Folate and iron difunctionalized multiwall carbon nanotubes as dual-targeted drug nanocarrier to cancer cells

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ABSTRACT

A nanomaterial, folate and iron difunctionalized multiwall carbon nanotube (FA-MWCNT@Fe), has been synthesized by conjugating folate and iron nanoparticles with oxidized multi-walled carbon nanotubes, and applied as a dual-targeted drug nanocarrier to deliver doxorubicin into HeLa cells with the assistance of an external magnetic field. The prepared FA-MWCNT@Fe was characterized by X-ray diffraction, transmission electron microscopy and infrared spectroscopy. This nanocarrier has a sufficient load capacity (doxorubicin/FA-MWCNT@Fe, 32 μg/mg) and a prolonged release property controlled by near infrared radiation. It also demonstrated both biologically (active) and magnetically (passive) targeting capabilities toward HeLa cells in vitro with ca. 6-fold higher delivery efficiency of doxorubicin than free doxorubicin.

1. Introduction

Carbon nanotubes (CNTs), as molecule carriers, exhibit potential in biological systems due to their distinct properties in cell membrane penetration, and loading and release of molecular cargoes [1-5]. Moreover, it has been discovered that CNTs could be metabolized by neutrophil myeloperoxidase [6] and the functionalized CNTs (f-CNTs) have less cytotoxicity [7]. In the past 10 years, a variety of f-CNTs including oxidized, aminated, surfactant-assisted dispersed, polymer-assisted dispersed, and bio-molecular modified CNTs have been discovered [8] and utilized as the effective bio-molecular carriers to deliver peptides, proteins, nucleic acids and small molecular drugs into cellular systems. For instance, Kam et al. have demonstrated the intracellular transportation of DNA [9], siRNA [10], and proteins [11] using functionalized single-walled carbon nanotubes (f-SWCNTs) as non-targeted drug carriers. Pantarotto et al. explored the delivery of bioactive peptides of K(TTCC)QRMLRQYELLK into 3T3 cells [12], methotrexate into Jurkat cells [13] and plasmid DNA into HeLa cells [14], using f-CNTs as non-targeted drug carriers. Additionally, f-CNTs have been applied as the non-targeted drug carriers to transport platinum(IV) [15], oligodeoxynucleotides [16] and boron [17] into cancer cells.

Although great improvements have been made to the application of f-CNTs as drug carriers, there are two major obstacles blocking further use of the f-CNTs in cancer chemotherapy. The first one is the non-specific toxicity of anticancer agents to the proliferation of normal cells, while the second one is the poor potency of the anticancer agents [18,19]. To achieve the discrimination of the natural and the cancerous tissues, the targeting ability is a crucial property for the f-CNTs as drug carriers. In order to develop the targeting ability of the f-CNTs, conjugation with targeted moieties of folate (FA), antibodies, receptors or nanoparticles (NPs) is necessary. Much research has been carried out, such as the
targeted delivery of cisplatin into Ntera-2 cells by FA-modified SWCNTs [20], cytochrome C into phagocytic cells by phosphatidylserine-modified SWCNTs [21], gemcitabine into lymph nodes by NPs-modified multiwall carbon nanotube (MWCNT) [22], doxorubicin (Dox) into WiDr cells by antibody-modified SWCNTs [23] and other related researches [24–27]. However, most of the f-CNTs were only designed as the mono-targeted drug carriers, while the much more efficient multi-targeted carriers, based on CNTs, have been reported to a less extent. On the other hand, during the past decade, diverse multi-targeted drug carriers have been prepared and tested in some cancer cell lines based on liposome or polymeric NPs. For example, a liposome-based dual-targeted drug delivery system with two probes of FA and the antibody of epidermal growth factor receptor (EGFR) has been prepared to improve the selectivity of targeting human KB cells, which over-expresses both FA and EGFR receptors [28]. Also, upon the changes of local temperature and pH value around certain pathological microenvironments of tumors, the thermo and pH-responsive NPs, consisting of the temperature-responsive polymer of poly(N-isopropylacrylamide) and the pH-responsive polymer of poly(N,N-dimethylacrylamide) [29], poly(methacrylic acid) [30] or (acrylic acid) [31], have been developed as anticancer drug carriers. Additionally, in order to better recognize cancer receptors, the FA receptor targeted nanocarriers have been conjugated with magnetic probes, which demonstrated to be an effective dual targeting nanoplatform for the delivery of anticancer drugs by guiding the nanocarriers to cancer sites efficiently using an external magnetic field [32].

