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Functional single-walled carbon nanotubes based on an integrin $\alpha_v\beta_3$ monoclonal antibody for highly efficient cancer cell targeting

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Abstract

The application of single-walled carbon nanotubes (SWNTs) in the field of biomedicine is becoming an entirely new and exciting topic. In this study, a novel functional SWNT based on an integrin $\alpha_v\beta_3$ monoclonal antibody was developed and was used for cancer cell targeting *in vitro*. SWNTs were first modified by phospholipid-bearing polyethylene glycol (PL–PEG). The PL–PEG functionalized SWNTs were then conjugated with protein A. A SWNT–integrin $\alpha_v\beta_3$ monoclonal antibody system (SWNT–PEG–mAb) was thus constructed by conjugating protein A with the fluorescein labeled integrin $\alpha_v\beta_3$ monoclonal antibody. *In vitro* study revealed that SWNT–PEG–mAb presented a high targeting efficiency on integrin $\alpha_v\beta_3$-positive U87MG cells with low cellular toxicity, while for integrin $\alpha_v\beta_3$-negative MCF-7 cells, the system had a low targeting efficiency, indicating that the high targeting to U87MG cells was due to the specific integrin targeting of the monoclonal antibody. In conclusion, SWNT–PEG–mAb developed in this research is a potential candidate for cancer imaging and drug delivery in cancer targeting therapy.

1. Introduction

Frequent challenges encountered by current cancer treatments fall into low specificity in cancer cell killing and low drug delivering efficiency as well as serious side effects on normal tissues [1, 2]. A rational strategy to couple the therapeutics to a targeted molecule that recognizes tumor-associated antigens, namely cancer targeting therapy, could increase the target selectivity of cancer cells and overcome the issues of deleterious side effects of traditional cancer therapy [3, 4]. On the other hand, the high impermeability of cell membranes to biological cargoes remains another barrier to the high efficiency of drug delivery. Hence, the pursuit of efficient and highly target-selective transporters to have better drug delivery and molecular imaging is an active topic in cancer targeting therapy.

Single-walled carbon nanotubes (SWNTs) have been considered potential biomedical materials because of their flexible structure and propensity for chemical functionalization; moreover, cargoes can be attached to the surfaces (inner or outer) or even packaged within the core of the tubes [5, 6]. The use of SWNTs in biological systems has opened up an entirely new and exciting field because of the special structural, electrical and optical properties of SWNTs [7]. Various research has been done focusing on the functionalization and immobilization of biomolecules on nanotubes for characterization, manipulation, purification, separation, and for device application such as sensors [8–11]. Recent studies showed that SWNTs can shuttle various molecular cargoes, including proteins [12, 13] and DNA oligonucleotides [14] as well as anticancer drugs [6, 15], crossing through the

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cellular membrane without cytotoxicity due to their distinct architecture, hollow interior and cagelike structures [16]. For example, the functionalized soluble SWNTs can serve as 'longboats' to carry Pt(IV) prodrugs into cells through clathrin-dependent endocytosis [6]. The unique properties of SWNTs as molecular transporters have a potential significance in the field of biomedicine, which needs to be fully developed.

Integrins, which consist of heterodimeric, transmembrane glycoprotein subunits (α and β), are a large family of cell surface receptors [17]. One of the most prominent members of this family is integrin $\alpha_v\beta_3$, which plays a key role in tumor proliferation, metastasis and tumor-induced angiogenesis [18]. Integrin $\alpha_v\beta_3$ was found to have high expressions on various cancer cells (glioma, melanoma and ovarian) and tumor neovasculature compared to normal tissues [19, 20], which makes integrin $\alpha_v\beta_3$ a promising candidate as a cancer cell targeting marker. Antagonists of integrin $\alpha_v\beta_3$, such as monoclonal antibodies (mAbs) and arginine–glycine–aspartic (RGD) peptide, have been found to inhibit tumor angiogenesis, cancer growth and cancer cell metastasis [21]. Although RGD peptides are commonly used as binding ligands to integrin $\alpha_v\beta_3$ due to their small molecular size, drawbacks have also been revealed: relatively low binding specificity was found due to their universal targeting ability to the other integrins ($\alpha_{11b}\beta_3$, $\alpha_v\beta_6$, $\alpha_5\beta_1$) [22, 23]; moreover, some RGD peptide containers are prone to degrade rapidly *in vivo* [24, 25]. Comparing with RGD peptides, integrin $\alpha_v\beta_3$ mAbs exhibit unique specificity to integrin $\alpha_v\beta_3$ due to their high selectivity to the antigenic target and relative stability *in vivo*. Therefore, they might play a potential role in effective drug delivery and target imaging in cancer targeting therapy.

