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Intracellular trafficking and therapeutic outcome of multiwalled carbon nanotubes modified with cyclodextrins and polyethylenimine

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ABSTRACT

Functionalized carbon nanotubes (CNTs) have been proposed in the last years as vectors for delivery of biomolecules, proteins and DNA into various cells. In this study, a new multiwalled carbon nanotube β-cyclodextrin platform (MWCNT-CD) modified with branched polyethylenimine (PEI) and doped with Rhodamine (Rhod), MWCNT-CD-PEI-Rhod, was designed and investigated as drug delivery system. The drug binding abilities of MWCNT-CD-PEI-Rhod towards Cidofovir (Cid) and DNA plasmid encoding enhanced green fluorescence protein (pCMS-EGFP) were investigated by complementary spectroscopic techniques. MWCNT-CD-PEI-Rhod showed no significative cytotoxicity and an excellent ability to entrap and delivery Cid. The present study broadens the spectrum of biological evaluation by investigating platform-treatment induced cellular response such as antiviral activity, transfection properties, cellular uptake, internalization mechanisms and cellular localization. The mechanism of cellular uptake was elucidated monitoring the dependence of intracellular red fluorescence from the assembly concentration, time and presence of specific uptake inhibitors. The biological results indicated that MWCNT-CD-PEI-Rhod loaded with Cid and/or pCMS-EGFP crossed the cell membrane via clathrin-dependent pathway and co-localized in lysosomal compartment. However, no green fluorescent protein expression of pCMS-EGFP was detected, whereas the efficient escape of Cid from lysosome and the release close to nuclear region prompted the antiviral activity.

1. Introduction

Since the discovery of carbon nanotubes (CNTs) in the early 1990s, their development for applications in nanomedicine has emerged as one of the most interesting fields [1–4]. Their ability to cross the cell membranes has boosted their use as nanomaterial for biological applications, including drug carriers [5], gene therapy [6,7], scaffold components in tissue engineering [8,9] vaccine delivery and vaccine adjuvants [10]. CNTs can be described as rolled-up sheets of graphene forming single or multiwalled seamless cylinders (SW- and MWCNTs, respectively); their diameters range from few to hundreds of nanometers, respectively. They generally present a length/diameter ratio higher than 10^6 and have exceptional electrical, thermal, mechanical and optical properties [11].

Despite intensive research on CNTs there is still a wealth of unanswered questions about their interactions with human cells and tissues and more detailed and systematic studies need to be undertaken in this direction [1]. Many experimental evidences have pointed out that CNTs endowed with a proper size and degree of functionalization do not exhibit any evident toxic effect and undergo an effective clearance during *in vivo* experiments [12–14].

CNT chemistry has been thoroughly explored and many synthetic procedures are available for CNT functionalization [15–17]. The initial oxidation step is the most important modification of CNTs for applications in nanomedicine because shortened CNTs are more hydrophilic and their aggregation into bundles is avoided.

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Moreover, the oxidation provides multiple points amenable to chemical modification allowing an extensive and multiple functionalization. The decoration of CNTs with β-cyclodextrins (CDs) has been employed to produce nanohybrids with combined CDs and CNTs properties [18].

β-Cyclodextrins are macrocycles composed of seven glucose units which are toroidal in shape with a hydrophobic inner cavity and a hydrophilic exterior. They can include selectively a large variety of organic molecules into their cavities to form stable host-guest inclusion complexes, showing high molecular selectivity and enantioselectivity [19].

Currently, many methods, including covalent sidewall coupling reactions and non-covalent exohedral interactions, have been developed to produce CDs/CNTs nanohybrids [18,20–22]. The research on CDs-CNTs as sensing materials [21,22] has increased exponentially in the last years while their potential applications in nanomedicine as platforms for drug/gene delivery are scarcely explored. Recently, we have developed an hybrid MWCNT-CD nanoplatform [18], for the entrapment and delivery of guanine based drugs; the dual contribute of CD cavity inclusion and CNT π-stacking interactions resulted in a host-guest strong affinity with values of association equilibrium constants higher than $10^4\ M^{-1}$.

Herein, we report a new MWCNT-CD hybrid platform, modified with branched polyethylenimine (PEI 1.8 kDa) and its evaluation as carrier of antiviral drug and DNA plasmid. These hybrid ternary materials composed of MWCNT, CD and PEI have been not reported till now and their interactions with drugs, nucleic acids and cells could have a great potential in multi-channel therapy as drug and/or gene vector. Furthermore, the MWCNT-CD-PEI platform was conjugated with Rhodamine (Rhod) to track *in vitro* the nanocarrier (Fig. 1) and to elucidate the uptake cellular mechanisms.

For our studies we have selected as model drug the broad-spectrum antiviral agent Cidofovir (Cid), approved for the treatment of cytomegalovirus retinitis in AIDS patients; in addition, several *in vitro* and clinical reports well demonstrated its activity as antineoplastic drug, in particular against virus-associated tumors [23]. Some drawbacks have hindered its spread clinic use, such as the limited cell uptake, due to the presence of negatively charged phosphonate groups and the high systemic toxicity, mainly the nephrotoxicity due to the saturation of the renal transporter hOAT1. Encapsulation into specific micro- or nanocarriers, able to modulate the release of the drug, may represent an effective strategy both to minimize the off-target organ exposure and to increase the concentration of Cid in the site of action [24,25].

