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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

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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).

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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

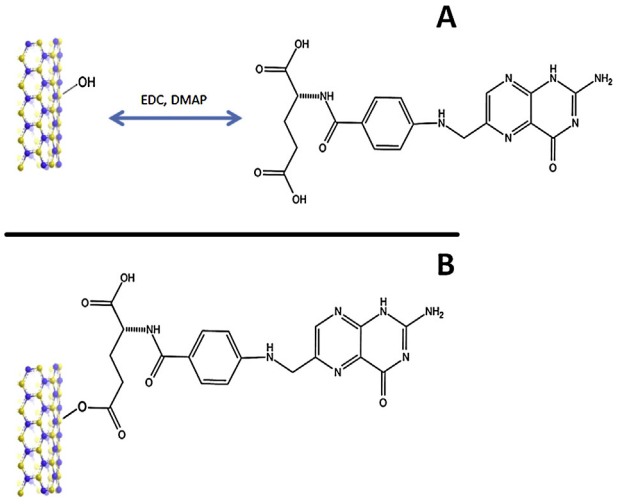

Fig. 1. Schematic representation of the chemical reactions involved in the functionalization of BNNTs with folic acid.

placing a drop of diluted nanoparticle solution on a silicon wafer substrate and allowing the substrate to dry before observation. The TEM procedure was performed on a Tecnai G2-20-FEI 2006 microscope, with an acceleration voltage of 200 kV.

The percentage of folate grafted on the BNNT surface was evaluated through thermogravimetric analysis (TGA). TGA measurements were taken with a Shimadzu DTG-60/60H at a temperature ranging from 25 °C to 600 °C. Approximately 4.0 mg of sample (both BNNTs and FA-BNNTs) were analyzed with a heating rate of 5 °C/min, under a nitrogen atmosphere flow of 20 mL/min and in open alumina cell.

2.3. Biological testing

2.3.1. Cell culture

In vitro assays were performed on HeLa cell line (ATCC CCL-2), derived from a human cervical adenocarcinoma. HeLa cells were plated on T-75 flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Medium was changed every two days and HeLa cells were splitted 1:4 every 4 days using 0.05% trypsin with 0.02% EDTA.

Viability tests on BNNTs and FA-BNNTs were performed by using WST-1 (2-(4-iodophenyl)-3-(4-nitophenyl)-5-(2,4-disulfophenyl)-