Previously, we have investigated the cellular uptake of oxidized CNTs [33], the detection of intracellular uptake of drug molecules carried by CNTs [4], and the targeted drug delivery of Dox to multidrug resistant leukemia cells by using antibody functionalized SWCNT as nanocarrier [34]. These studies have demonstrated the potential of CNTs to carry drugs into the cells efficiently via endocytosis. Herein, we developed a dual-targeted drug delivery system to transport Dox into HeLa cells using FA and iron difunctionalized MWCNT (FA-MWCNT@Fe) as drug nanocarrier. The purpose of this dual-targeted drug delivery system is first to guide the nanocarriers to the location of cancer cells with an external magnetic field, and then let the FA-encoded CNT target the FA-receptors over-expressed on the cancer cells. Due to the combination of the highly specific biological affinity probe of FA and the magnetically guided probe of iron NPs on the CNTs, the enhanced drug delivery efficiency of FA-MWCNT@Fe nanocarriers along with the controlled release performance assisted by near infrared radiation (NIR) are expected.

2. Materials and methods

2.1. Materials

The pristine MWCNT produced by chemical vapor deposition with a diameter of 10–20 nm and a purity of >98% was purchased from Shenzhen Nanoharbor, China. Dox and fluorescein isothiocyanate (FITC) was purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (PMS) were purchased from Dojindo laboratory (Kumamoto, Japan). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Pierce (Pierce, IL, USA). Phosphate buffered saline (PBS) contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 and 1.4 mM KH2PO4 with pH values of 7.4. The Fe3O4 NPs (10–20 nm) were prepared in our lab using the following literature procedure [35]. Other reagents were all analytical grade unless stated otherwise.

2.2. Cell culture

HeLa cells were obtained from the Institute of Blood, Chinese Academy of Medical Sciences (Tianjin, China). HeLa cells were seeded initially at a density of 1.0 × 104 cells/mL, cultured in 75-cm2 vented culture flasks at 37 °C with RPMI-1640 medium containing 10% calf serum in 5% carbon dioxide atmosphere, and split every 2–3 days.

2.3. Preparation of nanocarriers

The nanocarriers including FA-MWCNT@Fe, FA-(FITC)MWCNT@Fe and (FITC)MWCNT@Fe were prepared as follows. Firstly 1 g raw MWCNT were treated in 120 mL concentrated HNO3 and H2SO4 (1:3, v/v) solution at 120 °C in an oil bath for 30 min [34]. After dilution with water by 10-fold, the solution was filtered through a 0.45 μm filter. Then the product was re-dissolved in water. The resulting mixture was centrifuged at 5000 g to remove any large or unreacted MWCNT. The supernatant was collected and lyophilized where nearly 100 mg water soluble o-MWCNT was acquired. The o-MWCNT was subsequently added to an aqueous Fe(NO3)3 solution under stirring followed by ultrasonic treatment and further stirring for 2 h. Evaporation was first allowed under ambient conditions, followed by heat-treatment at 140 °C in air for 8 h and then at 350 °C in He for 3 h. This resulted in decomposition of Fe(NO3)3 to Fe2O3. The loading of Fe2O3 on the composites of Fe2O3 and MWCNT (denoted as o-MWCNT@Fe2O3) was ca. 8 wt.%. The obtained o-MWCNT@Fe2O3 was then reduced by flowing H2 at 350 °C [36].