A variety of research has been done using RGD as a targeting molecule; however, so far to our knowledge, no research has been done to conjugate the integrin $\alpha_v\beta_3$ mAb with SWNTs as a transporter for the detection of cancer cells. In this study, integrin $\alpha_v\beta_3$ mAb was conjugated with SWNTs: the antibody was used as a specific targeting component, aimed at integrin $\alpha_v\beta_3$-positive cancer cells via specific integrin $\alpha_v\beta_3$ binding; SWNTs were used as a molecular carrier.

2. Materials and methods

2.1. Materials

The major agents used in our research are: SWNTs, purchased from Chengdu Organic Chemicals Company Ltd of the Chinese Academy of Sciences; 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy (polyethylene glycol) 2000] (PL-PEG), purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA); integrin $\alpha_v\beta_3$ (23C6) monoclonal antibody (integrin $\alpha_v\beta_3$ mAb), purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); protein A, purchased from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China); fluorescein isothiocyanate (FITC), *N*-hydroxysulfosuccinimide (NHS) and 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDC), purchased from Sigma-Aldrich Co. Since all the reagents were of analytical grade and used without further purification.

2.2. Preparation of functional SWNTs based on integrin $\alpha_v\beta_3$ monoclonal antibody

2.2.1. Functionalization of SWNTs by PL–PEG. SWNTs were sonicated in an aqueous solution of PL–PEG (1 mg of SWNTs: 1 mg of PL–PEG: 1 ml of water) for 6 h. The mixture was then centrifugated at 10 000 g for 15 min. The sediment comprising of impurities, aggregates and bundles of nanotubes at the bottom of the centrifuge tube was discarded, then the supernatant was collected. Excess phospholipids were completely removed by repeated filtration using 100 kDa filters (Millipore) and rinsing with phosphate-buffered saline (PBS). Then SWNTs were resuspended in PBS by sonication for 1 h. Finally, the purified PL–PEG-functionalized SWNTs (SWNT–PEG) were composed and were resuspended in PBS.

2.2.2. Conjugation of protein A and SWNTs. The SWNT–PEG solution was activated by EDC/NHS to afford SWNT–PEG–NHS (molar ratio, SWNT–PEG: EDC: NHS = 1:1:1). SWNT–PEG–protein A was produced by incubating protein A with SWNT–PEG–NHS (pH 7.4) for 4 h. The solution was then filtrated using 100 kDa filters (Millipore) to remove excess protein A.

2.2.3. Fluorescent labeling of integrin $\alpha_v\beta_3$ monoclonal antibody. Integrin $\alpha_v\beta_3$ mAb was labeled with FITC by the procedures according to Wisdom [26]. Briefly, the solution of integrin $\alpha_v\beta_3$ mAb at a concentration of 40 nM in standard PBS was mixed with 50 μl of sodium bicarbonate solution. The solution was then mixed with FITC (13 mM, 100 μl) dissolved in DMSO (Aldrich). After incubating the mixture for 1 h at room temperature, protected from illumination, the conjugated integrin $\alpha_v\beta_3$ mAb–FITC was filtrated through 100 kDa filters (Millipore) to remove excess FITC. The resultant fluorescein-labeled protein solution was then diluted with PBS to a concentration of 8 nM.

2.2.4. Preparation of SWNT–PEG–protein A–integrin $\alpha_v\beta_3$ monoclonal antibody–FITC. The mixture of the solution of integrin $\alpha_v\beta_3$ mAb–FITC and the solution of SWNT–PEG–protein A was incubated at room temperature for 4 h (protecting from illumination) to produce SWNT–PEG–protein A–integrin $\alpha_v\beta_3$ monoclonal antibody–FITC (abbreviated as *SWNT–PEG–mAb*). Then the mixture was dialyzed against PBS by using a membrane (molecular weight cutoff = 500 kDa, PVDF) to remove unbound integrin $\alpha_v\beta_3$ mAb–FITC. To ensure complete removal, the dialysis lasted 3 to 4 days with frequent replacement of the PBS buffer until no fluorescence was detected in the rinse. Finally, purified SWNT–PEG–mAb was collected. The concentration of SWNTs in the solution after these processes was 0.3–0.4 mg ml^{-1}.

