Methods

BNNT Sample Preparation and Purification

BNNTs were synthesized by the hydrogen-assisted BNNT synthesis (HABS) method. Briefly, the method is an atmospheric pressure, high-temperature plasma process utilizing an hBN powder feedstock (70 nm; MK-hBN-N70, MK Impex Corp.) and a relatively high partial-pressure of hydrogen (15-30 vol.% H2 in Ar/N2/H2), which catalyzes the growth of BNNTs through formation of B-N-H intermediate species. The synthesis product, a combination of fibrous, sheet and powder-like material, is brown in color due to the presence of boron as an impurity. This material was homogenized by mechanical griding to produce the BNNT material referred to *as-produced* or AP-BNNTs.

AP-BNNTs were first purified by a gas-phase process to chemically remove the boron impurity. The resulting material, referred to here as *boron-removed* BNNTs (*BR-BNNTs*).

The BR-BNNTs were further purified by sequential washing of solvents and water and referred to as "*Washed 1*" or *W1-BNNTs*.

In addition to the series of progressively more purified BNNTs (AP to BR to W), a second washed sample, called "*Washed 2*" or *W2-BNNTs* was also utilized for the study.

Cell Culture

Human monocyte THP-1 cells (ATCC # TIB 202) were grown in growth media containing RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 µg/mL Penicillin-Streptomycin, and 50 µM of beta-mercaptoethanol. Cells were cultured in an incubator maintained at 37 °C and 5% CO2. THP-1 cells were differentiated into macrophages by treating them with growth media containing vitamin D3 at 150 nM for 48h and then 5 nM Phorbol 12-myristate 13-acetate (PMA) for 12 h. For inflammasome activation, the differentiated THP-1 cells were primed to induce the transcription of pro-IL-1β by co-treating them with 10 ng/ml Lipopolysaccharide (LPS) when challenging with the nanomaterial. NF-κB SEAP Reporter Monocytes (THP 1-Blue™ NF-κB Cells, Invivogen Inc, San Diego, CA) were cultured and differentiated like THP-1 WT cells described above. 10 µg/ml of Blasticidin (Invivogen Inc, San Diego, CA) was added to the growth media to maintain selection. Experiments were run in triplicate or quadruplicates and repeated three times. All experiments were conducted on cells with passage 20 or below. Cell cultures were tested and found to be negative for mycoplasma contamination.

Cytotoxicity and Membrane Damage

THP-1 monocytes were differentiated with Vit D3/PMA as described in the cell culture section and the differentiated macrophages were challenged with 100 µl of fresh growth media containing 0, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100µg/ml of Hexagonal Boron nitride, AP-BNNT, BR-BNNT, W1-BNNT and W2-BNNT for 24 h. Membrane damage was assessed by evaluating the LDH released using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Inc, Madison, WI). In parallel, cytotoxicity was due to the particulate exposure was assessed using cell's ability to reduce tetrazolium salt WST-1 (Sigma-Aldrich, St. Louis). Fresh media containing 10% WST-1 was given after 24 h of nanoparticle exposure and after 2 h of incubation, the WST-1 consumption was recorded by measuring the absorbance at 450 nm subtracted with absorbance at 660 nm to account for turbidity/background. Data was presented as % change from control cells with no particulate treatment. Total LDH in the cells was evaluated by lysing the cells with

0.1 % Triton X-100 (Sigma-Aldrich, St. Louis) for 1 h. Equal volume of cell culture media and CytoTox-ONE™ Reagent (Promega, Madison, WI) was reacted at room temperature and change in fluorescence was measured at excitation 560 nm and emission 590 nm. All experiments were repeated three times, and each measurement had quadruplicate technical replicates

Electron Paramagnetic Resonance

Acellular reactivity and test materials ability to generate free radical intermediates was evaluated by electron paramagnetic resonance (EPR) spin-trapping. The particulates were exposed to H_2O_2 and particle's ability to produce hydroxyl radicals ($^{\cdot}OH$) through a Fenton-like reaction was evaluated. 100 mM DMPO (5,5'-dimethylpyrroline N-oxide, Sigma-Aldrich), 5 mg/ml various BNNT's or hBN or potassium dichromate (2 mM), and 1 mM H_2O_2 suspended in PBS and mixed in the order listed. All reagents were mixed in test tubes for 3 min at room temperature, filtered through a Titan3 nylon 0.45 µm filter to halt the reaction and filter any particulate. EPR measurements were performed on a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA) using quartz flat cell. Samples were run in independent experiments n =3. 3 technical replicate scans were performed for each run and each scan was performed for 41 sec with a receiver gain of 1.0×10^4, a 40 msec time constant, 1.0 G modulation amplitude, 63.4 mW power, 9.751 frequency, and 3515 G magnetic field center. Signal intensity (peak height) from the 1:2:2:1 spectra, which is characteristic of $^{\cdot}OH$ was used to measure the relative amount of short-lived radicals trapped.