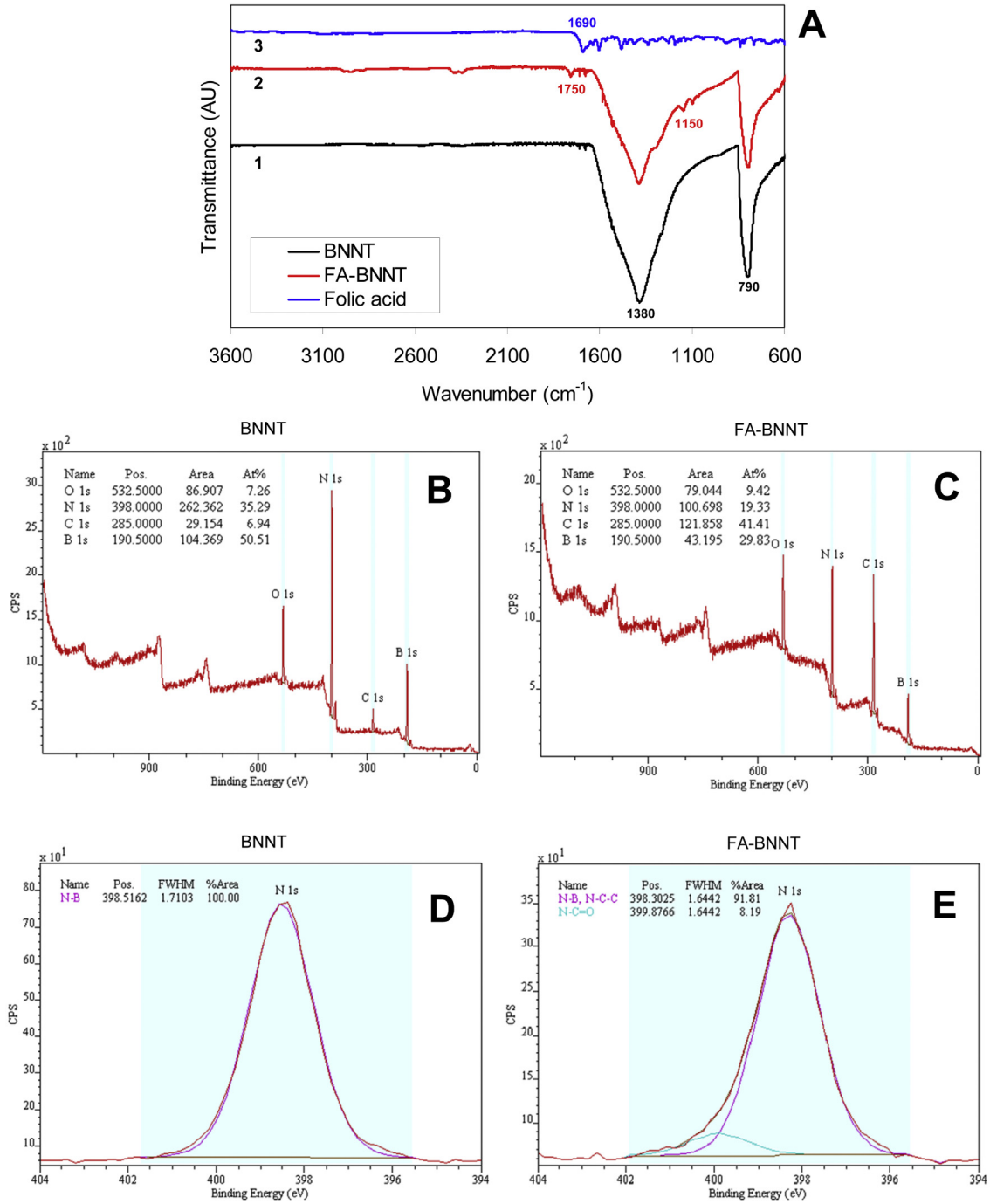

Fig. 2. (A) Infrared spectra of BNNT (1), FA-BNNT (2), and folic acid (3) samples; wide XPS spectra of pristine (B) and folate functionalized (C) BNNTs; narrow-scan high-resolution XPS spectra of N1s for BNNT (D) and FA-BNNTs (E).

2H-tetrazoilium monosodium salt, provided in a pre-mix electro-coupling solution from BioVision) metabolic assay. Cells were seeded at a density of 5000/cm^2 in 96-well plate wells (n = 6). After adhesion (about 12 h since seeding) cultures were treated with 0, 5, 10, 20, and 50 μg/mL of nanotubes for 24 and 72 h. At each time-point, cultures were treated with 100 μL of culture medium supplemented with 10 μL of the pre-mix solution for further 2 h. Absorbance at 450 nm was read by using a microplate reader (Victor3, PerkinElmer).

2.3.2. Cellular up-take investigation

For BNNT internalization experiments, HeLa cells were seeded on glass coverslips (5000 cells/cm^2) and incubated for 24 h. The cells were then treated for 6 h with BNNTs or with FA-BNNTs dispersed in culture medium corresponding to a final concentration of 20 μg/mL. Actin and nucleus staining was performed with FITC-conjugated phalloidin (Sigma) and DAPI (Millipore), respectively, according to standard procedures. Briefly, after the treatment with BNNTs or FA-BNNTs, cells were rinsed with PBS,