Prior to preparation of the dual-targeted drug carrier, FITC conjugated with hexamethylenediamine (FITC-HMDA) and folic acid conjugated with hexamethylenediamine (FA-HMDA) as the two monomers were synthesized in aqueous phase. For the synthesis of FITC-HMDA, 100 mg HMDA was dissolved in 40 mL aqueous NaHCO3–Na2CO3 buffer (0.1 M, pH 9.4). Next, 10 mL NaHCO3–Na2CO3 buffer containing 50 mg FITC was slowly added and reacted for 24 h under stirring. After the completion of the reaction, the mixture was lyophilized and stored for further purification. Additionally, FA-HMDA was synthesized via the diimide-activated amidation reaction as described elsewhere [33]. Briefly, 100 mg of FA, 500 mg EDC and 1 g NHS were added to 800 mL aqueous 2-(4-morpholino)ethanesulfonic acid (MES) buffer solution (50 mM, pH 6.0) and reacted for 2 h under stirring. Then 200 mg HMDS was added into the solution, and the mixture was stirred for 12 h. After that, the products were lyophilized. Finally, both of the raw FITC-HMDA and FA-HMDA were separated using liquid chro-
matography, where 30 mg of FITC-HMDA and 40 mg of FA-HMDA were collected and further characterized by mass spectrometry (see SI, SI-1 and Fig. S-1).

At last, these three nanocarriers of FA-MWCNT@Fe, (FITC)MWCNT@Fe and FA-(FITC)MWCNT@Fe were assembled via the diimide-activated amidation reaction between o-MWCNT@Fe and FA-HMDA or/and FITC-HMDA. In detail, 80 mg o-MWCNT@Fe, 200 mg EDC and 400 mg NHS were dissolved in 40 mL MES buffer and the mixture was stirred for 2 h. Then, the mixture was filtered through a 0.45 μm filter and fully rinsed with 50 mM MES buffer solution. Then the obtained products were divided into triplicate, and reacted with FA-HMDA, FA-HMDA/FITC-HMDA (m/m, 1/1) and FITC-HMDA, respectively. After 12-h reaction under stirring, the three NPs, FA-(FITC)MWCNT@Fe, FA-MWCNT@Fe and (FITC)MWCNT@Fe, were filtered through a 0.45 μm pore-size membrane filter, sufficiently washed with acetonitrile till no residual folate detected in filtrate by mass spectrum. All of the prepared NPs were lyophilized and stored 4 °C for further use. The schematic procedure for preparation of the three NPs was illustrated in Fig. 1. FA-MWCNT@Fe as a dual-targeted carrier was characterized by transmission electron microscopy (TEM, FEI Tecnai G2 T20 microscope set at an accelerating voltage of 120 kV), X-ray diffraction (XRD, Rigaku X-ray diffractometer set at scanning speed of 10°/min) and infrared spectroscopy (IR, Bruker FT-IR spectrometry, VECTOR22, set at a resolution set of 4 cm⁻¹).

2.4. NIR-stimulated release

The release of Dox from FA-MWCNT@Fe was performed as described in our previous report [34]. Briefly, a Philips PAR 38 infrared lamp (Philips, Eind-hoven, Netherlands) were utilized to supply the NIR, and was placed ca. 15 cm away from the dialysate level to make sure a 0.2-W/cm² power. Then, an optimized condition of 5-min NIR during each 35 interval (see SI-2, Fig. S-2b) was adopted to restrict the temperature of dialysate under 37 °C during the dialysis procedure.

