International Journal of Pharmaceutics 481 (2015) 56–63

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Folate-grafted boron nitride nanotubes: Possible exploitation in cancer therapy

Tiago H. Ferreira [a], Attilio Marino [b,c], Antonella Rocca [b,c], Ioannis Liakos [d], Simone Nitti [e], Athanassia Athanassiou [d], Virgilio Mattoli [b], Barbara Mazzolai [b], Edesia M.B. de Sousa [a,**], Gianni Ciofani [b,*]

[a] SENAN – Centro de Desenvolvimento da Tecnologia Nuclear – CDTN/CNEN, Av. Presidente Antônio Carlos 6627 – Campus da UFMG, Belo Horizonte, MG CEP 30270-901, Brazil
[b] Center for Micro-BioRobotics @SSSA, Istituto Italiano di Tecnologia, Viale Rinaldo Piaggio 34, Pontedera, Pisa 56025, Italy
[c] The BioRobotics Institute, Scuola Superiore Sant'Anna, Viale Rinaldo Piaggio 34, Pontedera, Pisa 56025, Italy
[d] Nanophysics Department, Istituto Italiano di Tecnologia, Via Morego 30, Genova 16163, Italy
[e] Nanochemistry Department, Istituto Italiano di Tecnologia, Via Morego 30, Genova 16163, Italy

ARTICLE INFO

Article history:
Received 1 December 2014
Received in revised form 22 January 2015
Accepted 27 January 2015
Available online 28 January 2015

Keywords:
Boron nitride nanotubes
Folic acid
HeLa cells
Nanovectors
Cancer therapy

ABSTRACT

Boron nitride nanotubes (BNNTs) have generated considerable interest among the scientific community because of their unique physical and chemical properties. They present good chemical inertness, high thermal stability, and optimal resistance to oxidation, that make them ideal candidates for biomedical applications, in particular as nanovectors for drug, gene and protein delivery into the cells. In this study, BNNTs were prepared through a synthesis based on a chemical vapor deposition (CVD) method, and thereafter chemically functionalized with folic acid. The obtained nanostructures have been characterized by Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), thermogravimetric analysis (TGA), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The characterization showed efficiently functionalized BNNTs of length of about 1 μm. Furthermore, confocal laser microscopy demonstrated that our nanotubes can be fluorescently-traced under appropriate excitation. Thanks to this property, it has been possible to investigate their internalization by HeLa cells through confocal microscopy, demonstrating that the BNNT up-take clearly increases after the functionalization with folate, a result confirmed by inductively coupled plasma (ICP) assessment of boron content inside the treated cell cultures.

1. Introduction

Boron nitride nanotubes (BNNTs) have been widely studied in the recent years, and many favorable physico-chemical features previously theoretically described have been step by step confirmed (Chang et al., 2008; Chiu et al., 2012; Lee et al., 2013; Tang et al., 2007; Yum and Yu, 2006): distinct mechanical, optical, and structural properties, jointly to a good chemical inertness and a high thermal stability, make BNNTs an extremely versatile material suitable for a wide range of applications in the nano-domain (Terrones et al., 2007; Zhi et al., 2010). Aiming at biomedical exploitation, these nanoparticles can provide significant advances in the area of medical imaging, molecular biology, and biomedical technology, contributing, theoretically, to the diagnosis and treatment of several diseases, including cancer (Ciofani et al., 2013, 2009).