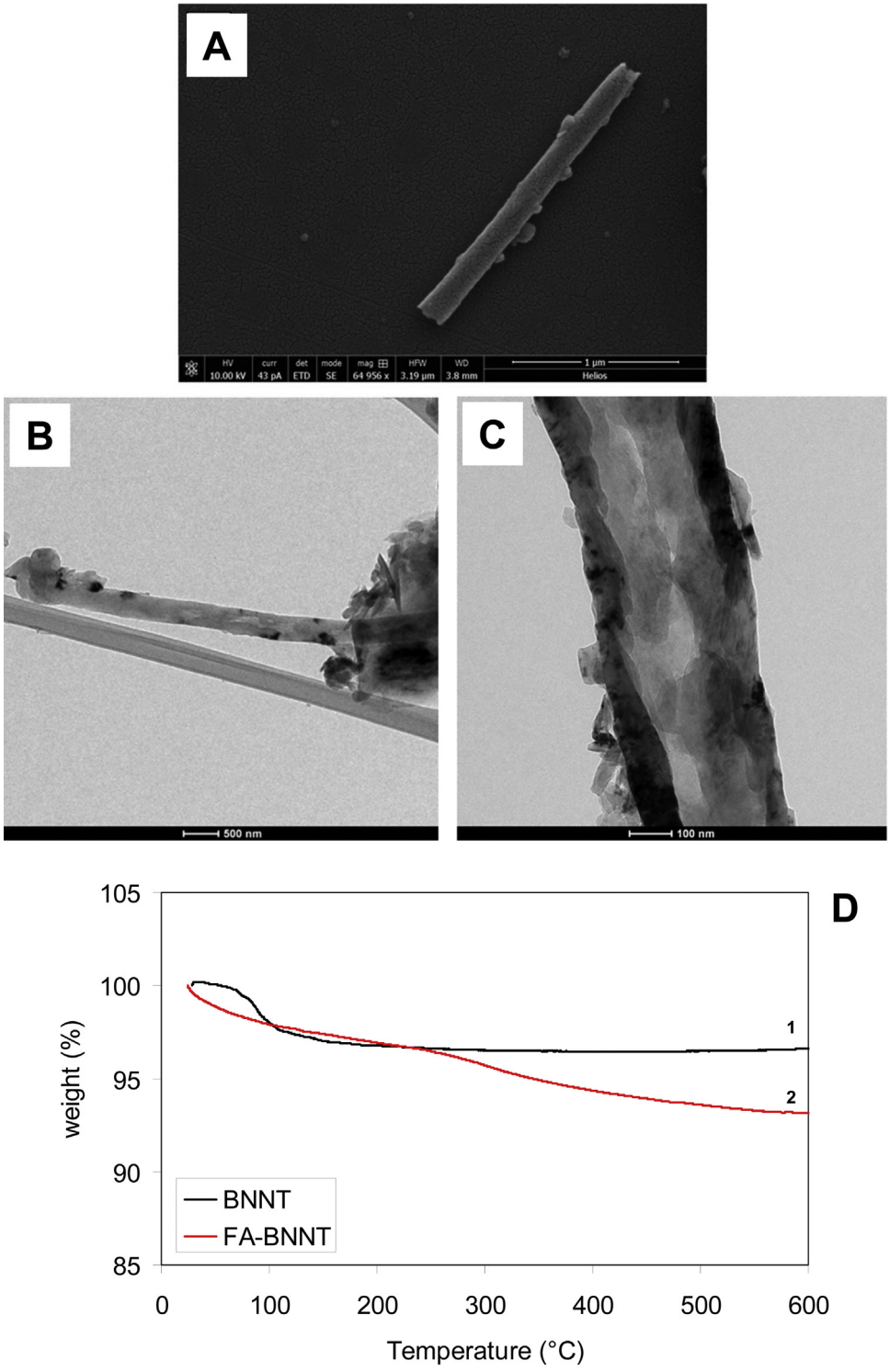

Fig. 3. SEM image (A) and low (B) and high (C) magnification TEM images of representative FA-BNNTs. TGA analysis on BNNT (1) and FA-BNNT (2) samples (D).

fixed with 4% paraformaldehyde (Sigma) solution in PBS for 20 min at 4 °C and then washed again with PBS to remove paraformaldehyde residues. Thereafter, cell membranes were permeabilized with a 0.1% Triton X-100 (Sigma) solution in PBS for 15 min. The staining was obtained with a solution of FITC-conjugated phalloidin (100 μM) and DAPI (1 μM) incubated for 30 min at room temperature. Samples were finally rinsed with PBS.