Fig. 1 – Synthesis scheme for NPs of FA-MWCNT@Fe, FA-(FITC)MWCNT@Fe and (FITC)MWCNT@Fe, including the oxidation of MWCNT, loading of Fe(NO₃)₃ onto o-MWCNT, thermal decomposition of Fe(NO₃)₃, reduction of Fe₂O₃ NPs, and diimide-activated amidation reaction between o-MWCNT@Fe and the intermediates.
2.5. **Observation under confocal laser scanning microscope (CLSM)**

HeLa cells were seeded on four chambered cover glasses and incubated at 37°C in 5% CO₂ atmosphere until cells were about 80% confluent. On the day of the experiment, the four cell samples were incubated with media containing (FITC)-MWCNT@Fe, FA-(FITC)MWCNT@Fe, Dox/(FITC)MWCNT@Fe and Dox/FA-(FITC)MWCNT@Fe (1 μg/mL Dox and 40 μg/mL NPs), respectively. At the end of incubation, the monolayer was washed three times with a PBS buffer to eliminate excess NPs which were not bound onto the cells. A TCS-SP2 CLSM (Leica, Wetzlar, Germany) with a 100× objective was used for the cell fluorescent imaging.

2.6. **Cytotoxicity assays**

The drug delivery efficiencies of the nanocarriers were evaluated by WST-1 assay. HeLa cells were seeded in a 96-well plate in 100 μL medium per well at a density of 5000 cells per well, and incubated for 24 h. After incubation with agents, the culture media in each well were removed. A 200 μL aliquot of the culture media containing WST-1 and PMS (200 μM WST-1, 1 μM PMS) was added to each well and incubated with HeLa cells for another 3 h. WST-1 absorbance was measured by Model 550 ELISA reader (BIO-RAD, Hercules, CA, USA) at 450 nm. A higher WST-1 absorbance indicates higher relative cell viability.

2.7. **Statistics**

All experiments were performed in triplicate and the results were analyzed by SPSS 15.0 software (SPSS, Chicago, IL, USA). A one-way ANOVA and a Student's t-test were applied to determine the statistical differences. The difference was considered significant at P value <0.05.

3. **Results and discussion**

3.1. **Synthesis and characterization of the dual-targeted drug nanocarrier**

For guiding the CNT-based drug delivery vehicles to cancer cells by applying a magnetic field, the incorporation of CNTs with magnetic moieties are required due to the absence of magnetism for CNTs per se. Iron NPs possess good magnetic property and thus have been used as the magnetic cores for some drug delivery purposes [37–39]. Previously, Chen et al. have reported the direct reduction of Fe₂O₃ to metallic iron on the inner and external surface of CNTs [36,40]. So, the composites of Fe/CNTs were hereby chosen as the original material for the design of dual-targeted drug delivery system in this work.

The synthesis scheme of the dual-targeted drug nanocarriers of (FITC)MWCNT@Fe, FA-(FITC)MWCNT@Fe and FA-MWCNT@Fe is illustrated in Fig. 1. The preparation procedure of these NPs comprises the synthesis of MWCNT (10–20 nm o.d. and 0.5–1 μm length, see SI, Fig. S-3), adsorption of Fe³⁺ onto o-MWCNT; growth of iron NPs onto o-MWCNT and the final modification of FA or FITC onto o-MWCNT@Fe.

The dual-targeted nanocarrier of FA-MWCNT@Fe was characterized by TEM, XRD and IR spectrum. Fig. 2a displays the TEM image of FA-MWCNT@Fe with iron NP size around 5–7 nm. The XRD spectrum of the sample (Fig. 2b) exhibits a typical peak of MWCNT (marked with triangle), a peak indexed to Fe [1 1 0] (marked with square) and diffraction lines corresponding to Fe₂O₃ [3 1 1], [4 2 2], [5 1 1] and [5 3 3] (marked with circle) [40]. This can be explained that the pure iron NPs were active and could be oxidized to iron oxide on the surface of iron NPs after exposure to air. The cover of iron oxide on iron NPs would thus decrease the surface activity of iron NPs and prevent the possible corrosions in further use. The conjugation of FA ligand on o-MWCNT was verified by infrared spectroscopy (IR). As shown in the spectra of FA-MWCNT@Fe (see SI, Fig. S-4a), the IR absorption peaks result from three moieties, including the o-MWCNT@Fe, the spacer arm (HMDA) between FA and o-MWCNT@Fe, and FA. The observed two peaks in the IR spectra of FA-MWCNT@Fe at 3433 and 1633 cm⁻¹ corresponding to the absorption peaks of o-MWCNT@Fe at 3436 and 1635 cm⁻¹ (see SI, Fig. S-4b), are attributed to the typical stretching vibrations of O–H and C=O, respectively, which indicate the presence of –COOH group. The absorption peaks at 2923 and 2846 cm⁻¹ imply both the symmetric and asymmetric stretching vibrations of C–H bond which arise from the spacer arm of HMDA on FA-MWCNT@Fe. While the peaks at the spectral regions of 1750–1000 cm⁻¹ of FA-MWCNT@Fe are similar with those of