2.3. Cell culture

U87MG human glioblastoma cancer cells and MCF-7 human breast cancer cells were cultured in Eagle's minimal essential medium (EMEM) and Dulbecco's modified Eagle's medium

(DMEM), respectively. The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in 5% CO_2, 95% air at 37 °C in a humidified incubator.

2.4. In vitro cytotoxicity assay of SWNT–PEG–mAb

A cell cytotoxicity assay of SWNT–PEG–mAb to U87MG was performed by colorimetric tetrazolium salt-based assay using Cell Counting Kit-8 (CCK8 Dojin Laboratories, Kumamoto, Japan). Cell viability was determined by a 96-well plate reader (INFINITE M200, Tecan, Switzerland) at an absorbance value of 450 nm (OD450). The viability of cells was calculated as: cell viability (% of control) = ODTre/ODCon × 100% (where ODTre is the absorbance value of treated cells; ODCon is the absorbance value of untreated cells). After culturing U87MG cells and MCF-7 cells in a chamber slide for 24 h, SWNT–PEG–mAb was added to each well at a final SWNT concentration of 3–6 μg ml^{-1}.

2.5. Characterization

2.5.1. Transmission electron microscope (TEM) measurement. To examine the dispersion of PL–PEG functionalized SWNTs, the SWNT–PEG sample was observed by a JEM-2010HR transmission electron microscope (TEM) by dropping 3 μl of the solution on top of a TEM grid.

2.5.2. Optical spectra measurements. To take advantage of the intrinsic optical properties of SWNTs, Raman spectroscopy was used to directly detect SWNT–PEG–mAb solution using capillaries without spinning or stirring during the measurements. An argon ion laser (514.5 nm) was used for excitation in combination with a 40× objective of an Olympus BX-41 microscope, a Acton spectro@2300i spectrometer system (Princeton Acton, USA) and a Pixis 256 CCD detector (Princeton Acton, USA). After focusing at the center of the capillary, the Raman spectrum of the sample with a resolution of 2 cm^{-1} (10 mW power, 20 s collection time) was recorded.

The optical absorbance and fluorescence characteristics of SWNT–PEG–mAb were investigated by UV–vis absorption spectra (Lambda-35 UV–vis spectrophotometer, Perkin-Elmer, USA) and fluorescence spectra (LS-55 fluorescence spectrophotometer, Perkin-Elmer, USA) with an excitation of 490 nm.

2.5.3. Confocal microscopy. After incubating the cells with SWNT–PEG–mAb at 37 °C (5% CO_2) for 2 h, the cells were rinsed by fresh culture medium and were imaged by a commercial laser scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Germany) equipped with a Plan-Neofluar 40×/1.3 NA oil DIC objective.

