Boron Nitride Nanotubes functionalized with glucosamine as a potential novel carrier system for radioisotope and drug delivery

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ABSTRACT

Different inorganic nanostructures constituted by metals, metal oxide, silica, semiconductors (quantum dots) and carbon nanotubes has been object of intense investigation as potential drug delivery systems, aiming early diagnosis or the treatment of different diseases including the cancer. In the present work, boron nitride nanotubes (BNNTs), a structural analog of a carbon nanotube where C atoms are substituted by alternating B and N atoms in a resonance hybrid, were synthesized and functionalized with glucosamine molecules. The nanostructures were physicochemical and morphologically characterized by Scanning Electron Microscopy (SEM), X-ray diffraction (XRD), Raman Spectroscopy and Photon Correlation Spectroscopy (PCS). Furthermore, *in vitro* tests were conducted aiming to evaluate the cytocompatibility of the nanostructures.The results showed that nanostructures have adequate characteristic to applied in *in vitro* studies with a good cytocompatible profile.

Keywords: boron nitride nanotubes, glucosamine functionalization, physicochemical and morphological characterization, cytocompatibility tests, MRC-5 fibroblast lung cells.

1 INTRODUCTION

In the last decade, several inorganic nanostructured systems such as metals, metal oxide, silica, semiconductors (quantum dots) and carbon nanotubes have been proposed as new drugs and radioisotopes delivery systems, aiming the diagnosis and treatment of many diseases, including cancer [1,2]. These inorganic nonosystems have the potential ability to deliver a relatively large amount of drug in specific tissues without any or minimal premature release until reached the specific target. Moreover, they can permeate through interstitial space, allowing the accumulation and retention of drugs or radioistopes on the pathological tissue [3]. The greatest challenge for *in vivo* applications of any nanostructure, including inorganic nanoparticles, is to give these nanosystems stealth features, preventing them being recognized and phagocytosed by cells of mononuclear phagocyte system [4,5]. This recognition leads the nanostructures to accumulate in the liver, spleen and marrow bone, which often are not the target tissues. Seeking to solve this problem, inorganic nanosystems have been the object of functionalization with different molecules, polymers, antibodies and aptamers [6,7]. The results shows that these functionalizing agents cause strongly influence the *in vivo* behavior and constituted an important strategy for the nanostructures reaches the target tissue with successful [8]. However, to achieve these results, the functionalized agents should possess biocompatibility, high hydrophilicity, and complete stability in different biological medium. Boron nitride nanotubes (BNNTs), a structural analog of carbon nanotubes, with atoms of boron and nitrogen arranged alternately, have demonstrated interesting characteristics to be applied in biomedical area due to its high stability and considerable biocompatibility. In this work, these nanostructures were synthesized and functionalized (coating) with glucosamine molecules aiming to evaluate its potentiality as a novel carrier system for radioisotope and drug delivery.

2 EXPERIMENTAL

2.1 Materials

Amorphous boron powder, ammonium nitrate, hematite and glucosamine (MW=2.5×105 Da, degree of deacetylation = 88%) were obtained from Sigma–Aldrich (São-Paulo-Brazil).

2.2 Methods

2.2.1 BNNTs preparation and functionalization

BNNTs samples were prepared from a synthesis developed in our laboratory [10]. NH_4NO_3 powders (95% w/w), amorphous boron (97% w/w), and hematite (95% w/w and particle size less than 50 nm) were mixed in a molar ratio of 15:15:1 respectively and heated up to 550°C in tubular furnace, using an alumina boat as support. This temperature was kept constant for one hour. After that, the temperature was raised until 1300°C under nitrogen gas flow. These conditions were maintained during 1 hour. In sequence, the gas nitrogen flow was interrupted and a flow ammonia gas (50 $cm^3.s^{-1}$) was added, with samples treated for one additional hour. After these steps, the BNNTs were successfully obtained with high yield.

The BNNTs were functionalized (noncovalent coated) with glucosamine, based in methods described by Ciofani et al., 2010 [11].

