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Boron Nitride Nanotubes and Nanoplatelets as Reinforcing Agents of Polymeric Matrices for Bone Tissue Engineering

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Abstract

This study investigates the mechanical properties and *in vitro* cytotoxicity of one- and two-dimensional boron nitride nanomaterials-reinforced biodegradable polymeric nanocomposites. Poly(propylene fumarate) (PPF) nanocomposites were fabricated using crosslinking agent N-vinyl pyrrolidone (NVP) and inorganic nanomaterials: boron nitride nanotubes (BNNTs) and boron nitride nanoplatelets (BNNPs) dispersed at 0.2 wt.% in the polymeric matrix. The incorporation of BNNPs and BNNTs resulted in a ~38% and ~15% increase in compressive (young's) modulus, and ~31% and ~6% increase in compressive yield strength compared to PPF control, respectively. The nanocomposites showed a time-dependent increased protein adsorption for only collagen-I protein. The cytotoxicity evaluation of aqueous BNNT and BNNP dispersions (at 1-100 µg/mL concentrations) using a representative murine MC3T3 preosteoblast cell line showed cytocompatibility of BNNTs and BNNPs (~73-99% viability). The cytotoxicity evaluation of media extracts of nanocomposites prior to crosslinking, after crosslinking and upon degradation (using 1X-100X dilutions) showed dose-dependent cytotoxicity responses. Crosslinked nanocomposites showed excellent (~79-100%) cell viability, cellular attachment (~57-67%), and spreading similar to cells grown on the surface of tissue culture polystyrene (TCPS) control. The media extracts of degradation products showed a dose-dependent cytotoxicity. The favorable cytocompatibility results in combination with improved mechanical properties of BNNT and BNNP nanocomposites opens new avenues for further *in vitro* and *in vivo* safety and efficacy studies for their bone tissue engineering applications.

Keywords

boron nitride; inorganic nanomaterials; biodegradable polymer; cytotoxicity; mechanical properties; bone tissue engineering

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1. Introduction

Tissue engineering of critical sized bone defects requires the development of 3D porous scaffolds with mechanical properties similar to native bone tissue. However, porous polymeric scaffolds generally possess low mechanical properties and are unsuitable for tissue engineering of load-bearing bones. Reinforcement of polymeric scaffolds with nanomaterials is a general strategy to improve their mechanical properties. Recently, in the field of tissue engineering, carbon nanomaterials such as fullerenes, carbon nanotubes (CNTs), and graphene have been employed as building blocks to fabricate three-dimensional (3-D) porous scaffolds [1, 2]. Over the last decade, pristine and functionalized formulations of these carbon nanomaterials [3-6] as well as inorganic nanomaterials such as alumoxane nanoparticles [7, 8], tungsten disulfide nanotubes [5] and molybdenum disulfide nanoplatelets [4, 9] have been investigated as reinforcing agents to improve the mechanical (compressive) properties of various biodegradable and biocompatible polymeric matrices for load bearing bone tissue engineering applications.

Various one- and two-dimensional (1-D and 2-D) carbon and inorganic nanomaterials such as carbon nanotubes, graphene, tungsten disulfide nanotubes and molybdenum disulfide nanoplatelets have been used for as therapeutic drug delivery, bioimaging, and stem cell tracking and as reinforcing agents to improve the mechanical properties of polymeric scaffolds [10-15]. Missing from the above list of reinforcing agents for biodegradable polymers for load bearing bone tissue engineering applications are boron nitrides nanoparticles, which have similar structural and in some cases superior physiochemical properties to carbon nanomaterials [16-18]. It has been reported that boron nitride nanotubes (BNNTs) and boron nitride nanoplatelets (BNNPs, exfoliated bulk boron nitride - less than ten atomic layers) exhibit excellent mechanical properties [18-20]. The mechanical properties of their nanocomposites can be improved various functional groups such as amines, nitriles, epoxides and oxides [21-23] with improvement in their dispersion in polymer matrices that facilitate better polymer-nanomaterial interaction and allow efficient load transfer from the polymer to the nanomaterial [19]. Additionally, it has been reported that presence of boron nitride nanomaterials (specifically exfoliated boron nitride nanosheets [24]) improves the thermal conductivity of polymers therefore may be beneficial for *in situ* thermal crosslinking of injectable polymer mixtures.