3. Results and discussion

3.1. Fabrication of SWNT–PEG–mAb

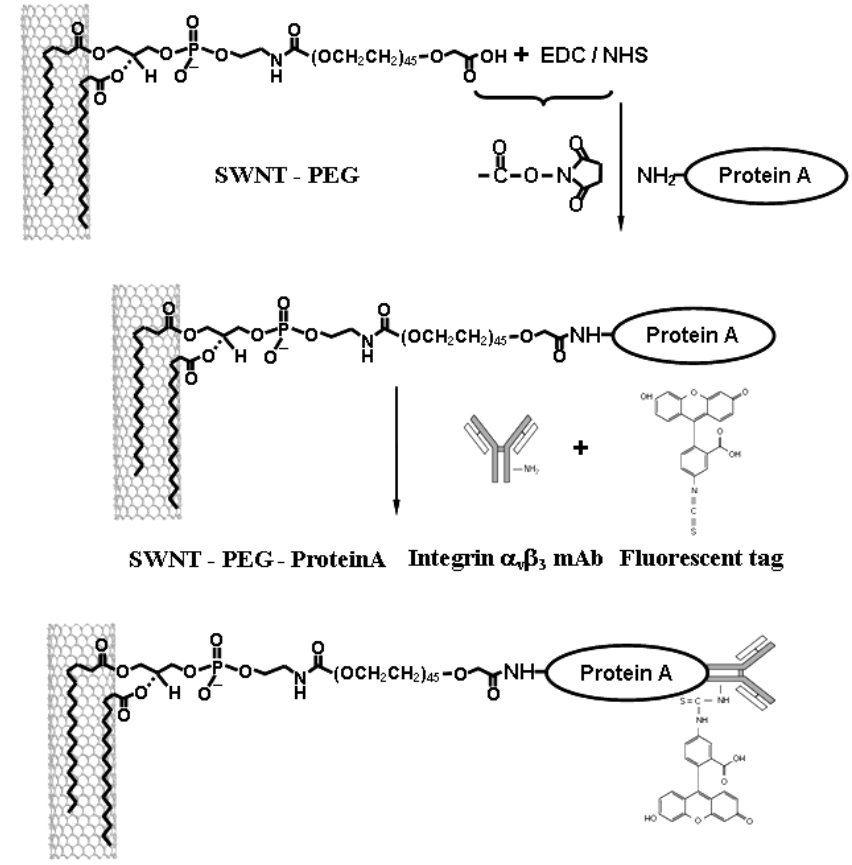
Figure 1(a) shows the schematic procedures of producing the SWNT–PEG–mAb. Firstly, SWNTs were solubilized in the aqueous phase by non-covalent adsorption of phospholipid molecules with PL–PEG chains and a terminal carboxylic acid group (PL–PEG–COOH). On the base of this PL–PEG functionalization, the carboxylic terminal of PL–PEG immobilized on SWNTs can then be used to conjugate with a wide range of biological molecules. Secondly, protein A was coupled to PEG–SWNT after activation with EDC/NHS based on the reaction between the NHS group of PEG–SWNT. Note that protein A was bound to the Fc portion of the antibody, allowing the functional portions (Fv) of the mAb to recognize integrin $\alpha_v\beta_3$. On the other hand, FITC was conjugated to integrin $\alpha_v\beta_3$ mAb via the primary amines. Finally, the SWNT–PEG–protein A was conjugated to integrin $\alpha_v\beta_3$ mAb–FITC to produce SWNT–PEG–mAb.

It is well known that carbon nanotubes are significantly hydrophobic: therefore, the transporting capability of SWNTs requires chemical functionalization with water-solubilizing moieties to increase its solubility. PEG is a well-established, benign bio-passivating material [10]. PL–PEG can be well conjugated to SWNTs via van der Waals and hydrophobic interactions between two PL alkyl chains and the SWNT sidewall [14], with the PEG chain extending into the aqueous phase to impart solubility in water. The typical diameter and length of SWNT–PEG were about 1–4 nm and 500 nm–1 μm (figure 1(b)). Previous publications showed that non-covalently functionalized SWNTs with PEG have a higher resistance to systemic clearance than that without PEG functionalization, because the sheath of PEG can prevent clearance of SWNTs by the reticuloendothelial system and can prolong their persistence in blood and humor circulation [10, 27].

3.2. Characterization of SWNT–PEG–mAb

Non-covalently functionalized SWNTs exhibit a strong resonance Raman shift at ∼1580 cm^{-1} (G band, characteristic of graphitic carbon) [28]. To confirm the successful composition of SWNT–PEG–mAb, Raman spectroscopy was used to directly detect SWNT–PEG–mAb in solution. As shown in figure 2, the G band peak area was integrated ∼1580 cm^{-1}. Raman spectroscopy clearly revealed the existence of SWNTs inside the final sample as indicated by the black color of the final sample (the inset of figure 2). Moreover, the suspensions of SWNT–PEG–mAb were well solubilized and stable in physiological media for at least two weeks at room temperature without any visible aggregation. The stabilization of SWNT–PEG–mAb revealed in our study should be attributed to non-covalent interaction between PL–PEG and SWNTs [27].

