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Interaction of carbohydrate modified boron nitride nanotubes with living cells

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ABSTRACT

Boron nitride nanotubes (BNNTs) are composed of boron and nitrogen atoms and they show significantly different properties from their carbon analogues (carbon nanotubes, CNTs). Due to their unique properties including low electrical conductivity, and imaging contrast and neutron capture properties; they can be used in biomedical applications. When their use in biological fields is considered, the route of their toxic effect should be clarified. Therefore, the study of interactions between BNNTs and living systems is important in envisaging biological applications at both cellular and sub-cellular levels to fully gain insights of their potential adverse effects. In this study, BNNTs were modified with lactose, glucose and starch and tested for their cytotoxicity. First, the interactions and the behavior of BNNTs with bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/Nutrient Mixture F-12Ham were investigated. Thereafter, their cellular uptake and the cyto- and genotoxicity on human dermal fibroblasts (HDFs) and adenocarcinoma human alveolar basal epithelial cells (A549) were evaluated. HDFs and A549 cells internalized the modified and unmodified BNNTs, and BNNTs were found to not cause significant viability change and DNA damage. A higher uptake rate of BNNTs by A549 cells compared to HDFs was observed. Moreover, a concentration-dependent cytotoxicity was observed on A549 cells while they were safer for HDFs in the same concentration range. Based on these findings, it can be concluded that BNNTs and their derivatives made with biomacromolecules might be good candidates for several applications in medicine and biomedical applications.

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1. Introduction

Boron nitride nanotubes (BNNTs) were first synthesized in 1995 by Chopra et al. [1] after the discovery of carbon nanotubes (CNTs) in 1991 by Iijima [2]. Although BNNTs are structural analogues of CNTs, they are reported to be more stable than the CNTs [3]. In fact, BNNTs are gaining increasing attention as novel nanomaterials because of their high oxidative properties, and mechanical and chemical resistance [4]. In addition, BNNTs are considered as good candidates for a wide range of biomedical and related applications such as orthopedic implants [5], biosensing [6], and drug and gene delivery [7].

The high chemical stability and hydrophobicity of BNNTs result with their poor dispersibility in aqueous media [8,9], which hinders their cellular uptake studies and reliable assessment of their adverse effects on living systems. Therefore, BNNTs were functionalized with several biomolecules such as gum Arabic, glycine, mesoporous silica and europium doped sodium gadolinium fluoride (NaGdF4:Eu) for cellular uptake studies for biomedical applications including drug delivery and tissue engineering [10–13]. The functionalization provides dispersibility in aqueous media and functional groups to visualize them by binding fluorescent dyes or quantum dots in fluorescence imaging studies [14,15].

The cytocompatibility of derivatized BNNTs and their interactions with the living systems were first evaluated by Ciofani et al. [14]. In another study, the glycol–chitosan (GC) non-covalently coated BNNTs exposed human neuroblastoma SH-SY5Y cells were viable up to100 μg/mL of GC-BNNTs [16]. In another study, the BNNTs were functionalized with organic hydrophilic agents including glucosamine (GA), poly(ethylene glycol)$_{1000}$ (PEG$_{1000}$) and chitosan (CH), and their toxicity was investigated [17]. The study found that the PEG$_{1000}$-BNNT and CH-BNNT were cytotoxic at high concentrations (100 μg/mL) while the GA-BNNT was not cytotoxic. Moreover, the hemocompatibility study of the unmodified BNNTs

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showed that they were hemocompatible on the malignant U87 (wild type p53) and T98 (mutant p53) glioblastoma, MCF-7 adenocarcinoma mammary gland cells and normal MRC-5 fibroblast lung cells [18].

In another study, the cytotoxicity of Tween 80 coated BNNTs was investigated with several cell lines including A549, murine alveolar macrophage cells (RAW 264.7), murine embryonic fibroblast cells (3T3-L1) and human embryonic kidney cells (HEK293) [19]. The results of that study demonstrated that BNNTs were cytotoxic at variable BNNT concentrations (0.2–20 μg/mL) in a cell-type-dependent manner. They were enormously cytotoxic especially on macrophage cells due to the high endocytosis capacity of these cells.

Furthermore, the biocompatibility of the glycol–chitosan modified BNNTs was investigated *in vivo*. The BNNTs were intravenously injected in rabbits at 1 mg/mL dose in a first experiment [20]; then, 5 and 10 mg/kg doses were adopted in a second experiment [21]. All the collected results indicated that the BNNTs have no significant adverse effect on the white blood cells, red blood cells and many other blood parameters of the rabbits upon their injection in the blood. In a recent study, the gum Arabic functionalized BNNTs was injected into planarian in order to investigate the influence of the modified BNNTs on the regeneration of planarians. The animals amputated below their heads showed no important morphological and progressive differences [22].

The inconsistent results in the literature cause confusions about the biocompatibility of BNNTs. It is clear that more data is needed to understand their behavior in biological systems. Therefore, the toxicity of the BNNTs should be further investigated by using many other cell models before concluding about their biocompatibility. In this study, the cellular uptake and the cyto- and genotoxicity of unmodified and carbohydrate-modified BNNTs were investigated. Their modification with carbohydrates was performed to increase their dispersibility in aqueous media. Then, their interactions with culture media contents, bovine serum albumin (BSA), DMEM, and DMEM/Ham's F-12, were investigated using Bradford assay. Finally their cellular uptake and toxicity were evaluated using HDFs and A549 cell lines.