Images were acquired with a confocal laser scanning microscope (C2s, Nikon), and NIS professional software was used for the 3D reconstruction of collected Z-stacks. BNNTs were observed by using an excitation wavelength of 642 nm and collecting the fluorescence emission from 670 nm to 750 nm.

Lysosome staining of cells treated for 6 h with 20 μg/mL of FA-BNNTs was performed by using a fluorescent acidotropic probe, the LysoTracker dye (Invitrogen). Specifically, cells were incubated for 2 h in a serum free growth medium with the appropriate dye dilution (1:2500).

To quantitatively assess the presence of BNNTs inside the cells, the amount of boron was measured in HeLa cultures grown in 75 cm^2 flasks and incubated with BNNTs (20 μg/mL), FA-BNNTs (20 μg/mL) or without nanoparticles (as control) for 6 h. After the treatment, cultures were washed three times with PBS and detached by trypsin. Cell suspensions were centrifuged, the supernatant removed, and 0.5 mL of a concentrated HCl/HNO_3 (Carlo Erba super-pure grade) solution was added to digest the pellets (the solution was left for 24 h). MilliQ grade water (18.3 MΩ) was then added (4.5 mL) to the samples, and boron concentration was measured by means of elemental analysis (ICP-OES spectrometer, iCAP 6500, Thermo). The most sensitive 249.7 nm boron emission line was used.

2.4. Statistical analysis

Analysis of the data was performed by analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* correction for test significance, which was set at 5%.

3. Results

3.1. BNNT characterization

Fig. 2A shows a comparison among IR spectra of BNNTs (1), FA-BNNTs (2) and folic acid (3). The samples (1) and (2) have the typical bands of BNNTs, which are a strong asymmetric band centered at 1380 cm^{-1}, corresponding to the bond B—N stretch, along with a less intense band at 790 cm^{-1} attributed to B—N—B bond (Zheng et al., 2008). The presence of a band centered at 1750 cm^{-1}, which can be ascribed to C=O absorption typical of saturated aliphatic esters, can be appreciated in the spectrum of FA-BNNTs (2). Furthermore, it is possible to notice the presence of an absorption peak at 1150 cm^{-1} which can be ascribed to C—O—C bond. Both the new bands stem from the presence of folic acid conjugated on the BNNT walls, as it can be easily deduced from the comparison with spectrum (3).

Wide XPS spectra of pristine BNNTs and folate functionalized BNNTs (FA-BNNTs) are reported in Fig. 2B and C. The peaks of boron and nitrogen are clear in both samples, located at 398.0 eV (N) and 190.5 eV (B), consistently with the literature (Zhi et al., 2010). The peaks of carbon (285.0 eV) and oxygen (532.5 eV) present in pristine BNNTs are due to the previously mentioned impurities. Concerning FA-BNNTs (Fig. 2C), it is possible to observe a consistent increment of the carbon peak intensity (from 6.9 to 41.4%) related to the introduction of folate molecules on the BNNT walls.

Narrow XPS spectra of N1s are shown in Fig. 2D and E. In pristine BNNTs (Fig. 2D) the peak is symmetric with respect to its center (398.5 eV) and corresponds to the B—N bond. In FA-BNNTs (Fig. 2E), the N1s peak becomes asymmetric after the modification, revealing a shoulder for higher binding energies. Peak deconvolution in fact shows a second Gaussian component at 399.87 eV (8.19% of the whole N1s peak area). Since free folate molecules were removed by centrifugation, this peak related to N—C=O bond could be attributed to folate molecules grafted on the BNNT walls, thus suggesting a successful functionalization.

