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Bottom-up synthesis of carbon nanoparticles with higher doxorubicin efficacy

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ABSTRACT

Nanomedicine requires intelligent and non-toxic nanomaterials for real clinical applications. Carbon materials possess interesting properties but with some limitations due to toxic effects. Interest in carbon nanoparticles (CNPs) is increasing because they are considered green materials with tunable optical properties, overcoming the problem of toxicity associated with quantum dots or nanocrystals, and can be utilized as smart drug delivery systems. Using black tea as a raw material, we synthesized CNPs with a narrow size distribution, tunable optical properties covering visible to deep red absorption, non-toxicity and easy synthesis for large-scale production. We utilized these CNPs to label subcellular structures such as exosomes. More importantly, these new CNPs can escape lysosomal sequestration and rapidly distribute themselves in the cytoplasm to release doxorubicin (doxo) with better efficacy than the free drug. The release of doxo from CNPs was optimal at low pH, similar to the tumour microenvironment. These CNPs were non-toxic in mice and reduced the tumour burden when loaded with doxo due to an improved pharmacokinetics profile. In summary, we created a new delivery system that is potentially useful for improving cancer treatments and opening a new window for tagging microvesicles utilized in liquid biopsies.

1. Introduction

Nanoparticle technology is an attractive field at the forefront of research and plays important roles in medicine, agriculture and electronics. Nanoparticles have wide applications in medicinal fields as nanocarriers for drug delivery and agents for multifunctional diagnosis, for example [1,2]. Recently, a new class of carbon nanomaterials, including nanodiamonds [3] and fluorescent carbon nanoparticles (CNPs) [4], have been widely investigated due to their high hydrophilicity, excellent biocompatibility, good cell permeability, high photostability and flexibility in surface modification as a result of the presence of different functional groups (carboxyl, hydroxyl and amino groups), allowing the covalent conjugation of chemotherapeutic and targeting agents [5]. Particularly, fluorescent CNPs have wide applications in areas such as bioimaging, drug delivery [6–10], sensors [11–14], optoelectronics [15] and photocatalysis [16]. CNPs are comparable to quantum dots (QDs) and organic dyes [17]. QDs are semiconductor nanostructures with unique optical and electrical properties and great flexibility in their bright and tunable photoluminescence. The blinking effect is a problem with QDs that can be overcome by surface passivation or core-shell formation [18]. QDs are composed of heavy metal precursors such as selenium (Se) and cadmium (Cd), which are toxic at low concentrations in the human body and environment [17,19]. The use of CNPs in place of QDs might overcome the above mentioned problems. Notably, CNPs have attracted considerable interest, as they offer

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potential advantages over the other carbon nanomaterials such as carbon nanotubes [20–22] and Halloysite nanotubes [23,24] including their small size, simple and inexpensive synthetic routes, high aqueous solubility, their fluorescence property which make them useful for cell imaging and their high cargo loading.

In recent years, much progress has been made in terms of the synthesis, properties and applications of CNPs [17,25]. The synthesis of CNPs can be classified in two groups: chemical and physical methods. Chemical methods include electrochemical synthesis [26], acidic oxidation [4,6,27], thermal/hydrothermal synthesis [28–31] and microwave/ultrasonic synthesis [12,17,28,32]. Physical methods include arc discharge [33], laser ablation [34] and plasma treatment [35]. Chemical oxidation was commonly used to prepare fluorescent CNPs, which almost always originate from carbon-based nanomaterials. This method is easier, avoids multi-step synthesis and introduces carboxyl and hydroxyl groups on the CNP surface, making the particles negatively charged and hydrophilic. As a result, a variety of fluorescent CNPs have been prepared using food waste [36], carbon nanotubes [37], candle soot [4], carbohydrates (sucrose, glucose) [30,38], active carbon [32], orange juice, polyphenol [39,40] and honey [41]. Although numerous synthetic approaches have been developed, those that are eco-friendly and inexpensive are in demand. Furthermore, large-scale synthesis and size-controlled CNPs remain unmet technological needs.

In the field of drug delivery, carbon nanomaterials have gained considerable attention as nano-carriers due to their high surface area, enhanced cellular uptake and easy conjugation with therapeutics [42–45]. CNPs are spherical and composed of an sp^2 carbon core, which can be conjugated with chemotherapeutic drugs and biomolecules through covalent or noncovalent interactions (π–π stacking or electrostatic interactions) and used for *in vitro* and *in vivo* drug delivery applications [43,46]. However, most of the published papers to date on this topic have focused on the optical properties and *in vitro* biocompatibility of CNPs [47–50], and few have studied CNPs as delivery agents in depth [9,51,52]. Therefore, clinical application remains a challenge.

In this report, we present a green source, "black tea", as a suitable precursor for the synthesis of CNPs by nitric acid (HNO_3) oxidation. This synthesis is simple and economical because of the selection of an inexpensive carbon source. These CNPs are non-toxic; easily synthetized in large-scale production with tunable optical properties up to red spectra, which can be utilized for multiplexing applications; and can efficiently deliver doxorubicin (doxo). The biodistribution, pharmacokinetics (PK) profiles and kinetics of release suggest that CNPs-doxorubicin (Cdoxo) is an optimal drug delivery vector for cancer therapy.

