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Characterization of ester- or thioamide-functionalized single-walled carbon nanotube-azithromycin conjugates

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ABSTRACT

Functionalization of single-walled carbon nanotubes (SWCNTs) with nitrile groups, followed by further reactions allowed direct attachment of azithromycin and its N-demethyl derivative to the side-walls of SWCNTs for the first time. With these approaches, the cleavable ester or thioamide bonds are formed to connect azithromycin to SWCNTs resulting in azithromycin-SWCNT conjugates. These cleavable bonds are able to control molecular release from nanotube surfaces which are generally applicable to a variety of hybrid materials based on SWCNTs. A non-covalent azithromycin-SWCNT has also been compared with azithromycin-SWCNTs conjugates. Thermogravimetric analysis (TGA), Fourier-transformed infrared (FT-IR), UV–vis, and Raman spectroscopies give hints on the characterization of azithromycin-SWCNT. Both drug release and antimicrobial activity of azithromycin-SWCNT conjugates were also tested.

1. Introduction

Rapid development in the field of nanomedicine is bringing novel opportunities for improved disease diagnosis and drug delivery. Nanomaterials exhibit interesting physical properties, presenting new opportunities for research and applications in various areas including biology and medicine. Among nanomaterials, carbon nanotubes (CNTs) have been extensively explored for a wide range of applications in biology and medicine [1–5]. CNTs have shown promise in the intracellular delivery of small drug molecules, DNA plasmids, short-interfering RNA (siRNA), and proteins, for applications in disease treatment such as (*in vitro* and *in vivo*) cancer therapies [6–15]. Due to their unique one-dimensional structure, CNTs cross cell membranes easily to deliver peptides, proteins, and nucleic acids into cells [16–21]. On the other hand, the unique optical and electrical properties of CNTs make them attractive platforms to detect various biological molecules [22,23]. Moreover, CNTs with the excellent mechanical properties have also found applications as potential tissue engineering scaffold materials [24,25]. Single-walled carbon nanotubes (SWCNTs) exhibit many intrinsic optical properties which can be used in different biological imaging modalities [26–29].

The chemistry of SWCNT offers the possibility of introducing more than one function on the same tube, so that targeting molecules, contrast agents, drugs, or reporter molecules can be used at the same time. The ability of functionalized SWCNT to penetrate into the cells offers their potential as vehicles for the delivery of small drug molecules [30–33]. These innovative carriers present a low toxicity, a fact that boosts their potential for biomedical applications [34–37].

In this regard, our research group strives to rationally design nano-hybrid structures in order to benefit from the combination of specific properties of organic and inorganic components in one structured material. Accordingly, we have recently reported new functionalized SWCNTs [38,39].

The aim of the current study is the attachment of azithromycin to the side-walls of SWCNTs by cleavable ester or thioamide bonds. The incorporation of cleavable bonds into the functionalization enables controlled molecular release from nanotube surfaces, thus creating 'smart' nanomaterials with useful functionality in chemical and biological settings. Antimicrobial activity and drug release of SWCNT-azithromycin conjugation are also tested.

2. Experimental

2.1. Materials

The pristine single-walled carbon nanotubes (p-SWCNT) used in the current investigation (Nanocyl-1100 series) are commercially available (Nanocyl Inc., Sambreville, Belgium).

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3′-N-desmethyl azithromycin (AZ-2) was prepared by analogous procedure used for 3′-N-desmethyl erythromycin [40]. SWCNT-1 [38] was synthesized based on our previous report.

2.2. Purification of pristine single-walled carbon nanotubes (p-SWCNT)

Purification begins with a 45 h reflux in concentrated hydrochloric acid to remove metal catalyst particles (typically 1 L of acid per 10 g of raw material). Weight loss is 20% after 24 h with little further weight loss after this time. Following the reflux the black solution is centrifuged (20,000 × *g*/20 min) leaving a black sediment at the bottom of the centrifuge bottle and a clear, brownish yellow supernatant acid, which is decanted off. The sediment still contains substantial trapped acid which is removed by repeatedly re-suspending the sediment in deionized water (by shaking vigorously), centrifuging, and decanting the supernatant liquid (for 10 g of starting material 3–4 such washings usually suffice). In the following, unless otherwise specified, a starting batch of 10 g may be assumed. With each such washing and centrifugation cycle, as the solution becomes less acidic, it is observed that the supernatant solution (following centrifugation) which was clear on the first cycle, becomes darker. The nearly neutral solution is completely black, remaining black even if longer centrifugation times are used.

2.3. Procedure for the synthesis of SWCNT-2

10 mg of SWCNT-1 was reacted overnight at 80 °C with aq. KOH (10 mL, 40%, w/w) and abs. EtOH (10 mL). After cooling to 25 °C, the mixture was acidified to pH 3 (HCl sol.) and diluted with EtOAc. The obtained paste was filtered through a PTFE membrane (0.2 μm pore size). The collected solid was first washed with copious amount of water and then further washed using sequential cycles of sonication in acetone (30 min each), followed by filtration until the filtrate became colorless. The resulting purified SWCNT-2 was dried in a vacuum oven overnight.