2.2.2 BNNTs physicochemical characterization

2.2.2.1 Scanning electron microscopy - SEM

The procedure was performed in a scanning electron microscope (JEOL JSM, 840A) operating at 15kV. Samples (5µL) were deposited and spread on silicon substrate and dried with an argon stream.

2.2.2.2 X-ray diffraction - XRD

The samples were analyzed by XRD for identification of crystalline phases present. The high-angle XRD patterns were obtained using a Rigaku Geigerflex-3034 diffractometer with a Cu-Ka tube. The identification of crystalline phases was obtained by comparing the X-ray diffractogram of the samples with the database of ICDD - International Center for Diffraction Data / Joint Committee on Powder Diffraction Standards - JCPDS (Sets 01 to 50, 2000).

2.2.2.3 Raman Spectroscopy

Raman spectroscopy was used for preliminary evidence of the nanotubes formation in the sample. The analysis of Raman scattering were carried out in an equipment brand Horiba Jobin Yvon model IHR 550, which is equipped with monochromator, confocal microscope (Olympus BH-2) and CCD detector. The scattering was excited using laser 2.4 eV (λ = 514.25 nm). The objective lens used was 50x. The accumulation time was 5 x 7 seconds, with power of 2.67 mW. Spectrum was collected with a wavelength from 1000 to 1700 cm-1.

2.2.2.4 Photon correlation spectroscopy and Zeta Potential analysis

This analytical procedure allows one to determine the mean lengh of BNNTs and the Polydispersity Index (P.D.I). It was conducted in a Zetasizer Nanoseries Zs (Malvern Instruments, Malvern, UK) apparatus after its adequate dilution in ultra-pure MilliQ® water. The results are expressed as mean ± standard deviation for at least three different batches of each nanotubes preparation. The zeta potential was determined by Laser Doppler Anemometry (LDA) in same Zetasizer equipment.

2.2.3 Cytocompatibility tests

The MTT colorimetric assays were employed to determine the cytotoxicity of pure and functionalized BNNTs. The cells (MCR-5) were seeded at 1500 cells/well in 96-well flat-bottomed plates and incubated for 24 h in incubator with atmosphere of 5% CO2 at 37ºC. The cells were treated with samples, varying the concentrations from 0.1 to 200.00.1 to 200.0 µg/mL. After 48 h of incubation at 37ºC, MTT reagent was added to each well and after more 4 hours of incubation, dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan precipitates and absorbance was measured at 570 nm. The fraction of surviving cells in treated groups was calculated as a percentage of control groups and the absorbance in control considered 100% survival.

For analysis of changes in chromosomal DNA, MRC-5 cells were cultured in 96-well plates and treated with silica and functionalized silica nanoparticles at different concentrations ranging from 50.0 to 200.0 µg/mL during 48 h. After, the cells were fixed in methanol and incubated with DAPI (4 ', 6 - diamidine-2'-phenindole dihydrocloride) at a concentration of 400 ng/mL in PBS. After incubation, the supernatant containing DAPI was removed and the cells were washed 5 times with PBS. Cell nuclei stained with DAPI were observed by fluorescence microscopy (Nikon, 385-410 nm).

3 STATISTICAL ANALYSIS

All experiments were performed in triplicate and expressed as mean ± standard deviation, unless otherwise stated. Mean size and zeta potential data within each time period were compared by means of the ANOVA test, using the software prism 5.0 and considering a probability of 5% as significant.

4 RESULTS AND DISCUSSION

4.1 BNNTs physicochemical characterization

Figure 1 shows two different cuvettes where BNNTs samples were added to a volume of 500µL of water. On the right cuvette, the non-functionalized BNNTs is presented in a gross dispersion. It is possible to verify the lower dispersivity of nanostructures in aqueous solution, forming aggregates of particles visible to the naked eye and with a strong tendency to deposit on the bottom of flask. The cuvette on the left present samples BNNTs functionalized with glucosamine. The coating with the polymer improved significantly the dispersity of the nanostructures, thus enabling *in vivo* applications.