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** – (a) TEM and (b) XRD characterization of FA-MWCNT@Fe. The iron NPs can be clearly observed on both the inner and outer surfaces of MWCNT (marked with arrows). The XRD pattern displays the peaks of iron NPs (marked with squares) and their oxidized products (marked with circles).
FA (see SI, Fig. 4c) and can be explained as follows: (i) the peaks in the region of 1750–1630 cm\(^{-1}\) are attributed to the presence of various C=O groups from -COOH, -CONH\(^{-}\) and -CON=; (ii) the peaks in the region of 1600–1450 cm\(^{-1}\) represent the characteristic absorption of aromatic compounds from phenyl and pyrazinyl; (iii) the region of 1400–1000 cm\(^{-1}\) corresponds to the stretching vibration of C–N from amide, aromatic amine, pyrazinyl and pyrimidinyl.

### 3.2. Drug loading and release performance of FA-MWCNT\(^{\odot}\)Fe

Both the desirable adsorption capability to poorly water-soluble drugs by \(\pi-\pi\) stacking and the strong absorption ability to NIR are the two distinguished features ofCNTs, which are advantageous to loading drug molecules and releasing them into cancer cells [41–44]. Previously, we have demonstrated that antibody-functionalized SWCNTs possess expected adsorption capacity and release performance for Dox [34]. The loading and release performances of the FA-MWCNT\(^{\odot}\)Fe nanocarriers were investigated and compared with a commonly used magnetic carrier, Fe\(_3\)O\(_4\) NPs [45–47].

To investigate the loading performance of nanocarriers of FA-MWCNT\(^{\odot}\)Fe and Fe\(_3\)O\(_4\) NPs in vitro, both nanocarriers (each of 200 \(\mu\)g) were dissolved into 2 mL Dox solutions with concentrations ranged from 0 to 50 \(\mu\)g/mL. After the solutions were vortexed for 1 h at 25 \(^\circ\)C, FA-MWCNT\(^{\odot}\)Fe NPs were filtered through a 0.45 \(\mu\)m pore-size cellulose membrane, while Fe\(_3\)O\(_4\) NPs were separated from solutions by a magnet. And finally the Dox concentrations in the supernatants were detected by a fluorescence spectrophotometer (FS). The amounts of Dox adsorbed on NPs were the amount difference between the original Dox solutions and the filtrates. Fig. 3a shows the resulting adsorption isotherms of Dox on FA-MWCNT\(^{\odot}\)Fe and Fe\(_3\)O\(_4\) with the maximum loading capacities of ca. 32 and 24 mg/mg, respectively, which could be approximated by the Langmuir isotherms. The higher Dox loading capacity of FA-MWCNT\(^{\odot}\)Fe vs. Fe\(_3\)O\(_4\) is consistent with the expectation that CNTs not only provide the sufficient surface area but also the \(\pi-\pi\) stacking interaction for molecule adsorption.

