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## Boron Nitride Nanotubes Are Noncytotoxic and Can Be Functionalized for Interaction with Proteins and Cells

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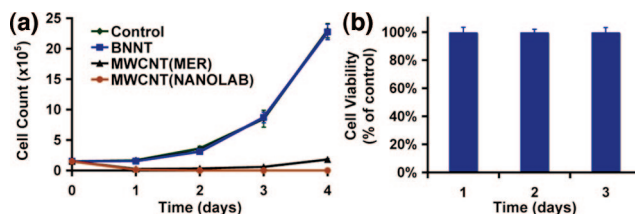
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Carbon nanotubes (CNTs) have been widely explored for use in biological applications including biosensing,<sup>1</sup> imaging,<sup>2</sup> intracellular delivery,<sup>3,4</sup> and cancer cell targeting.<sup>5</sup> However, their inherent cytotoxicity has imposed limitations on their use as biological probes and in therapeutic composites.<sup>6–9</sup> The cytotoxicity of CNTs can be reduced by surface functionalization,<sup>5,10–12</sup> but the possibility of in situ desorption brings considerable risk to their use in living organisms. Here we report that boron nitride nanotubes (BNNTs), isosteres of CNTs with unique physical properties, are inherently noncytotoxic. Furthermore, BNNTs can be surface functionalized with biological epitopes that mediate protein and cell binding. Finally, we show that BNNTs can deliver DNA oligomers to the interior of cells with no apparent toxicity. This work suggests that BNNTs may be superior to CNTs for use as biological probes and in biomaterials.

Boron nitride is isoelectronic to carbon and has a stable hexagonal structure analogous to that of graphite. The existence of BNNTs was predicted theoretically in 1994,<sup>13,14</sup> and they were synthesized shortly thereafter.<sup>15</sup> In addition to their structural similarity, BNNTs and CNTs have similar mechanical properties and thermal conductivity.<sup>16,17</sup> However, BNNTs are distinct in several key aspects. First, BNNTs are wide band gap semiconductors whose electrical properties are independent of geometry, while CNTs may be metal or semiconducting depending on chirality and diameter. Second, BNNTs are more chemically inert and structurally stable than CNTs. This latter property prompted us to investigate the properties of BNNTs in biological systems where the toxicity of CNTs is troublesome.

Pristine multiwalled BNNTs were synthesized by a chemical vapor deposition process adapted from a previously reported method.<sup>18</sup> Slight modifications of experimental procedures were made to produce highly pure BNNTs to fulfill the requirement for cellular studies. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) investigations revealed high-purity and -quality multiwalled BNNTs with an outer diameter of ~20–30 nm and a length of up to 10  $\mu$ m (Figure S1). Similar to CNTs, BNNTs aggregate into entangled bundles.

HEK 293 cells were cultured with BNNTs (at 100 mg/mL) for 4 days. For comparison, the cells were cultured similarly with two types of commercially available multiwalled CNTs (MWCNTs) with diameters and lengths similar to BNNTs, purchased from MER



**Figure 1.** BNNTs are not cytotoxic. (a) BNNTs do not inhibit HEK 293 cell proliferation. (b) BNNTs have no effect on cell viability. HEK 293 cells were cultured with BNNTs or with media alone. Cell viability is expressed as the percentage of viable cells compared to untreated controls. Error bars represent the standard deviation for three replicates.

corp. (AZ, USA) and NanoLab (MA, USA). The MWCNTs were used directly without further purification. Both BNNTs and MWCNTs formed aggregates in culture media. In control experiments, the cells were cultured with media alone. Live cells were counted each day (Figure 1a). Cells cultured with BNNTs were indistinguishable from cells grown with media alone. In sharp contrast, cells cultured with either type of MWCNTs were unable to expand during the course of the experiment. Similar results were obtained using CHO cells (not shown).

CNTs have been shown to induce apoptosis in HEK 293 cells.<sup>7</sup> We therefore tested the viability of cells cultured with BNNTs using the Annexin V-FITC/propidium iodide (PI) assay. HEK 293 cells incubated with BNNTs showed no increased staining with Annexin V-FITC or PI for at least 4 days (Figure 1b). Thus, BNNTs do not appear to inhibit cell growth or induce apoptotic pathways in the cells. It should be noted that the high purity and quality of BNNTs are crucial for their nontoxicity.

Similar to CNTs, BNNTs are not soluble in aqueous media, a limitation that must be overcome for biological implementation. Although some methods for solubilizing BNNTs have been reported,<sup>19,20</sup> general functionalization strategies for interfacing BNNTs with biomolecules and cells are lacking. We therefore explored the use of amphipathic dendritic structures similar to those we recently reported as coatings for CNTs.<sup>21</sup> As shown in Figure S2, the dendrimers comprise synthetic carbohydrate ligands at the chain ends that enable specific binding to receptors in solution. A pyrene group at the dendrimer focal point allows adsorption to CNT surfaces through  $\pi$ -stacking and hydrophobic interactions. We hypothesized that these dendrimers might also interact with the isoelectronic BNNT surface, permitting specific binding to carbohydrate-binding proteins (Figure 2a).