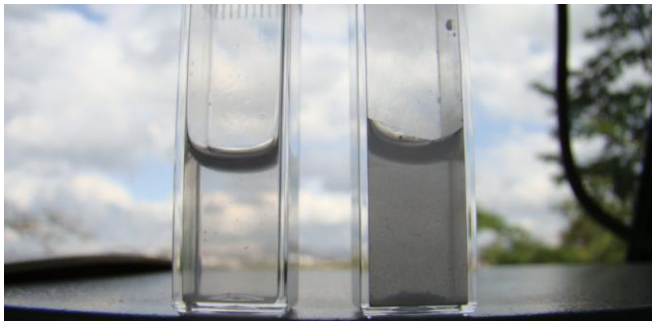

Figure 1 – Samples of BNNTs added in aqueous solution. On the right cuvette is presented the BNNTs non-functionalized. On the left cuvette, sample with functionalized BNNTs.

4.1.1 Scanning electron microscopy - SEM

Figure 2a shows an image of non-functionalized BNNTs obtained in our laboratory, which dimensions are about 10

µm in length and diameter of about 70 nm. Notably, they are usually entangled together. Figure 2b shows that obtained glucosamine coating BNNT dispersions resulted in remarkably stable and well dispersed pieces of approximately 400 nm in length and diameter of about 80 nm. This considerable increase in the BNNT diameter indicates the efficiency of the functionalization process.

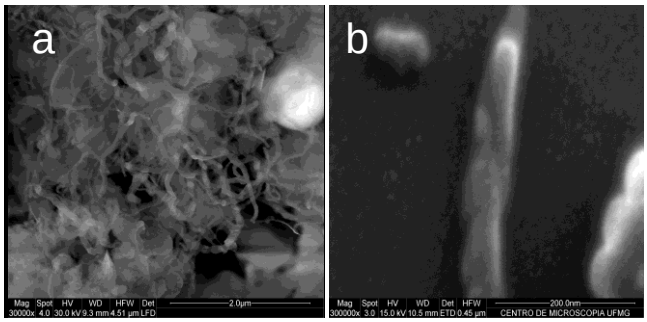

Figure 2- SEM images of BNNTs before (a) and after the functionalization process (b).

4.1.2 X-ray diffraction - XRD

Figure 3 shows the diffractogram of the sample containing BNNT. It is possible to clearly identify the presence of peaks typical of hexagonal phase of boron nitride in 2θ = 26.75 °, 2θ = 41.58 °, 2θ = 50.16 ° and 2θ = 75.86 °, which are perfectly aligned with the pattern of h-BN JCPDS database, card no. 9-12.

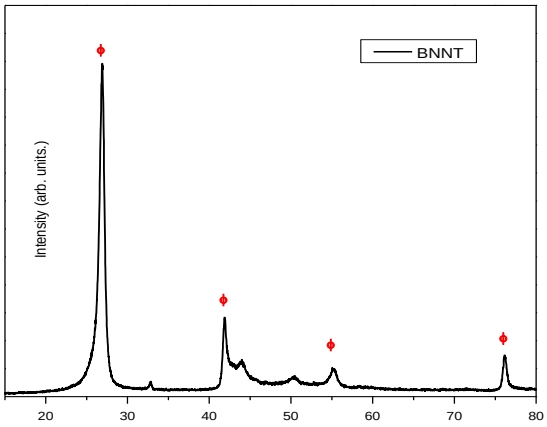

Figure 3 - XRD diffraction patterns of BNNT sample.

This result indicates that the boron nitride is being formed in a satisfactory manner and that impurities from the synthesis process are present in very small quantities in the sample.

4.1.3 Raman Spectroscopy

From the analysis of Figure 4 it is possible to observe the presence of a single peak at 1370 cm^{-1} which is characteristic of the tangential vibration mode BNNT as described in the literature [12]. The high background observed is due to the fluorescence of the sample. From this technique it was possible to confirm that the boron nitride is structured in the nanotubes form.