Most of the studies carried out so far have explored BNNTs as reinforcing agents to improve tensile mechanical properties of non-polymers (hydroxyapatite [25], alumina [26] and aluminum [27]) and a limited number of polymers (polylactide-polycaprolactone [28] and epoxy [29]). Boron nitride nanotubes have been shown to be cytocompatible [30] and bioactive [31], however their application as a part of a nanocomposite bone graft has not been thoroughly investigated. Herein we have investigated the efficacy of BNNTs and BNNPs as reinforcing agents to improve the compressive mechanical properties of biocompatible, and biodegradable PPF polymer; widely investigated for load bearing bone tissue engineering applications [32-34]. Additionally, along with efficacy studies, *in vitro* cytotoxicity and *in vivo* biocompatibility of nanomaterials-incorporated polymers also need to be thoroughly investigated. As a first step, we have thoroughly examined the *in vitro* cytocompatibility of BNNT and BNNP nanocomposites before and after polymer

crosslinking, and upon polymer degradation. Additionally, we have characterized cell attachment and spreading, and protein adsorption on BNNT and BNNP nanocomposites.

2. Materials and methods

2.1. Polymer

PPF was synthesized in accordance with a previously reported protocol and characterized using an Oxford (1H NMR, 500Hz) proton nuclear magnetic resonance spectroscope (Oxford, UK). Briefly, a two-step trans-esterification of polypropylene glycol and di-ethyl fumarate was used to synthesize PPF polymer. Purification of PPF (3 batches) and eliminating the chains with lower molecular was performed through washing with brine solution, and ether and dissolving/solvent removal using methylene chloride. Figure S-1 displays the NMR spectrum of the synthesized PPF polymer (supplementary information) with NMR peaks consistent with the literature [35].

2.2. Nanomaterials and their characterization

BNNTs (~100 nm diameter and 1-2 μm length with traces of Si, Cr and Fe according to the energy dispersive x-ray (EDX) spectroscopy) of and BNNPs (diameter ~ 200 – 1800 nm, specific surface area ~ 35 m^2/g , boron oxide content 0% and B/N ratio of 0.99) were purchased from Daikin University (Daikin, Victoria, Australia) and PHmatter (Columbus, OH, USA), respectively. As-received nanomaterials were characterized using Raman spectroscopy and transmission electron microscopy (TEM). Nanomaterials were used for nanocomposite fabrication without any further processing.

2.2.1. Raman spectroscopy of nanomaterials—A ProRaman-L spectroscope (TSI, Shoreview, MN, USA) was used to acquire Raman spectra of BNNTs and BNNPs in 100-3000 cm^{-1} wavenumber range. The nanomaterials were dispersed in a 50:50 aqueous mixture of ethanol in distilled water, bath sonicated for 15 minutes (FS30H, Fisher Scientific, Madison, CT, USA), and probe sonicated for 2 minutes (2 sec ‘on’, 1 sec ‘off’ cycle, LX750, Cole-Parmer, Vernon Hills, IL, USA) in microcentrifuge tubes (Eppendorf AG, Schönenbuch, Switzerland). Next, the tubes were subjected to centrifugation at 10,000 rpm for 5 minutes and 20 μL of supernatant was drop casted onto freshly cleaved silicon wafers (Ted Pella, Redding, CA, USA), air-dried, and used for Raman spectroscopy.

2.2.2. Transmission electron microscopy of nanomaterials—Transmission electron microscopy (TEM) was used for morphological characterization of nanomaterials as described previously [36]. Briefly, the nanomaterial dispersions prepared for Raman spectroscopy were drop casted on TEM grids (mesh size: 300, holey lacey carbon grid, Ted Pella, Redding, CA, USA). The sample coated TEM grids were air-dried, vacuum dried (overnight), and used for TEM. Imaging was carried out using a TECNAI BioTwin G² TEM (FEI Technologies, Hillsboro, OR, USA) at an accelerating voltage of 80 kV.