The optical absorbance and fluorescence characteristics of SWNT–PEG–mAb were investigated by UV–vis absorption spectra and fluorescence spectra. Since the unbound integrin $\alpha_v\beta_3$ mAb–FITC was removed by dialysis in all of the final samples, the optical absorbance and fluorescence emission detected were the result of SWNT–PEG–mAb alone. Figure 3(a) shows the absorbance peak of SWNT–PEG–mAb is at 490 nm (thick curve) (on top of the characteristic SWNT–PEG absorption spectrum (dotted curve)); the peak



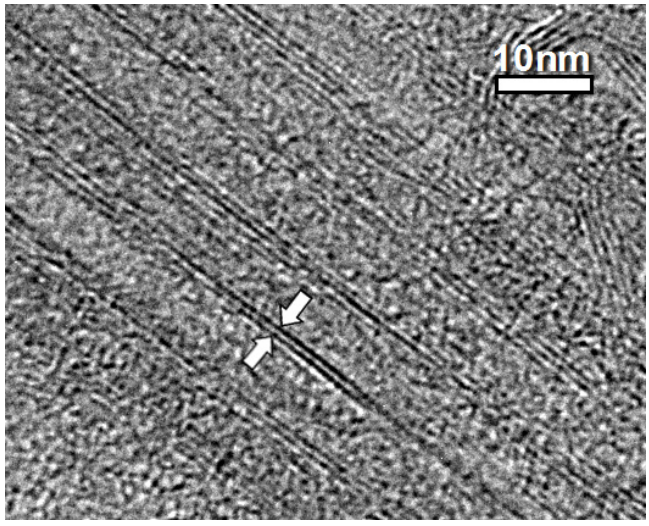


Figure 1. (a) An illustration of the procedures of producing SWNT–PEG–mAb. (b) A typical TEM image of SWNT–PEG.

of free integrin $\alpha_v\beta_3$ mAb–FITC (thin curve) is quite similar to that of SWNT–PEG–mAb, confirming a fine conjugation between integrin $\alpha_v\beta_3$ mAb–FITC and PL–PEG functionalized SWNTs. With an excitation of 490 nm, the fluorescence generated from SWNT–PEG–mAb exhibited a much weaker fluorescence than free integrin $\alpha_v\beta_3$ mAb–FITC at the same concentration (figure 3(b)). The fluorescence quenching of FITC was probably due to the energy transfer between FITC and SWNTs, which is analogous to the previous results of SWNT-bound pyrene [29, 30]. The fluorescence quenching in SWNT–PEG–mAb further confirms integrin $\alpha_v\beta_3$–FITC was well coupled on PEG-functionalized SWNTs.

3.3. Cytotoxicity of SWNT–PEG–mAb

An important concern of SWNT–PEG–mAb *in vivo* applications is its cellular cytotoxicity. To investigate the cytotoxicity

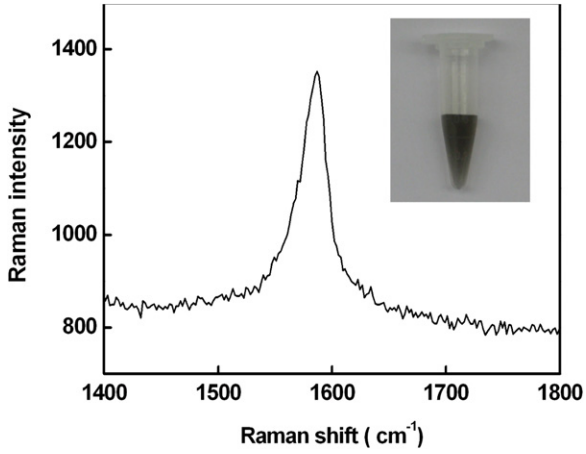


Figure 2. Raman spectra of SWNT–PEG–mAb and a photograph of stable SWNT–PEG–mAb solution (inset).

of SWNT–PEG–mAb, U87MG were cultured in a 96-well microplate (1×10^3 per well) for 24 h and were then incubated with SWNT–PEG–mAb at different concentrations (1.5, 3, 6 and 12 μg ml^{-1}) for 12 h. After rinsing with PBS, the cells were cultured for another 72 h at 37 °C. The viability of cells was calculated and no obvious toxic effect of SWNT–PEG–mAb on U87MG cells was observed compared with the non-addition of SWNT–PEG–mAb on U87MG cells, suggesting the low toxicity of SWNT–PEG–mAb.