Fig. 3A reports a SEM image of a typical BNNT, after the grafting procedure (FA-BNNTs), showing its typical shape and morphology with a well-defined tubular structure. Fig. 3B shows a representative low-magnification TEM image of a FA-BNNT, whilst Fig. 3B shows a TEM image at high-magnification, where we can appreciate the hollow inner channel of the nanotube, preserved after the modification steps.

The thermogravimetric curves shown in Fig. 3D present a characteristic mass loss in two stages. The first one occurs within a temperature range of 25–180 °C, and it can be attributed to adsorbed water. The second stage, within 180–600 °C, can be attributed to the decomposition of organic groups grafted on the BNNT walls. Pristine BNNTs (1) lose mass only in the first region, confirming the thermal stability of the material. On the other hand, FA-BNNTs (2) present a mass loss even in the second region, which is due to the degradation of folate bound to the BNNTs. By considering the different weight drops due to the organic component, the fraction of grafted folate resulted to be about 5% (w/w).

3.2. Biological testing

Specificity, selectivity, and biological effects are essential characteristics that make nanoparticles suitable for specific uses. Because of the high expression of folate receptors by HeLa cells (Parker et al., 2005), it is expected that BNNTs functionalized with folic acid present a stronger interaction with this cell line in comparison to the pristine BNNTs. Therefore, following assays were performed to verify an increment of internalization of the functionalized nanovectors.

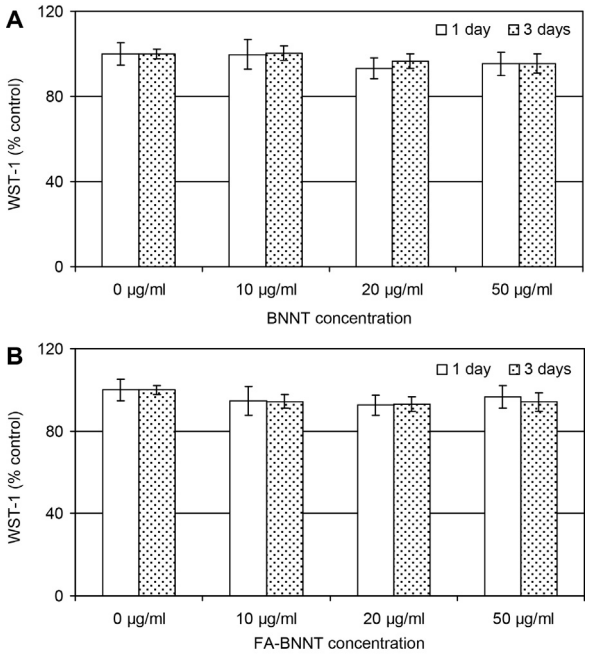

Fig. 4. WST-1 metabolic assay on HeLa cells incubated for 1 and 3 days with increasing concentrations of BNNTs (A) and FA-BNNTs (B).