Nuclear Factor Kappa B (NF-κB) Activation

NF-κB activation due to particulate exposure was evaluated using THP1-Blue™ NF-κB cells (InvivoGen Inc, San Diego, CA). These cells are derived from human THP-1 monocyte cell line by stable integration of an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct. NF-κB activation was determined by measuring the induced SEAP levels in the cell culture supernatants. The differentiated macrophages were exposed to 100µl of 0, 6.25, 25 and 100 µg/ml of hBN, AP-BNNT, BR-BNNT, W1-BNNT and W2-BNNT for 12 hours. The supernatants were centrifuged and SEAP in the supernatants was quantified by reacting the supernatant with QUANTI-Blue Solution (InvivoGen Inc, San Diego, CA) and measuring the absorbance at 660 nm.

Inflammasome Activation

An Inflammasome model was used to assess hBN and BNNT's potential for inflammasome induction. In this in vitro model the macrophages are co challenged with LPS along with the nanoparticles. Differentiated THP-1 macrophages were challenged with fresh media containing 10 ng/ml of LPS and 0, 6.25, 25 and 100 µg/ml of the various BNNT's or hBN for 24 h. The nanoparticles were cotreated with LPS to prime the macrophage monocultures to induce the transcription of pro-IL-1β and promote inflammasome activation. IL-1β and IL-18, markers for Inflammasome activation were assessed using human IL-1β ELISA kit (R&D Systems, Minneapolis, MN, #DLB50) and IL-18 ELISA kit (MBL International, MA, # 7620) following the manufacturers' recommendations.

Inflammatory Protein Secretions

Differentiated THP-1 macrophages were challenged with growth media containing 0, 6.25, 25 and 100 µg/ml of the various BNNT's or hBN for 24 h. 48 protein multiplex cytokine analysis was performed on the supernatants by Eve technologies (Eve Technologies, Calgary, Alberta, Canada). The 48-plex consisted of

soluble CD40 ligand (sCD40L), epidermal growth factor (EGF), Eotaxin, fibroblast growth factor 2 (FGF2), Fms-like tyrosine kinase receptor 3 ligand (Flt-3 ligand), Fractalkine, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), growth regulated oncogene α (GROα), human interferon alpha-2 (IFNα2), interferon gamma (IFNγ), interleukins IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1/CCL2), monocyte chemotactic protein-3 (MCP3), macrophage colony-stimulating factor (M-CSF), macrophage derived chemokine (MDC/CCL22), monokine induced by gamma (MIG), macrophage inflammatory protein 1 alpha (MIP-1α/CCL3), macrophage inflammatory protein 1 beta (MIP-1β), platelet-derived growth factor AA (PDGF-AA), platelet derived growth factor AB/BB (PDGF-AB/BB), regulated upon activation, normal T cell expressed and presumably secreted (RANTES), transforming growth factor alpha (TGF-α), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ) and vascular endothelial growth factor A (VEGF-A). The assay sensitivities for these markers ranged from 0.1 – 33.3 pg/mL.

Macrophage Phagocytosis Assay

Differentiated THP-1 cells were exposed to 0, 6.25, 25 and 100 µg/ml of hBN and various purity grade BNNTs for 24 h and were further challenged with fresh medium containing Escherichia coli GFP (ATCC # 25922GFP) at multiplicity of infection (MOI) of 1:25. In order for the bacteria to reach the cells at the bottom of the well, the plate containing the cells and bacteria was centrifuged at 300 G for 10 min. Before placing them in an incubator at 37 °C. After 2 h of challenge, the cells were washed with PBS, harvested by trypsinization and scraping, centrifuged at 1000 g for 5 min and resuspended in PBS. The cell associated bacteria was quantified using a BD LSR II flow cytometer (BD Biosciences, San Diego, CA). All experiments were performed using triplicate samples and at least 10,000 cells were analyzed per treatment.