When it comes to inorganic materials for bioapplications, a recurring concern is related to the biological response of these systems. Over the latest few years, several studies have been carried out to evaluate the effects of BNNTs *in vitro* (Ciofani et al., 2010, 2008c; Ferreira et al., 2013; Horváth et al., 2011; Lahiri et al., 2010) and *in vivo* (Soares et al., 2011). Despite of some discrepancies in obtained results because of different experimental approaches due to the intrinsic complexity of the nanomaterials, most of results indicated very good response of cells and organisms toward BNNTs. This is an outstanding starting point, as lack of

* Corresponding author at: Italian Institute of Technology, Center for Micro-BioRobotics @SSSA, Viale Rinaldo Piaggio 34, 56025 Pontedera (Pisa), Italy. Tel.: +39050883019; fax: +39050883497.
** Corresponding author at: Laboratório de Materiais Nanoestruturados para Bioaplicações, Centro de Desenvolvimento da Tecnologia Nuclear, Av. Pres. Antonio Carlos, 6627 Campus da UFMG, Pampulha, 31270-901 Belo Horizonte, MG, Brazil. Tel.: +553130693223; fax: +553130693164.
E-mail addresses: sousaem@cdtn.br (E.M.B. de Sousa), gianni.ciofani@iit.it (G. Ciofani).

http://dx.doi.org/10.1016/j.ijpharm.2015.01.048
0378-5173/

adverse side effects on healthy cells is absolutely mandatory, even envisaging anti-cancer applications. Once functionalized, BNNTs may further improve their interaction with the biological environment (Chen et al., 2009; Ciofani et al., 2012; Nakamura et al., 2010; Yang et al., 2011), and, moreover, can be theoretically targeted to specific tissues and organs. Thus, BNNTs can be exploited as effective nanocarriers, providing safe and efficient targeted release of biomolecules into desired cells and tissues (Lacerda et al., 2008, 2007; Liu et al., 2011).

Folic acid receptors have been demonstrated to be over-expressed on the surfaces of several kind of human tumors, including ovarian, brain, endometrial, kidney, and breast cancer cells (Parker et al., 2005; Zwicke et al., 2012). Having folic acid (FA) a high binding affinity to the FA receptors on the cell surface of FA-positive tumors (Ciofani et al., 2008a; Xia and Low, 2010), its covalent conjugation to drug carriers could result in a selective targeting to cancer cells (Atluri et al., 2013).

Boron nitride nanotubes can be internalized by the cells through an energy-dependent process (Ciofani et al., 2008b), and they were also proven to efficiently deliver DNA inside the cells with no apparent toxicity (Chen et al., 2009). Therefore, it could be expected that BNNTs functionalized with folic acid can be easily internalized by tumor cells though an endocytosis process mediated by folate receptors. As a consequence, this system could become an important approach for the delivery of proteins, drugs, or genes in cancer treatment. To date, to assess whether cells can bind and internalize folate-conjugated nanocarriers, tracking of folate-linked imaging agents (like luminescent nanoprobes or radiotracers) has been efficiently exploited (Lu and Low, 2012; Song et al., 2009). In our case, taking advantage of a significant signal from BNNTs when excited at appropriate wavelength, it has been possible to follow this process through confocal microscopy.

Summarizing, in the present work, BNNTs synthesized as described in previous works (Ferreira et al., 2011) were covalently functionalized with folic acid (FA), fully characterized, and *in vitro* tested in order to validate their potential as theranostic nanotools in cancer treatment.

2. Methods

2.1. BNNT chemical modification and characterization

Boron nitride nanotubes were prepared through a chemical synthesis based on a chemical vapor deposition (CVD) method. This process was developed and optimized at the Centro de Desenvolvimento da Tecnologia Nuclear, Brazil, and previously published (Ferreira et al., 2011). Briefly, powders of ammonium nitrate NH_4NO_3 (95% w/w), amorphous boron (97% w/w), and hematite (95% w/w and particle size less than 50 nm) were mixed at a molar ratio of 15:15:1, respectively, placed in tubular furnace and subjected to a heat treatment. The obtained material was purified with hydrochloric acid solution (3 M) at 90 °C for 10 min, and then the sample was collected by filtration and dried at 40 °C. Purity of samples was confirmed with energy dispersive X-ray spectroscopy (EDX, Bruker) performed on a scanning electron microscope (SEM Helios NanoLab 600i, FEI), that provided a composition characterized by N (45.6 ± 6.4%), B (37.4 ± 6.2%), C (9.8 ± 2.0%), O (6.5 ± 1.3%), and traces of Na, Cl and Ca (collectively about 0.8 ± 0.1%).