The DNA plasmid encoding enhanced green fluorescence protein (pCMS-EGFP) was used as a model system to study the gene delivery ability of MWCNT-CD-PEI platform. The structure, morphology and chemical composition of the platform was investigated by TEM, STEM and TGA analyses. In an attempt to elucidate its structural organization in aqueous solution, ζ-potential and Dynamic Light Scattering measurements at various pHs were carried out. The interactions of Cid with MWCNT-CD-PEI-Rhod platform, with or without co-entrapped pCMS-EGFP, were investigated by complementary spectroscopic techniques.

Cellular responses (cytotoxicity, inhibition of plaque formation, expression of the green fluorescent protein) of MWCNT-CD-PEI-Rhod, MWCNT-CD-PEI-Rhod/Cid and MWCNT-CD-PEI-Rhod/Cid/pCMS-EFGP assemblies were evaluated in Vero and Hep-2 cells. The mechanisms of cellular uptake were elucidated monitoring the dependence of intracellular red fluorescence from the assembly concentration, time, presence of specific inhibitors of caveole- or clathrin-dependent endocytosis (filipin or sucrose). Finally, the localization of MWCNT-CD-PEI-Rhod platform into lysosomal compartment has been determined using Cell Light lysosomes-GFP.

2. Materials and method

2.1. Synthesis of MWCNT-CD (6)

(MWCNT-Ox **1**) were obtained by oxidation of MWCNTs as previously reported [26]. Briefly, 500 mg of MWCNTs were dispersed in 100 mL of sulfuric acid:nitric acid (3:1 v/v, 98% and 69% respectively), sonicated in a water bath (60W, 35 kHz) and stirred at 60 °C for 6 h. De-ionized water (500 mL) was added and the mixture was filtered under vacuum on a 0.1 μm Millipore membrane and carefully washed with deionized water until neutral pH. The residue was dried under vacuum at 50 °C to give 490 mg of MWCNT-Ox **1** with a mean diameter of 8 nm (short diameter 5 nm; long diameter 10 nm) and an average length of 0.2–1.0 μm. A sample of MWCNT-Ox **1** was treated with sodium hydroxide (0.01 N), stirred for 48 h and centrifuged at 10,000 rpm for 15 min. Unreacted NaOH was titrated with 0.01 N hydrochloridric acid to determined total acidity. The amount of acidic surface groups was found to be 1.8 mmol/g. MWCNT-Ox **1** (400 mg) were dispersed in *o*-dichlorobenzene (100 mL) and sonicated for 15 min at room temperature. A solution of *p*-(2-propylnyloxy)-benzamine [18] **2** (2 g) in dry acetonitrile (200 mL) was added and the mixture was sonicated for 30 min at room temperature. Then 3.0 mL of isoamyl nitrite **3** were added and the reaction was stirred at 60 °C for 24 h under argon flow. The cooled mixture was diluted with ethanol and filtered under vacuum on Millipore membrane of 0.1 μm. The residue was washed with water, ethanol, chloroform and each time sonicated for 5 min. Then it was dried at 60 °C to give 390 mg of MWCNT-Alk **4**. 100 mg of **4** were dispersed by sonication in 15 mL of DMF and treated with mono-6-deoxy-6-azido-β-cyclodextrin [18] **5** (125 mg 0.125 mmol), $CuSO_4$ (15 mg, 0.085 mmol) and Na ascorbate (35 mg, 0.17 mmol) under argon flow. The reaction mixture was sonicated for 10 min and heated at 85 °C for 48 h. After, the reaction was cooled to room temperature, diluted with ethylacetate (50 mL) and filtered under vacuum (Millipore membrane of 0.1 μm). The residue was washed with different solvents (water, ethanol, ethyl ether) and each time sonicated for 5 min. The residue was dried at 60 °C to give 80 mg of MWCNT-CD **6.** From TGA analyses the degree of functionalization was estimate to be ≈19.4% wt corresponding to 0.148 mmol/g of grafted CD.

2.2. Synthesis of MWCNT-CD-PEI-Rhod (8)

The MWCNT-CD-PEI platform **7** was synthesized from MWCNT-CD **6** and polyethyleneimine (PEI, Mn ≈ 1.8 KDa by GPC) using a weight ratio of 1:5. First, 745 mg of PEI were solubilised in 5 mL of deionised water by stirring the mixture at room temperature for 1 h. MWCNT-CD **6** (150 mg) in 20 mL of deionized water was sonicated for 30 min to obtain a homogeneous suspension, then EDC (124 mg, 0.64 mmol) and NHS (93 mg, 0.80 mmol), were added to black suspension and the reaction mixture was stirred at room temperature. After one hour, PEI solution was added and the reaction was stirred overnight. The mixture was washed 5 times with deionised water by centrifugation at 5000 rpm for 30 min. The precipitate was dried at 60 °C to give 617 mg of MWCNT-CD-PEI **7**. The NH_2 loading on MWCNT-CD-PEI was found to be 0.25 mmol/g by Kaiser test (see SM).

MWCNT-CD-PEI **7** (83 mg, 0.0208 mmol of free NH_2) was sonicated in 10 mL of dry DMF for 30 min and triethylamine (0.6 mL, 0.0043 mmol) and Rhodamine B isothiocyanate (Rhod) (10 mg, 0.018 mmol) were added. The temperature was increased to 50° C and the reaction was stirred overnight in the dark. The mixture was washed for several times with a solution of water/ethanol (1:1) by centrifugation at 5000 rpm for 20 min to ensure complete removal of non-covalent bonded Rhod. The residue was dried at 60 °C to give 84 mg of MWCNT-CD-PEI-Rhod **8**. The NH_2 loading