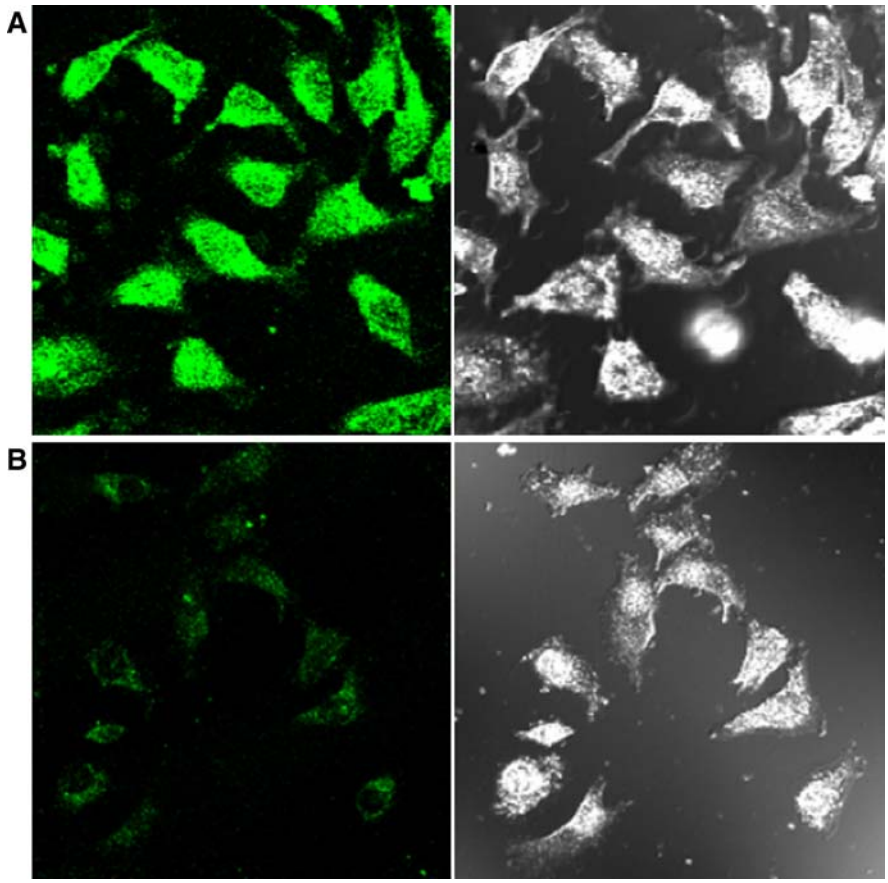


Fig. 4 Confocal fluorescence images of SWNTs-dsDNA-FAM–PL-PEG–FA with the final concentration of 0.006 mg/mL after incubated with Hela cells treated with different method at 37 °C for 1 h. A. The Hela cells were cultured in FA-free RPMI-1640 medium; B. The Hela cells were cultured in normal RPMI-1640 supplemented with 10% FBS

This indicates the intracellular uptake of fluorescently tagged DNA molecules without nanotube transporters is neglectful and the functionalized SWNTs promote dsDNA-FAM into tumor cells effectively. Also, the noncovalent conjugations between dsDNA-FAM and SWNTs are sufficiently strong for entry as carrier–cargo complexes into living cells (Fig. 3).

Targeted delivery of the functionalized SWNTs by Hela

The tumor cell-targeting property of the folic acid conjugated functionalized SWNTs were further studied by confocal microscopy for two different treatment Hela cells after incubation with SWNTs-dsDNA-FAM–PL-PEG–FA. It is known that the FA-starved cells overexpress FRs on the cell surface. Therefore, Hela cells of experimental group were cultured in the FA-free RPMI-1640 medium, and Hela cells of control group were cultured in normal medium of RPMI-1640 supplemented with 10% FBS. As shown in Fig. 4, the fluorescence level of SWNTs-dsDNA-FAM–PL-PEG–FA in the FA-starved Hela cells is much higher than that in the normal Hela cells. Thus the folic acid modified functionalized SWNTs can be delivered into Hela cultured in folic acid free medium quickly and effectively. This indicates that the functionalized SWNTs can be delivered into the targeted tumor cells which overexpress FRs.