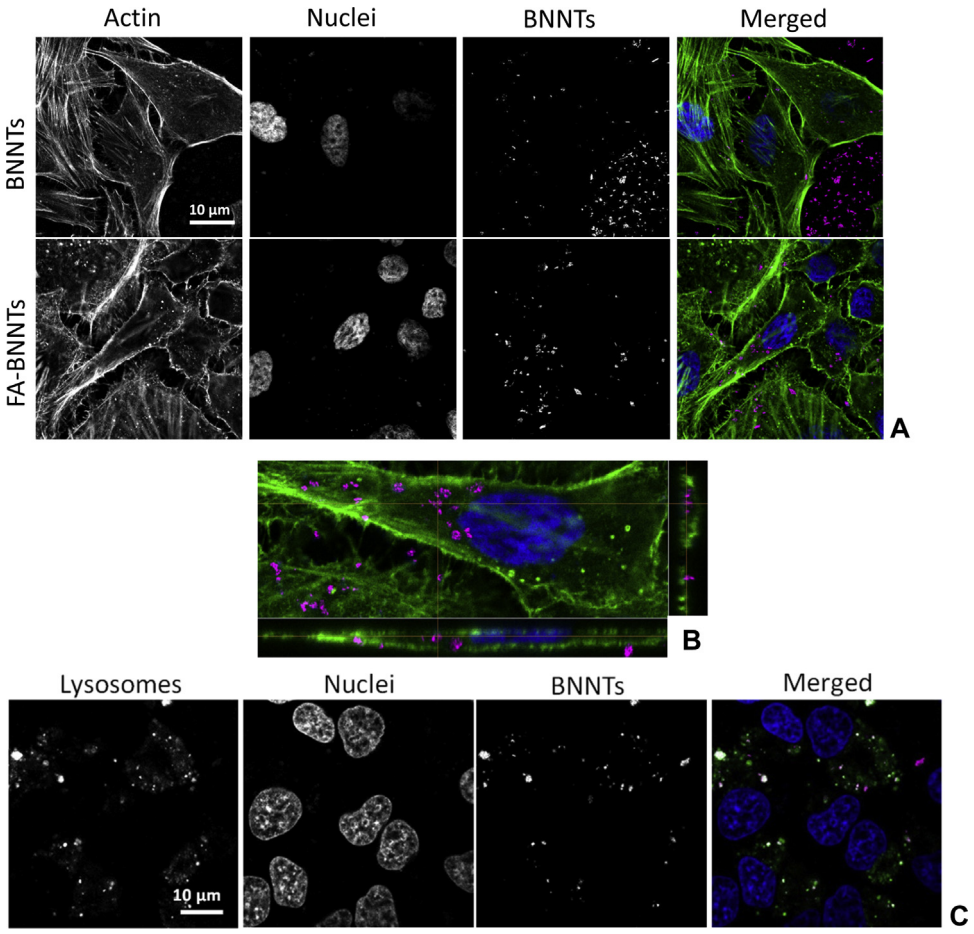

Fig. 5. Confocal images of HeLa cells following treatment with BNNTs and FA-BNNTs (A); side projections of a confocal Z-stack confirming FA-BNNT internalization by HeLa cells (B); BNNTs are showed in pink, cytoskeletal f-actin is stained in green and cell nuclei are counterstained in blue. Lysosome staining (in green) for FA-BNNT (in pink) co-localization assessment (C).

Before internalization assessment, absence of cytotoxic effects of both BNNTs and FA-BNNTs has been verified: after both 1 and 3 days of incubation, no adverse effects were found on HeLa cultures in terms of metabolic activity (Fig. 4) in a concentration range of 0–50 μg/mL.

Fig. 5A shows confocal images of HeLa cells incubated with BNNTs and FA-BNNTs. Both separated channels (f-actin, nucleus and BNNT signals), and merged images are reported. As above mentioned, it has been possible to clearly visualize the nanoparticles through confocal microscopy by excitation at 642 nm; the reason of this phenomenon is still unclear, and it could involve the strong scattering signal presented by BNNTs. Merged images allow for a direct comparison between BNNT and FA-BNNT interaction with HeLa cells. Pristine not-folate-functionalized BNNTs are preferentially located outside the cells, while functionalized FA-BNNTs, following the same incubation conditions, are highly internalized by the cells, thus suggesting an active role played by folate during the up-take process.

A clearer hint of FA-BNNT enhanced up-take is provided by a 3D acquisition: Fig. 5B reports side projections of a confocal Z-stack demonstrating extensive FA-BNNT internalization by HeLa cells.

Finally, lysosomal staining (Fig. 5C) demonstrated a pretty high co-localization (Pearson's index 0.65 ± 0.04) between FA-BNNTs (pink) and lysosomes (green).