2.4. Synthesis of SWCNT-4

10 mg of SWCNT-2 were stirred in 20 mL of $SOCl_2$ [containing 1 mL of dimethylformamide (DMF)] at 70 °C for 24 h. After centrifugation, the brown-colored supernatant was decanted and the remaining solid was washed with anhydrous THF. After centrifugation, the pale yellow-colored supernatant was decanted.

The remaining solid (SWCNT-3) was dried at room temperature under vacuum. A mixture of the resulting SWCNTs and 2 g of azithromycin (AZ-1) was heated at 90–100 °C for 96 h. After cooling to room temperature, the AZ-1 excess was removed by washing four times with dichloromethane. The remaining solid was dissolved in dichloromethane, and after filtration, the black-colored filtrate was taken to dryness on a rotary evaporator. The resulting black solid was dried at room temperature under vacuum. The yield of SWCNT-4 is about 60%.

2.5. Synthesis of SWCNT-5

Caution: Experiments should be carried out in an efficient hood to avoid exposure to noxious vapors of hydrogen sulfide.

In a typical experiments, a mixture of N-desmethyl-azithromycin (AZ-2) (8 g, 12 mmol), and sulfur (768 mg, 12 mmol) in 40 mL of DMF in an open pyrex glass flask was refluxed for 15 min. Then, sodium sulfide (360 mg, 1.5 mmol) was added [41] and the reaction mixture was refluxed for 1 h. SWCNT-1 (20 mg, 10 mmol) was added to the cooled reaction mixture and then refluxed overnight.

After cooling to 45 °C, the reaction mixture was filtered through a PTFE membrane (0.2 μm pore size). The collected solid was washed with DMF until the filtrate became colorless. Dispersing the solid in DMF by sonication followed by filtration afforded purified SWCNT-5. DMF was removed by washing with acetone, water and finally diethyl ether. SWCNT-5 was dried in a vacuum oven overnight.

2.6. Drug release method

SWCNT-4 or SWCNT-5 was put in a 100 mL beaker containing 100 mL of buffer solution of pH 6.8 and mechanically stirred with a magnetic stirrer. Samples were periodically withdrawn from the beaker using a 1 mL pipette and the volume of the withdrawn samples was replaced with distilled water. Buffer solution of pH 6.8 was prepared by taking 50 mL of 0.2 M KH_2PO_4 and 39.1 mL of 0.2 N NaOH in volumetric flask to make a total volume of 200 mL with distilled water [42].

Then samples were analyzed spectrophotometrically at 215 nm wavelength on a UV–vis spectrophotometer.

3. Results and discussion

3.1. Azithromycin-SWCNT conjugation

Azithromycin 1 (AZ-1) is an antibiotic derived from erythromycin A with improved biological and pharmacodynamic properties over the parent compound. In this contribution, we considered azithromycin (AZ-1) and 3′-N-desmethyl azithromycin (AZ-2) which the latter, unlike the former, lack antibacterial activity. As shown in Fig. 1, AZ-2 is prepared by simple demethylation of AZ-1 [40].

The preparation of azithromycin-SWCNT conjugates (SWCNT-4 and SWCNT-5) was achieved by two different synthetic approaches, *i.e.* the acylation and the thioamidation process on the nitrile groups. The procedure for the preparation of SWCNT-4 included three steps; first SWCNT-1 are converted to the corresponding carboxylic acid (SWCNT-2) [38], then the formation of acyl chloride intermediate (SWCNT-3), followed by the esterification of the acyl chloride with the hydroxyl group of AZ-1.

Based on our long experience on the Willgerodt–Kindler reaction [43–50], SWCNT-5 was also prepared from the reaction of benzonitrile moiety on SWCNT-1 with sulfur and secondary amine of AZ-2 in DMF under reflux conditions (Fig. 2).

The workup of the reaction mixtures involved a simple filtration with the 0.2 μ Teflon membrane filter, which was thoroughly washed with acetone, water, followed by diethyl ether and vacuum dried overnight.

By employing a combination of spectroscopic and microscopic techniques like Raman, Fourier-transformed infrared (FT-IR), thermogravimetric analysis (TGA), scanning electron microscopy (SEM) and UV–vis spectrometry, the structures of SWCNT-4 and SWCNT-5 were elucidated.

As shown in Fig. 3, Raman spectroscopy has provided essential and quick information for evaluation of the covalent sidewall modification of the SWCNT-1 [38]. It can be clearly seen that relative intensities of D-band (1340 cm^{-1}) increased after nitrile group functionalization. The increase in the D band is an indication of covalent side-wall functionalization, reflecting the conversion of the hybridization of some carbon atoms on the nanotube wall from sp^2 to sp^3. This indicates that the diazotization coupling was carried out successfully on SWCNTs' side walls. Calculations reveal that there is a remarkable increase in D:G ratio on functionalization (p-SWCNT = 0.26 and SWCNT-1 = 0.67). Raman spectroscopy of a physical mixture of SWCNT and benzonitrile was also measured.