A panel of glycodendrimers (generation 2 and 3 [G-2] and [G-3]) displaying various glycans were synthesized,<sup>21</sup> and data from studies using the [G-2] dendrimer with  $\alpha$ -mannose moieties ([G-2] Man, Figure S2) are shown in Figure 2. The [G-2] Man-coated BNNTs were stable in aqueous solution for weeks, while the unfunctionalized BNNTs precipitated very quickly (within 1 h) in water

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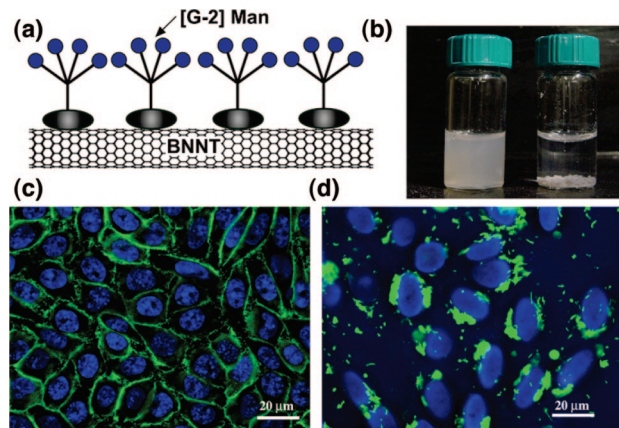
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**Figure 2.** (a) Schematic assembly of glycodendrimers on BNNT surface in aqueous media. (b) Photographs of vials containing BNNT suspensions: [G-2] Man-BNNTs (left) and uncoated BNNTs (right). (c) CHO cell surface binding of [G-2] Man-BNNTs. FITC-conjugated Con A was prebound to [G-2] Man-BNNTs. FITC-Con A-[G-2] Man-BNNT conjugates were incubated with CHO cells and imaged by fluorescence microscopy. The cell nuclei were counterstained with DAPI. (d) CHO cells after internalization of BNNTs coated with FITC-labeled DNA. CHO cells were incubated with FITC-DNA-BNNTs overnight and stained with DAPI prior to microscopy analysis.

(Figure 2b). TEM images of the coated BNNTs confirmed the presence of the glycodendrimers as an amorphous surface layer (Figure S3). Similar results were obtained with other glycodendrimers (not shown).

On cell surfaces, carbohydrates serve the dual role of specific molecular recognition via carbohydrate-binding receptors (lectins) and resistance to biofouling.<sup>22</sup> We evaluated whether the glycodendrimer coating we created on the BNNTs would mimic these functions. To test specific binding, BNNTs coated with [G-2] Man were incubated with the  $\alpha$ -mannose-specific receptor *Canavalia ensiformis* agglutinin (Con A) conjugated to fluorescein (FITC). Unbound lectin was removed by dialysis, and the labeled BNNTs were analyzed by fluorescent spectroscopy (Figure S4). Significant fluorescence was associated with Con A-bound [G-2] Man-BNNTs, while only background fluorescence was observed when the BNNTs were labeled with FITC-conjugated *Helix pomatia* agglutinin (HPA), a GalNAc-specific lectin that does not recognize mannose. Thus, glycodendrimer-functionalized BNNTs can bind to proteins via ligand–receptor interactions while resisting nonspecific binding of irrelevant proteins.

We used the glycodendrimer coating to specifically bind BNNTs directly to cell surfaces, an even more rigorous test of their biocompatibility. We previously showed that ConA is capable of cross-linking [G-2] Man-coated CNTs to cells by virtue of its tetravalent nature.<sup>21</sup> Using the same approach, we bound Con A-labeled [G-2] Man-coated BNNTs to the surface of CHO cells. Fluorescence microscopy analysis revealed robust cell surface fluorescence from FITC (Figure 2c). As a control, BNNTs coated with a similar [G-2] dendrimer bearing galactose residues ([G-2] Gal), which do not bind to Con A, showed no fluorescent labeling of the cells (Figure S5). Importantly, we did not see any cellular toxicity even when BNNTs were directly bound to the cell surface.

One of the most exciting applications of CNTs is to serve as a molecular transporter to deliver biological molecules such as proteins and DNA into living cells.<sup>4,5</sup> Given their similar dimensions, BNNTs might be similarly employed but without any unwanted toxic side effects. We explored their use as cell delivery agents using single-stranded DNA (ssDNA) as cargo. We loaded a synthetic 20-mer DNA oligomer conjugated to FITC onto the surface of BNNTs by passive adsorption, as previously achieved with CNTs.<sup>23</sup> BNNTs were sonicated with the FITC-labeled ssDNA (FITC-DNA) in aqueous solution, and the resulting suspension was

stable in water and physiological buffers for at least several days. CHO cells were then incubated with the FITC-DNA-BNNTs for 12 h. Fluorescence microscopy revealed that FITC-DNA-BNNTs were internalized by the cells in a manner dependent on the carrier BNNT (Figure 2d). In a control experiment, cells treated with FITC-DNA alone, without a BNNT carrier, showed no significant fluorescence above background (Figure S6).

In summary, we have showed that highly pure BNNTs are not cytotoxic, suggesting that their use in therapeutic or diagnostic applications should be seriously considered. Furthermore, we demonstrated that BNNTs can be surface functionalized with bioactive conjugates by noncovalent adsorption. This simple process enables the surface display of glycodendrimers capable of interacting with proteins and cells. Other biological epitopes such as proteins, DNA, and RNA can be displayed in a similar fashion. The method should facilitate applications of BNNTs in biosensing and bioimaging without limitations imposed by cytotoxicity. More generally, the observed properties of BNNTs are highly encouraging for their application in biocompatible materials.

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**Supporting Information Available:** Supporting figures; materials and methods; complete ref 9. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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