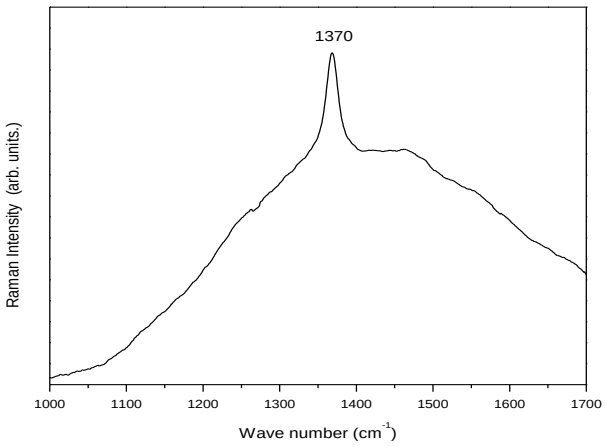

Figure 4 - Raman Spectroscopy of the BNNTs sample.

4.1.4 Photon Correlation Spectroscopy and Zeta Potential analysis

The photon correlation spectroscopy (PCS) was conducted on 3 samples at different times. The results were calculated from three independent samples by software provided by the manufacturer, which revealed the length of nanotubes nearly constant at 378 nm (Table 1). The polydispersity index (P.D.I.) found in this study is presented in Table 1. All samples presented a P.D.I. of above 0.3. These results indicate that the process used in nanotubes preparation and functionalization allows one to obtain a monodispersed system (<0.3). The Zeta potential of nanotubes cans predicting its fate of in vivo, given that the presence of electrical charges minimizes agglomeration and fusion phenomenon. In Table 1 the Zeta potential from the formulation exhibited a negative charge, with values of -12 ± 3.7 mV

Table 1- Physicochemical characteristics of the functionalized BNTTs

Mean Size ± S.D. (nm) (PCS)[a]	**Polydispersity Index[b]**	**ζ potential ± S.D. (mV)[c]**
378 ± 15	0.29 ± 0.15	- 7.6 ± 2.6

Nanostructures containing significantly positive or negative Zeta Potential suffer fewer aggregations, due to the appearance of electrostatic repulsion. Thus, particles with significantly Zeta potential are more stable. Furthermore, the superficial charge of nanostructures is shown to be an important pharmacokinetics regulatory property. For example, studies show that cationic or anionic

liposomes activate the complement system through classic or alternative pathway respectively, leading to a blood serum protein opsonization and subsequent phagocytosis by SMF cells [13].

4.2 Cytocompatibility tests

Samples were not cytotoxic to the cell line MRC-5, normal human fibroblast, in tested concentrations of 0.1 to 200 µg/mL. Furthermore, the cytotoxicity results indicated that functionalizing agents (glucosamine), in used concentrations, were unable to induce significant cytotoxicity to tested cells. The treatment did not affect the process of cell differentiation and caused no significant morphological changes in cells (Figure 5a). The analysis of chromosomal alterations and evaluation of possible changes in morphology cells, treated with 200 µg/mL of BNNTs, compared with controls, showed that no significant increase of chromosomal aberrations or significant changes in cells morphology when compared with the untreated control (Figure 5b).

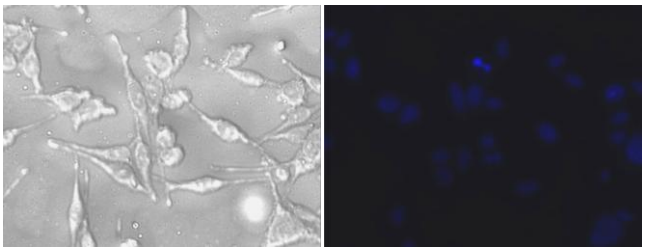

Figure 5 - Cell culture image of the bright field (A) and DAPI (B) assays in 200 µg/mL of BNNTs functionalized with glucosamine

5 CONCLUSION

From the described procedures it was possible to synthesize with successfully boron nitride nanotubes functionalized with glucosamine. These nanostructures demostrated characterists adequated to be used as potential carriers of drugs or radioisotopes in the therapy of diseases such as cancer.

6 ACKNOWLEDGMENTS

The authors wish to thank FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais), CNPQ (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and CAPES (Comissão Aperfeiçoamento de Pessoal de Nível Superior) for their financial support, and the Microscopy Center - UFMG for technical support during electron microscopy work.

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