3.4. Cancer cell targeting of SWNT–PEG–mAb

To investigate the targeting efficiency of SWNT–PEG–mAb, integrin $\alpha_v\beta_3$-positive cells (U87MG) and integrin $\alpha_v\beta_3$-negative cells (MCF-7) were incubated with SWNT–PEG–mAb, respectively. The fluorescence of fixed cells was detected by confocal microscopy: much higher fluorescence signals were observed in U87MG cells (figure 4(a)), whereas no obvious fluorescence was observed in MCF-7 cells (figure 4(b)), indicating the specific targeting of integrin $\alpha_v\beta_3$-positive U87MG cells was due to the specific recognition of integrin $\alpha_v\beta_3$ on the cytomembrane by the integrin $\alpha_v\beta_3$ monoclonal antibody. Since the cytomembrane is impermeable to FITC [12], the high fluorescence signals in figure 4(a) correspond to FITC labeled on the SWNT–PEG–mAb conjugation taken up by U87MG cells. Because of low integrin $\alpha_v\beta_3$ expression in MCF-7 cells, almost no targeting of SWNT–PEG–mAb at MCF-7 cells was observed. To utilize the potential molecular target, SWNT–PEG–mAb can selectively recognize integrin $\alpha_v\beta_3$-positive cancer cells with integrin $\alpha_v\beta_3$ mAb, and afford no destruction of integrin $\alpha_v\beta_3$-negative cancer cells and normal cells.

In order to have further confirmation that the main uptake mechanism of SWNT–PEG–mAb is due to the integrin $\alpha_v\beta_3$ receptor-mediated delivery pathway, U87MG cells were treated with SWNT–PEG–mAb in the presence of integrin $\alpha_v\beta_3$ antibody (5 nM) and characterized by confocal microscopy. As shown in figure 4(c), the targeting of SWNT–PEG–mAb was significantly blocked by integrin $\alpha_v\beta_3$ mAb: the fluorescence level detected in

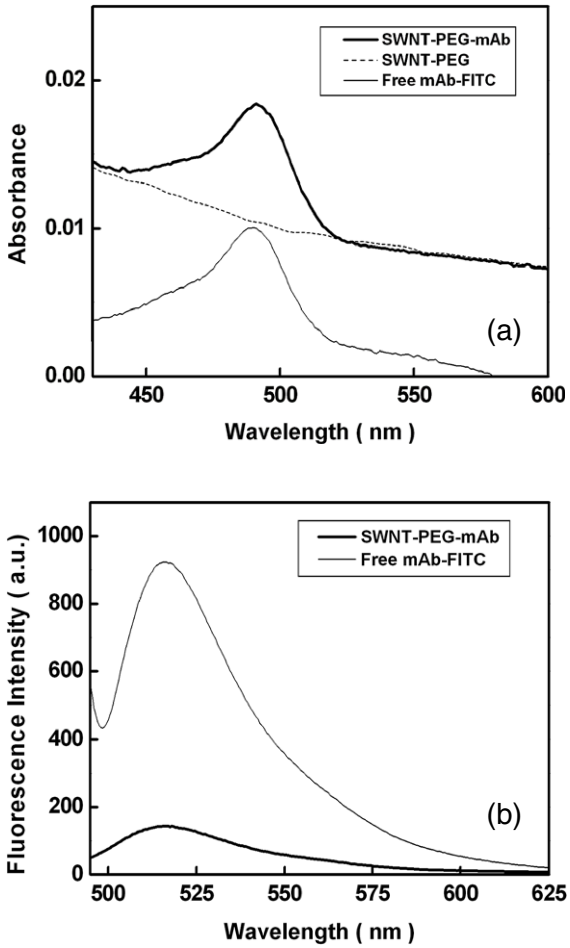


Figure 3. (a) Absorbance spectra and of SWNT–PEG–mAb (thick curve), SWNT–PEG (dotted curve) and mAb–FITC (thin curve); (b) fluorescence emission spectra of SWNT–mAb–FITC (thick curve) and mAb–FITC (thin curve) in PBS with an excitation of 490 nm.

the control group was significantly lower than that of the experiment group, indicating that the main delivery pathway of SWNT–PEG–mAb is via integrin $\alpha_v\beta_3$ acceptor-mediated endocytosis [31–34]. Moreover, the block result provides evidence of the existence of integrin $\alpha_v\beta_3$ mAb on the surface of SWNTs. Hence, the confocal microscopy results together with the Raman, absorbance and fluorescence results above confirmed that SWNT–PEG–mAb conjugation was successfully prepared, as shown in figure 1. The results above hint that the SWNT–PEG–mAb system with an efficient cancer targeting delivery ability has the potential to function as a transport matrix for various biological cargoes into cancer cells.