The in vitro release of Dox from FA-MWCNT\(^{\odot}\)Fe and Fe\(_3\)O\(_4\) NPs was examined by dialyzing Dox/NPs composites in PBS buffer solutions with an interval exposure of NIR, the carbon nanotube is the good energy absorption material for near-infrared radiation [41]. With NIR exposure, FA-MWCNT\(^{\odot}\)Fe will adsorb the energy thus consequently improve the release of Dox from FA-MWCNT\(^{\odot}\)Fe via an endothermic process [34]. In detail, both nanocarriers of FA-MWCNT\(^{\odot}\)Fe and Fe\(_3\)O\(_4\) (each of 1 mg) were incubated with 2 mL PBS buffer solutions containing 100 \(\mu\)g/mL of Dox for 1 h, and then separated and dialyzed in 2 mL PBS buffer solutions at 25 \(^\circ\)C for 4 days. During the 4-day dialysis, NIR was applied on the solutions of Dox/NPs conjugates every 35 min, while the PBS buffer solutions were refreshed every 2 h. The dialyzates were collected and the concentrations of Dox in dialyzates were monitored by FS. For comparison, the same release experiments were carried out but without the exposure of NIR. The release of Dox from NPs could be evaluated by the ratio of accumulative content of Dox in dialyze and the adsorption capacity. As shown in Fig. 3b, after exposure to NIR, the release of the Dox from FA-MWCNT\(^{\odot}\)Fe within 24 h was increased from 23% to 59%, while the release of the Dox from Fe\(_3\)O\(_4\) NPs only had a little increase, indicating the preserved advantage of CNTs in absorbing NIR. In addition, nearly 95% of Dox was released from Fe\(_3\)O\(_4\) NPs within 10 h, which is a burst procedure and disadvantageous to drug efficiency. Whereas, the release of the Dox from FA-MWCNT\(^{\odot}\)Fe nanocarriers is a prolonged procedure, with only 40% of the Dox released within 10 h. Even after 4-d release experiment, there were still ca. 35% of the Dox adsorbed on FA-MWCNT\(^{\odot}\)Fe. These release results suggest that FA-MWCNT\(^{\odot}\)Fe nanocarriers possess the prolonged as well as NIR controlled release properties.

### 3.3. Active targeting capability of FA-MWCNT\(^{\odot}\)Fe as nanocarrier

To observe the intracellular bio-distribution of FA-MWCNT\(^{\odot}\)Fe and o-MWCNT\(^{\odot}\)Fe, the FITC-labeled FA-MWCNT\(^{\odot}\)Fe and (FITC)MWCNT\(^{\odot}\)Fe, were applied and monitored by CLSM. The obtained CLSM images are illustrated in Fig. 4, where
the red and green fluorescence represent the Dox and the encoding FITC on these nanocarriers, respectively.

After incubation with 40 μg/mL of FA-(FITC)MWCNT@Fe or (FITC)MWCNT@Fe at 37 °C for 1 h, the cell samples were observed under CLSM. In Fig. 4a, the green fluorescence representing the (FITC)MWCNT@Fe in HeLa cells is inconspicuous, suggesting that only a little of (FITC)MWCNT@Fe entered into HeLa cells within this one hour incubation. Instead of using the FA-encoded FA-(FITC)MWCNT@Fe, as shown in Fig. 4b, the bright green circles were observed, indicating that the intracellular uptake of FA-(FITC)MWCNT@Fe was extremely enhanced due to the conjugation of FA on (FITC)MWCNT@Fe. Also, the FA-(FITC)MWCNT@Fe nanocarriers were mainly localized on the membrane of HeLa cells. Both of the fluorescent images from CLSM and their bright field images (see Fig. S-5) indicate that the FA-(FITC)MWCNT@Fe can automatically recognize HeLa cells rather than the (FITC)MWCNT@Fe because of the high affinity of the FA moiety on FA-(FITC)MWCNT@Fe toward the highly-expressed FA receptors on the membrane of HeLa cells. In addition, the intracellular uptake mechanism of FA-(FITC)MWCNT@Fe was also investigated by incubating FA-(FITC)MWCNT@Fe with HeLa cells at 37 or 4 °C. The flow cytometry measurement of HeLa cells incubated at these two temperatures suggests a clear endocytosis uptake mechanism (see SI, SI-3 and Fig. S-6).