2. Materials and methods

2.1. Hydroxylation and glucose, lactose and starch modification of hydroxylated BNNTs

BNNTs were synthesized from colemanite based on a chemical vapor deposition (CVD) method, and purified as reported earlier [23]. For hydroxylation, 100 mg of pure BNNTs were added into 10 mL of 30% H_2O_2 solution, and the mixture was sonicated at 25 °C for 1 h. Then, the mixture was refluxed for 48 h at 110 °C while stirring. The obtained hydroxylated BNNTs (h-BNNTs) were precipitated by centrifugation (15 min, 10,000 rpm) and washed with ddH_2O five times and dried at 60 °C.

Glucose, lactose and starch-modified BNNTs(m-BNNTs) were synthesized using a previously reported method [24]. Briefly, a suspension was prepared by dispersing 100 mg of *h*-BNNTs in 10 mL of deoxygenated H_2O, and sonicated for 30 min. 10 mL of 5% w/v glucose, 5% w/v lactose or 2% w/v starch solution were added to the suspension. This suspension was then incubated for 48 h on a magnetic stirrer at 37 °C after adding 500 μL of 10% v/v glutaraldehyde. The obtained *m*-BNNTs were precipitated by centrifugation (30 min, 14,000 rpm) and washed with ddH_2O five times and dried in a vacuum oven at 30 °C. The *h*-BNNTs and *m*-BNNTs were analyzed with Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analysis (TGA).

2.2. Interaction of the BNNTs with media and their contents

A 1 mg of unmodified or modified BNNTs was added into 1 mg/mL of BSA solution, DMEM or DMEM/Nutrient Mixture F-12Ham (Sigma–Aldrich, Germany), and the mixture was sonicated for 2 h in an ice bath. After the sonication, they were shaken in a cold room (4 °C) overnight. Thereafter, they were centrifuged at 14,000 rpm for 30 min at 4 °C, and the supernatant was discarded. This washing procedure was repeated three times. After the washing steps, 1 mL of ddH_2O was added into the BNNTs–BSA, BNNTs–DMEM and BNNTs–DMEM/nutrient mixture F-12Ham, respectively. A 100 μL of samples were added into 400 μL of Bradford reagent in a 24-well plate, and the mixture was allowed to incubate for 30 min at room temperature.

2.3. Cell culture

HDF and A549 cell lines were utilized to assess the cytotoxicity and the genotoxicity of BNNTs. The HDFs were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin ampicillin (PSA) antibiotics. The A549 cells were cultured in (DMEM F-12), supplemented with 1% L-glutamine in addition to 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin Ampicillin (PSA) . The cells were incubated in water-jacketed incubator in a 5% CO_2, 95% air atmosphere at 37 °C.

2.4. Cellular uptake assays

The cells were seeded on cover slips in a 6-well plate and incubated for 24 h. A 5-μL of diamidino-2-phenylindole (DAPI) was added to 1 mL of suspension containing a 50 μg/mL of BNNTs, *h*-BNNTs and *m*-BNNTs, respectively. They were kept at room temperature for 30 min, and then centrifuged at 14,000 rpm for 30 min at 25 °C. The supernatants were discarded and the precipitates were washed for three more times. 1 mL of cell medium was used to resuspend the BNNT–DAPI, *h*-BNNT–DAPI or *m*-BNNT–DAPI pellets at the end of the washing steps. A 5 μL DAPI was added into 995 μL of medium for the control experiments. The BNNT-DAPI, h-BNNT-DAPI or m-BNNT-DAPI included medium were added on the cells that were seeded on the cover slips and incubated for 24 h. After removing the medium from the cells, 1 mL of PBS was added onto the cells and shaken for 5 min in a shaker at room temperature. A 2.5% glutaraldehyde solution was prepared in PBS. After discarding PBS from the cells, 2.5% glutaraldehyde solution was added and incubated for 30 min at 4 °C. After the fixation, the cells were dehydrated through ethanol washing series (50, 70, 90, and 100%) for 5 min per solution. Samples were examined with a Zeiss LSM 700 confocal laser-scanning microscope.

2.5. Biocompatibility assays

2.5.1. Cell viability assay

The cytotoxic effects of the modified and unmodified BNNTs on cells were quantified with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) colorimetric assay. First, HDFs were seeded at 5×10^3 cells/well and A549 cells were seeded at 1×10^4 cells/well in 96-well plates, and incubated for 24 h. The cells were then treated with the BNNTs, *h*-BNNTs and *m*-BNNTs at increasing concentrations (5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL). The concentration of the modified BNNTs was adjusted based on the TGA data (see Fig. 2c). After 1–3 days of incubation the culture medium in 96-well plates was replaced with fresh culture medium containing WST-1 reagent with 1:10 ratio and incubated for a further 1 h. The percentage of living cells is calculated by measuring

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