4. Conclusion

In conclusion, with a combination of integrin $\alpha_v\beta_3$-mAb (an ideal targeting molecular) and SWNTs (a molecular carrier), an effective target delivering molecular transporter, SWNT–PEG–mAb, was successfully developed. SWNT–PEG–mAb shows high aqueous dispersibility and stability as well as

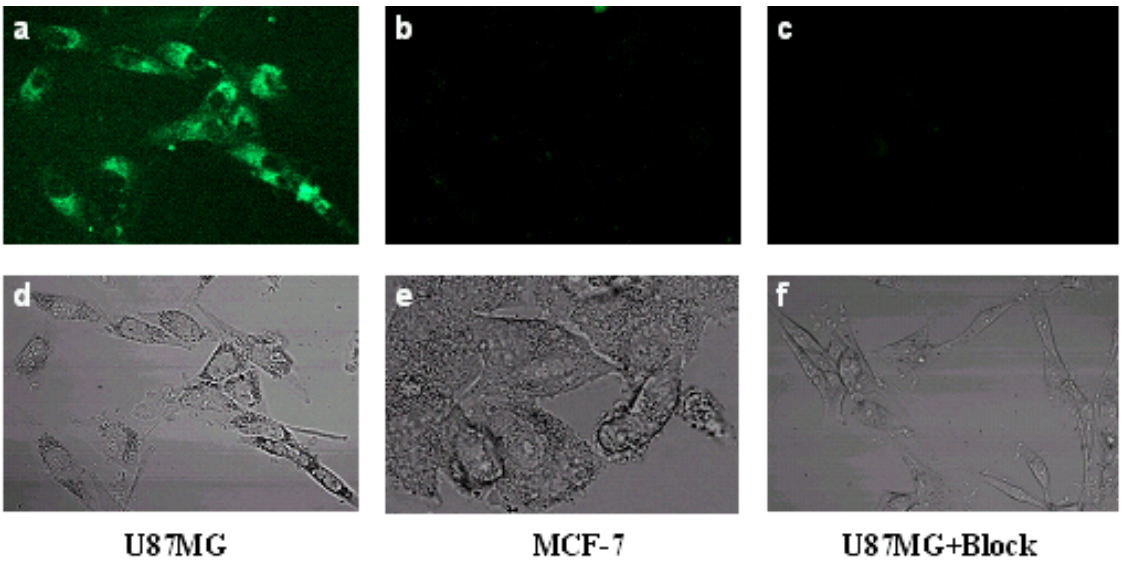


Figure 4. Confocal microscopic images of U87MG ((a), (d)) and MCF-7cells ((b), (e)) incubated with SWNT–PEG–mAb ((c), (f)) is the control group showing blocking of integrin $\alpha_v\beta_3$ on U87MG cells with integrin $\alpha_v\beta_3$ monoclonal antibody.

minimal cytotoxicity. Furthermore, specific targeting of SWNT–PEG–mAb on integrin $\alpha_v\beta_3$-positive cancer cells due to the high affinity of the integrin $\alpha_v\beta_3$-mAb was also observed. All these results suggest the possibilities of developing versatile drug delivery systems and cancer imaging systems that have highly specific cancer cell targeting using the combination of integrin $\alpha_v\beta_3$ monoclonal antibody and SWNTs. Thus, the transporting capabilities of carbon nanotubes combined with the suitable functionalization chemistry of SWNTs can open up exciting new venues for drug delivery and tumor targeting in cancer targeting therapy.

However, there are plenty of spaces for future explorations of the SWNT–PEG–mAb system in biological applications. Further optimization studies of SWNT–PEG–mAb and direct imaging of SWNT–PEG–mAb in cells as well as *in vivo* toxicity are further pursuits of our efforts. On the other hand, for the high expression of integrin $\alpha_v\beta_3$ in tumor neovasculature compared with normal blood vessels, photoacoustic molecular imaging with integrin $\alpha_v\beta_3$ antibody-functionalized SWNTs in early tumor detection are underway in our laboratory.

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