Moreover, the intracellular delivery of Dox to HeLa cells was investigated to examine the targeting and delivery performance of the FA-(FITC)MWCNT@Fe nanocarriers toward cancer cells. Thus HeLa cells were incubated with Dox/FA-(FITC)MWCNT@Fe or Dox/(FITC)MWCNT@Fe (1 μg/mL of Dox and 40 μg/mL of NPs in media) at 37 °C for 6 h. During this incubation, a 5 min NIR radiation was periodically applied on these cells with the break interval of 2 h to enhance the release of Dox into cells from the nanocarriers. As shown in Fig. 4c, the red Dox spots are rather dim for HeLa cells incubated with Dox/(FITC)MWCNT@Fe, as compared with the much bright Dox spots for cells treated by the Dox/FA-(FITC)MWCNT@Fe (Fig. 4d). The enhanced Dox spots indicate the increased intracellular delivery of Dox by FA-(FITC)MWCNT@Fe rather than (FITC)MWCNT@Fe, further confirming the targeting ability of the FA-encoded nanocarriers toward cancer cells. Also, the intracellular delivery of Dox was detected by capillary electrophoresis coupled with laser induced fluorescence (CE-LIF), and a significant enhancement of the Dox delivered by FA-MWCNT@Fe was also detected (see SI, SI-3 and Fig. S-7).

Fig. 4 – CLSM images of HeLa cells treated by (a) (FITC)MWCNT@Fe, (b) FA-(FITC)MWCNT@Fe, (c) Dox/(FITC)MWCNT@Fe and (d) Dox/FA-(FITC)MWCNT@Fe. Green spots represent the FITC labeled nanocarriers, and red spots represent Dox under CLSM. Compared to (FITC)MWCNT@Fe (panel a), the intracellular uptake of FA-MWCNT@Fe (panel b) is significantly enhanced due to the over-expressed FA receptor on the membrane of HeLa cells. Also, more Dox is delivered and released into HeLa cells by FA-MWCNT@Fe (panel d) than by o-MWCNT@Fe (panel c). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)
3.4. Intracellular delivery efficiency of Dox by FA-MWCNT@Fe

In cancer chemotherapy, the drug delivery efficiency is of essential importance for drug delivery systems. Though the biological affinity probes on nanocarrier such as FA can enhance the drug delivery efficiency by targeting the receptors on cell membrane, the binding amounts of nanocarriers on a target cell actually depend on the concentration of nanocarriers in extracellular circumstance. Magnetic field guiding can help to improve the transfer efficiency of drug vectors and thus elevate the circumstance concentration of the biological affinity probe once it possesses the magnetism. In this work, we have made dual-targeted nanocarriers possessing both magnetic and biological targeting capabilities. Thus, as shown in Fig. 5, the FA-MWCNT@Fe nanocarrier was expected to be anchored at the site of cancer cells induced by a magnetic field. Then the nanocarriers would recognize the cancer cells and deliver Dox into the cells by the biological affinity interaction between the FA and the FA receptor. As a result, the drug delivery efficiency would be greatly improved.

Hereby, we carried out the cytotoxicity assays to investigate the drug delivery efficiency of FA-MWCNT@Fe. Before that, the biological safety of o-MWCNT, o-MWCNT@Fe, FA-MWCNT and FA-MWCNT@Fe was evaluated in advance. HeLa cells were seeded on a 96-well plate and incubated with all of these NPs for 48 h, and the cellular viabilities were tested by the WST-1 assay. The obtained results suggest that these NPs have no significant cytotoxicity to HeLa cells and are safe for biological applications (see SI, Fig. S-8).