The nanotubes were functionalized with folic acid through a covalent approach. The first step consisted in the introduction of hydroxyl groups on the surface of BNNTs through an oxidation process. In a typical preparation, 30 mg of BNNTs were dispersed in 30 mL of HNO_3 (65% w/w) with an ultrasound treatment for 1 h and, thereafter, subjected to stirring overnight at 70 °C. After this process, the material (OH-BNNTs) was purified and dried. In the second step, 30 mg of folic acid was dispersed in 60 mL of *N*,*N*-dimethylacetamide (99.8%), and then mixed with 30 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and 20 mg of 4-(dimethylamino) pyridine (DMAP) under stirring for 20 min. The dried OH-BNNTs were then added to this solution and stirred overnight. In this way, the carboxyl groups of folic acid readily reacted with the hydroxyl groups on the nanotube surface produced through oxidation, thus covalently grafting folate molecules on the BNNT walls.

The functionalization product (FA-BNNTs) was washed five times with deionized water by centrifugation (10,000 rpm for 10 min), and finally freeze-dried in order to obtain a dry powder.

The schematic diagram presenting the steps of the functionalization process is shown in Fig. 1. The acid treatment promotes the oxidation of BNNT walls and the formation of B—OH groups (Fig. 1A). Thanks to EDC and DMAP, the carboxyl groups of folic acid readily react with the hydroxyl groups on the nanotube surface, grafting FA on the BNNT walls (Fig. 1B).

2.2. Physicochemical characterization of functionalized BNNTs

The modification of BNNTs was characterized by Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS). The FTIR analysis was carried out with a Thermo Nicolet 6700 spectrophotometer, ranging from 4000 to 600 cm^{-1} and with a resolution of 4 cm^{-1}. The XPS measurements were taken using a Specs Lab2 electron spectrometer equipped with a monochromatic X-ray source set at 1253 eV and with a Phoibos analyzer Has 3500 (Emispherical Energy Analyzer). The applied voltage of the Mg Kα X-ray source was 7.5 kV and the applied current 9.5 mA. The pressure in the analysis chamber was approximately 2×10^{-9} mbar. Small area lens mode was used for both wide and narrow scans. For the wide scan, the energy pass was 90 eV, the energy step was 0.5 eV, and the scan number was 1. For the narrow high-resolution scan, the energy pass was 30 eV, the energy step was 0.2 eV, and the scan number was 10. The spectra were then analyzed using CasaXPS software.

The morphological features of functionalized BNNTs were investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM observation was performed on a NanoLab 600i FEI microscope with an acceleration voltage of 10 kV. The sample was prepared by

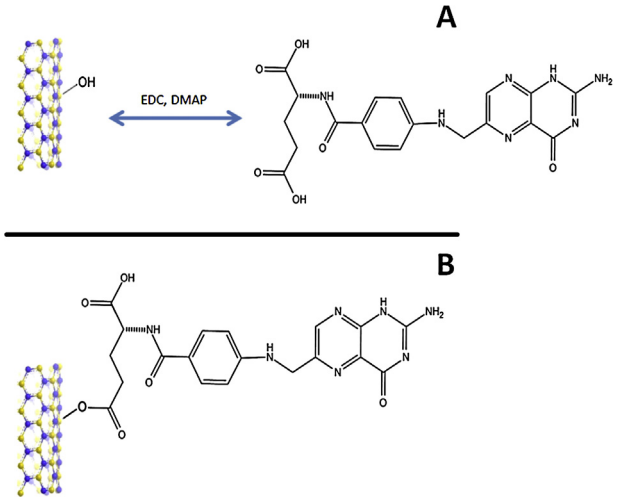

Fig. 1. Schematic representation of the chemical reactions involved in the functionalization of BNNTs with folic acid.