Conclusions

The functionalized SWNTs were prepared by loading FAM-labeled dsDNA onto the positively charged SWNTs in order to increase the loading efficiency. Then folic acid modified phospholipids were used to encapsulate the SWNTs loaded with FAM-labeled dsDNA to prepare the tumor cell targeted functionalized SWNTs. These multi-functionalized SWNTs

can target effectively mouse and mammalian tumor cells in which FRs overexpress. Since the structure of dsDNA chains are very similar to that of siRNA, the multi-functionalized SWNTs are also good vectors for tumor-associated siRNA targeted therapy.

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References

Chen J, Hamon MA, Hu H et al (1998) Solution properties of single-walled carbon nanotubes. Science 282:95-98

Far S, Kossanyi A, Verchere-Beaur C et al (2004) Bis- and tris-DNA intercalating porphyrins designed to target the major groove: synthesis of acridylbis-arginyl-porphyrins, molecular modelling of their DNA complexes, and experimental tests. Eur J Org Chem 1781–1797

Han G, Tamaki M, Hruby VJ (2001) Fast, efficient and selective deprotection of the *tert*-butoxycarbonyl (Boc) group using HCl/dioxane (4 m). J Pept Res 58:338–341

Lv X, Du F, Ma YF (2005) Synthesis of high quality single-walled carbon nanotubes at large scale by electric arc using metal compounds. Carbon 43:2020–2022

Liu J, Rinzler AG, Dai H et al (1998) Fullerene pipes. Science 280:1253–1256

Ni S, Stephenson SM, Lee RJ (2002) Folate receptor targeted delivery of liposomal daunorubicin into tumor cells. Anticancer Res 22:2131–2135

Pantarotto D, Briand JP, Prato M et al (2004) Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chem Commun (Camb) 1:16–17

Ratnam M, Hao H, Zheng X et al (2003) Receptor induction and targeted drug delivery: a new antileukaemia strategy. Expertop Biol Ther 3:563–574

Shi Kam NW, Connell M, Wisdom JA et al (2005) Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. Proc Natl Acad Sci USA 102:11600–11605

Shi Kam NW, Dai H (2005) Carbon nanotubes as intracellular protein transporters: generality and biological functionality. J Am Chem Soc 127(16):6021–6026

Shi Kam NW, Liu Z, Dai H (2006) Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. Angew Chem Int Ed 45:577–581

Singh R, Pantarotto D, Lacerda L et al (2006) Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers. Proc Natl Acad Sci USA 103(9):3357–3362

Singh R, Pantarotto D, McCarthy D et al (2005) Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: toward the construction of nanotube-based gene delivery vectors. J Am Chem Soc 127:4388–4396

Turk MJ, Waters DJ, Low PS (2004) Folate-conjugated liposomes preferentially target macrophages associated with ovarian carcinoma. Cancer Lett 213(2):165–172

Wu W, Wieckowski S, Pastorin G et al (2005) Targeted delivery of amphotericin B to cells by using functionalized carbon nanotubes. Angew Chem Int Ed 44:6358–6362

Weiman SD, Weinberg AG, Coney LR et al (1992) Cellular localization of the folate receptor potential role in drug toxicity and folate homeostasis. Cancer Res 52:6708–6711

Zhu Y, Peng AT, Carpenter K et al (2005) Substituted carborane-appended water-soluble single-wall carbon nanotubes: new approach to boron neutron capture therapy drug delivery. J Am Chem Soc 127(27):9875–9880

Zhou W, Yuan X, Wilson A et al (2002) Efficient intracellular delivery of oligonucleotides formulated in folate receptor-targeted lipid vesicles. Bioconjug Chem 13:1220–1225