2. Experimental section

2.1. Materials and instrumentation

2.1.1. Reagents

Commercially available Brooke Bond Taaza tea was utilized. HNO_3 (70%) and sodium hydroxide (NaOH) were purchased from Sigma Aldrich (St. Louis, Missouri, US), doxo was obtained from Accord Healthcare Ltd. (Durham, NC, US) and daunorubicin was purchased from Teva Pharmaceutical Industries Ltd. (Petah Tikva, Israel). All reagents were used as received without further purification. Minisart® syringe filters with a pore size of 0.2 μm were from Sartorius Stedim Biotech (Concord, CA, US), and a dialysis membrane (MWCO 0.5–1 kDa) was purchased from Spectrum Laboratories (Rancho Dominguez, CA, US) for CNP purification. LysoTracker® deep red probe was purchased from Life Technologies (Carlsbad, CA, US). Exosomes were prepared from exosome-depleted medium conditioned for 48 h and purified with an AB cell culture-nanovesicle solution according to the instructions (AB ANALITICA, Padova, Italy) [53].

DLD-1 and LoVo (colon) and MDA-MB-231 (breast) and HeLa (cervical) cancer cells were grown as indicated by the supplier (ATCC, Manassas, VA, US). Nude and FVB mice were purchased from Harlan Laboratories (Udine, Italy); the procedures were approved by the Italian Ministry of Health n°788/2015-PR and performed in accordance with the institutional guidelines. Data are reported as the mean and standard error.

2.1.2. Equipment

Water was obtained from a Milli-Q water purification system (18.2 Ω; EMD Millipore, Billerica, MA, US). UV–Vis absorption spectra were collected using a NanoDrop 2000c (Thermo Fischer Scientific, Waltham, MA, US). Fluorescence spectra were collected on an Infinite M1000 PRO and cell viability analyzed using an Infinite 200 PRO (Tecan, Männedorf, Switzerland). X-ray diffraction (XRD) data were collected on a Philips X'Pert vertical goniometer with Bragg-Brentano geometry. Transmission electron microscopy (TEM) was carried out using a Philips EM 208 microscope (Philips, Amsterdam, Netherlands). Fourier transform infrared (FT-IR) spectra were obtained on a NEXUS FT-IR spectrometer implementing a Nicolet Avatar diffuse reflectance accessory. X-ray photoelectron spectroscopy (XPS) was performed on a PHI Quantera SXM spectrometer using monochromatic Al-Kα X-ray sources at 1486.6 eV and 24.8 W with a beam diameter of 100.0 μm, a 1.2 V and 20.0 μA neutralizer, and FAT analyzer mode. Zeta potential (ζ) measurements were collected on a Zetasizer ZS90 (Malvern Instruments, Malvern, UK) using a 632 nm He-Ne laser as the light source. Fluorescence microscopy was carried out using a Nikon microscope at 20× and 40× magnification (Nikon, Chiyoda, Tokyo, Japan). The PK and biodistribution were evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a 4000 QTRAP MS/MS system equipped with a Turbo ESI source (AB Sciex, MA, USA). The exosome particle size was determined with an L10 NanoSight instrument (Malvern Instruments Ltd., UK).

2.1.3. Preparation of CNPs

CNPs were synthesized from tea in the following steps: (1) carbonization of commercial tea followed by (2) oxidation with HNO_3. The carbonized carbon was prepared by heating the commercial black tea at 200 °C for approximately 3 h, followed by evaporation of water and heating again at 200 °C for approximately 5 h. The so-formed carbonized tea powder was cooled to room temperature, dried on rotary evaporator and stored in a glass bottle. Then, 500 mg of the carbonized carbon was dispersed in HNO_3 (0.065 mol, 5 M, 13 ml) and refluxed at 80 °C for 20 h under vigorous stirring. Then, the orange solution was cooled to room temperature and centrifuged (4300*g*, 25 min, room temperature) to separate out any unreacted carbon. The orange supernatant was collected, neutralized by 5 M NaOH and filtered through a 0.2 μm Minisart® syringe. To remove salts and impurities, the raw solution was dialyzed against Milli-Q water using a dialysis membrane (MWCO 0.5–1 kDa) for at least 2 days. Finally, the obtained golden-yellow solution was dried on a rotary evaporator and used for further characterization (yield: 26%).

2.1.4. Fluorescence imaging

A droplet of an aqueous CNP dispersion (25 mg/ml) was imaged on a Nikon fluorescence microscope under different filter sets (nm), Ex 350/Em 460 (blue), Ex 490/Em 520 (green), Ex 550/Em 570 (red) and Ex 630/Em 670 (violet), at 20× magnification.

2.1.5. CNP cellular localization

The CNP cellular internalization was evaluated by plating HeLa cells at a density of 7.5×10^4 cells/slide. The next day, the cells were marked with 50 nM LysoTracker® deep red probe (Thermo Fisher, MA, US) for 2 h at 37 °C. After incubation, the cells were washed twice with 1× PBS and incubated for 24 h with 2 mg/ml CNPs. After incubation, the cells were washed twice with 1× PBS and fixed with 4% PFA for 10 min, and the slides were mounted with Alexa FluorSave solution (Thermo Fisher Scientific, Waltham MA, US). The images were obtained