Based on the results of the biosafety test, the cytotoxicities induced by the conjugates of NPs and Dox were thus tested. The nanocarriers (40 μg/mL NPs) loading Dox (0–5 μg/mL) were initially added into the 96-well plates and incubated with HeLa cells. Two parallel cytotoxic tests, with or without the presence of magnetic field, were carried out. The culture media was refreshed every 8 h to simulate the in vivo clearance during the cellular incubation procedures. In order to enhance the release of Dox from the Dox/NPs conjugates, a 5 min NIR (0.2 W/cm²) exposure was applied to the cells every 8 h during 48-h incubation period. Fig. 6 represents the Dox-induced cytotoxicities (1 μg/mL Dox) for free Dox, Dox/o-MWCNT, Dox/FA-MWCNT, Dox/o-MWCNT@Fe and Dox/FA-MWCNT@Fe. It can be seen that the cytotoxicity induced by free Dox, Dox/o-MWCNT and Dox/FA-MWCNT are not influenced by the magnetic field. Among these three agents, the Dox/FA-MWCNT exhibits the highest cytotoxicity due to the specific cellular recognition property of the FA moiety to cancer cells. Whereas, the cellular viability is only suppressed by ca. 45% because of the limited drug delivery efficiency of the mono-targeted drug delivery system. The introduction of a magnetic targeted moiety, iron NPs, onto MWCNT, shows 2–3 times enhancements of cytotoxicities induced by Dox/o-MWCNT@Fe or Dox/FA-MWCNT@Fe in the presence of a magnetic field than no magnetic field applied. It could be concluded that the magnetically targeted drug delivery system, a passive targeted drug delivery system, would enhance the drug delivery efficiency with the assistance of the magnetic field. Importantly, it’s the Dox/FA-MWCNT@Fe that exhibits the highest cytotoxicity among the other five agents in the presence of the magnetic field, which is ca. 6-fold higher than that induced by the clinically used Dox. From this it can be concluded that the biologically and magnetically dual-targeted nanocarrier of FA-MWCNT@Fe is able to greatly

Fig. 5 – Scheme for dual-targeted drug delivery of Dox by FA-MWCNT@Fe. It is expected that FA-MWCNT@Fe nanocarrier could be initially guided to anchor at the sites of cancer cells by an external magnetic field, and then recognize the cancer cells by the biological affinity probe of FA.
enhance the drug delivery efficiency because of the incorpo-
ration of FA moiety and iron NPs onto MWCNT, or the combi-
nation of active and passive targeted properties.

4. Conclusions

A novel dual-targeted drug delivery system, FA-MWCNT@Fe, has been synthesized by covalently conjugating folic acid on α-MWCNT loaded with iron NPs. Due to the strong non-specific adsorption of CNTs, Dox could be simply loaded on FA-MWCNT@Fe by π–π stacking interaction, with a larger loading capacity to Dox as compared to Fe3O4 NPs. Besides, the release of loaded drugs (Dox) from this nanocarrier is a NIR-assisted controllable procedure. Because of the incorporation of both the FA moiety and the magnetic iron NPs onto α-MWCNT, the FA-MWCNT@Fe could recognize cancer cells via FA by an active targeted manner, and anchor at the sites of cancer cells via iron NPs by a passive targeted manner. This novel nanocarrier provides excellent cancer specificity and greatly enhanced drug delivery efficiency, which could be applied as a promising dual-targeted drug nanocarrier in cancer chemotherapies.

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Appendix A. Supplementary data

Purifications and characterizations of FA-HMDA and FITC-HMDA, TEM images for α-MWCNT and FA-MWCNT@Fe, IR spectrums of α-MWCNT@Fe, FA and FA-MWCNT@Fe, study on intracellular pathway and detection of the intracellular uptake of Dox by CE-LIF, images of treated HeLa cells from CLSM and cytotoxicity assays of the NPs. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbon.2011.01.003.

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