A quantitative indication of BNNT and FA-BNNT internalization by HeLa cells was obtained by measuring the boron amount inside the cultures through ICP analysis. The results presented in Fig. 6 show a considerable up-take of pristine BNNTs by cells, confirming that also non-functionalized nanotubes can be internalized through non-specific routes. However, a huge increment in boron content (95%, $p < 0.01$) was found in cells treated with FA-BNNTs, quantitatively demonstrating a significant higher FA-BNNT uptake mediated by the folate receptor.

4. Discussion

In this work, we proposed BNNTs covalently functionalized with folic acid, with the objective of providing enhanced internalization by cancer cells over-expressing folate receptors.

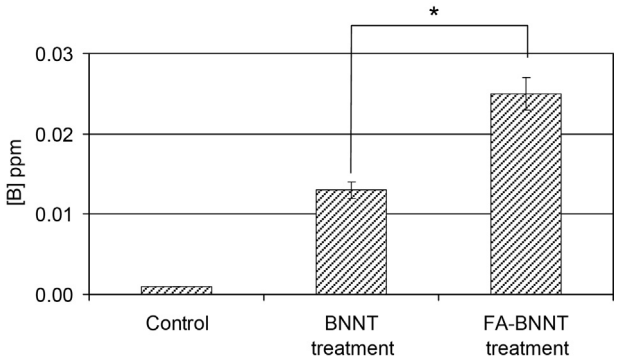

Fig. 6. ICP analysis showing B amount inside HeLa cultures treated with BNNTs and FA-BNNTs compared to non-treated control cultures. $^{*}p < 0.01$.

The morphological analysis of the functionalized BNNTs performed with SEM and TEM showed the typical tubular structure of nanotubes, surrounded by an amorphous layer ascribable to the presence of folate on their surfaces (Maguer et al., 2009; Zhi et al., 2008); the functionalization process was characterized through FTIR, XPS (Ciofani et al., 2012; Zhi et al., 2005), and TGA analysis, the latter highlighting a folate grafting of about 5% (w/w).

Thanks to their peculiar optical properties, confocal analysis of internalization was possible without conjugation with any other fluorescent probe that could affect nanoparticle behavior. Although a very useful property, the reason of this phenomenon is indeed not clear, and will deserve future dedicated investigations.

Nanoparticle targeting is a really important issue to be considered in order to optimize cancer therapies (Heinemann et al., 2013); an improvement in terms of nanoparticle internalization is expected following folate conjugation (Graham et al., 2013; Kam et al., 2005), and indeed our results confirmed a 2-fold FA-BNNTs higher up-take by HeLa cells with respect to pristine BNNTs. Furthermore, the co-localization with lysosomes suggests a receptor-mediated endocytosis as the process enabling FA-BNNT enhanced internalization by cancer cells (Barz et al., 2010). This observation is important when envisaging, for example, a drug delivery based on pH-responsiveness (Panariti et al., 2012).

Finally, we have to stress as FA-BNNTs can be used as drug *per se* in boron neutron capture therapy, as cargos of B atoms. This is a radiotherapy treatment based on the accumulation in the tumor of ^{10}B-enriched molecules and subsequent irradiation with low energy neutrons, which leads to the decay of ^{10}B to ^{7}Li and an α particle, causing the death of the neoplastic cells where the ^{10}B-enriched compounds accumulate (Achilli et al., 2013): the possibility to efficiently accumulate BNNTs just into cancer cells would thus represent an actual “magic bullet” against tumors.

5. Conclusion

In this paper, we have proposed, synthesized and characterized BNNTs chemically functionalized with folic acid, with the objective of providing greater affinity for tumor cells. *In vitro* testing on HeLa cells demonstrated an increased cellular up-take of the FA-BNNTs with respect to the pristine BNNTs, most probably because of an enhanced receptor-mediated endocytosis. Furthermore, their optical properties allowed for a direct investigation of the up-take process, revealing a high co-localization with the lysosomes. Even if preliminarily, the collected evidences are encouraging, and suggest that FA-BNNTs are worth to be further investigated as suitable nano-tools in cancer therapy.

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