2.3. Fabrication nanocomposites

PPF nanocomposites with 0.2 wt. % loading concentration of BNNTs and BNNPs were prepared as previously reported [4, 5]. The nanomaterials dispersed in chloroform were

subjected to bath sonication for 30 minutes at 1 W/cm² power output (FS30H sonicator, Fisher Scientific) followed by probe sonication for 2 minutes using a 2 sec 'on' and 1 sec 'off' cycle (LPX-750, 20 kHz sonicator, Cole Parmer, Vernon Hills, IL, USA) at a 20% intensity (approximately 360 W/cm²). The nanomaterial dispersions were then added to a 50:50 mixture of polypropylene fumarate (PPF) polymer and N-vinyl pyrrolidone (NVP) crosslinker, and the polymer-nanomaterial mixture was subjected to bath sonication for 15 minutes. Next, chloroform was removed using a rotavapor (R-215, Büchi, New Castle, DE, USA) and thermal cross-linking of nanocomposites was initiated by addition of 1 wt. % benzoyl peroxide (BP, free-radical initiator, Sigma Aldrich, St. Louis, MO, USA). Finally, the nanocomposite mixture was poured into custom machined Teflon molds (McMaster, Princeton, NJ, USA) and cured overnight at 60°C to fabricate 10 cylindrical specimens (n=10) with a diameter of 6.5 mm and height of 16 mm. For *in vitro* studies, the specimens were cut into discs of 0.5 mm thickness using a low-speed diamond saw (Model 650, South Bay Technology, Redondo Beach, CA, USA). Three batches of PPF nanocomposites were prepared.

2.4. Transmission electron microscopy characterization of nanocomposites

The dispersivity of nanomaterials in the polymer matrix was analyzed by transmission electron microscopy (TEM). A batch of four nanocomposite specimens (n=4) were cut into 500 nm thick slices using an ultra-microtome and mounted on Copper sample grids specific for TEM (mesh size: 400, Ted Pella, Redding, CA, USA). Imaging was performed using a TECNAI BioTwin G² TEM (FEI Technologies, Hillsboro, OR, USA) at an accelerating voltage of 80 kV.

2.5. Compressive mechanical testing

Compressive mechanical testing of the crosslinked nanocomposite cylinders was carried out using an uniaxial mechanical testing system (Instron 4010, Norwood, MA, USA) according to American Society of Testing Materials (ASTM) standard D695-08 [37]. The cylindrical specimens were compressed along their longitudinal axis at 0.1 mm/min strain rate with a 1 KN load cell. The force-displacement data was recorded and converted to stress-strain curve based on the length and cross section area of each specimen. The compressive modulus was determined as the slope of the initial linear portion of stress-strain curve whereas the compressive yield strength was determined as the maximum recorded stress for each specimen before plastic deformation. The yield strength was calculated using an offset method by drawing a parallel line from 0.2 % strain (with respect to linear region) and considering the intersection with stress-strain curve. A batch of three specimens (n=3) of each experimental group was used for the mechanical testing.

2.6. Sol fraction analysis

Sol fraction analysis was used to determine the crosslinking density of nanocomposites in the presence of nanomaterials. The crosslinked nanocomposite samples were crushed and approximately 2.5 g of each sample was placed inside a sealed vial containing 20 mL of Methylene chloride. The vials were then kept on a shaker plate at 100 rpm for 14 days at room temperature. After 14 days, nanocomposite samples were harvested using filtration

(Whatman® No.40, Madison, CT, USA) and their weights were recorded (accuracy 0.0001 g). The sol fraction was calculated using the following equation:

$$\text{Crosslinking density (\%)} = \frac{W_{f+p} - W_p}{W_i} * 100$$

Where W_i is the initial weight of nanocomposite, W_{f+p} is the weight of filter paper and recovered nanocomposite specimen after 14 days, and W_p is the weight of filter paper. PPF polymer served as a baseline control. A sample size of three ($n=3$) was used for each experimental group in sol-fraction analysis.

2.7. Protein adsorption

A well-established solution depletion method was used to characterize protein adsorption on the surface of crosslinked nanocomposites using Bicinchoninic acid (BCA) assay [38]. The bicinchoninic acid present in BCA assay chelates with Cu^+ ions that form due to the reduction of Cu^{2+} ions from cupric sulfate reagent. The reduction of Cu^{2+} ions is directly proportional to protein concentration in the solution. The formation of Cu^+ ions results in a color change of the solution from green to purple which can be quantified using a plate reader [39]. Solutions of bovine collagen-I, human fibronectin and mouse fibrin (Sigma Aldrich, St. Louis, MO, USA) were prepared (each at concentration of 400 $\mu\text{g/dL}$). Briefly, collagen-I was dissolved in 1 mL acetic acid (Fisher Scientific, Madison, CT, USA) and fibrin was dissolved in a 0.01 N sodium hydroxide solution (Fisher Scientific, Madison, CT, USA). Fibronectin was soluble in phosphate buffer saline solutions (DPBS) and thus did not require any additional dissolving steps. The dissolved collagen-I, fibrin and fibronectin were then added to DPBS to prepare experimental protein solutions. A batch of six ($n=6$) discs from each experimental were placed inside 24-well plates and incubated at 37°C with protein solutions for 1, 5 and 9 days. After each time point, 15 μL of supernatant was transferred to a fresh 96-well plate for quantification of protein levels using BCA assay (Thermo Scientific, Madison, CT, USA). For BCA assay, 200 μL of working solution was added to each well, incubated for 30 minutes, and their absorbance at 562 nm was recorded using a Spectramax-M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was prepared according to the manufacturer's protocol using Bovine Serum Albumin (BSA) standards. The fraction of adsorbed proteins on the crosslinked samples was calculated using the following equation:

$$\text{Fraction of adsorbed protein (\%)} = \left(\frac{C_i - C_s}{C_i} \right) * 100$$

Where C_i is the initial concentration of protein in the solution and C_s is the concentration of protein in the solution after incubation. For protein adsorption studies, regular polystyrene (PS) 24-well plates (not modified using plasma treatment) were used. Wells containing protein solutions served as positive controls, wells containing DPBS served as negative controls, and PPF polymer served as a baseline control. Number of six wells ($n=6$) were tested for each of the nanocomposite and PPF discs.

2.8. Cell culture

MC3T3 pre-osteoblasts were used for *in vitro* cytotoxicity studies. MC3T3 cells (passages 10-14) were suspended in Minimum Essential Medium-Alpha (MEM- α , Gibco, Grand Island, NY, USA) supplemented with 10 vol. % Fetal Bovine Serum (FBS, Gibco, Grand Island, NY, USA) and 1 vol. % antibiotics (Penicillin-Streptomycin, Gibco, Grand Island, NY, USA) and seeded inside a 10 cm diameter tissue culture polystyrene (TCPS) petri-dish (Sarsdedt®, Newton, NC, USA). The cells were incubated in a humidified atmosphere at 37°C with 5% carbon dioxide (95% air). For cytotoxicity studies, MC3T3 cells were washed using Dulbecco's Phosphate Buffer Saline (DPBS, Gibco, Grand Island, NY, USA), trypsinized using 0.05% Trypsin-EDTA (Gibco, Grand Island, NY), and seeded in 96-well plates (BD Falcon, Franklin Lakes, NJ, USA) at a density of 5000 cells/well. The cells were incubated for 24 hours before commencement of the assays. For cell attachment study, MC3T3 cells were seeded at a density of 50,000 cells/well in 6-well plates (Corning Inc., Y, USA) for 24 hours.

2.9. *In vitro* assays

2.9.1. Presto Blue® assay—Cell viability was determined using a resazurin-based Presto Blue® assay (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. The enzymatic reduction of cell permeable resazurin dye into highly fluorescent pink resorufin dye by the cytoplasmic environment of living cells is quantified as a measure of cell viability by Presto Blue® assay [40]. The experiments were performed as follows: number of 6 wells (n=6) after seeding cells were first incubated with the experimental media for 24 hours. Next, 10 μ L of Presto Blue® working solution was added into each well of 96-well plates. After 2 hours of incubation in the dark, fluorescence spectra of plates were recorded using a Spectramax-M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 560 and 590 nm, respectively. Cell numbers were determined using a standard curve. Wells containing viable cells served as live (positive) controls whereas blank wells containing only media (without cells) served to correct the background effect. Six wells (n=6) were tested for each experimental group. The fraction of live cells for each experimental group was normalized to live control calculated using the following equation:

$$\text{Viability } (\%) = \frac{F_s - F_b}{F_c - F_b} * 100$$

Where F_s is fluorescence of tested specimen, F_b is the background fluorescence (for the blank media) and F_c is the fluorescence of live (positive) control.

2.9.2. Lactate dehydrogenase assay—The membrane integrity of cells was determined using Lactate Dehydrogenase assay (LDH-TOX7; Sigma Aldrich, St. Louis, MO, USA) according the manufacturer's protocol. This assay measures the cytotoxicity for the cells by quantifying the amount of intracellular LDH enzyme released in the media by apoptotic or necrotic cells [41, 42]. The assay was performed as follows: Number of six wells (n=6) were treated with each experimental media inside 96-well plates and after 24 hours of exposure of the cells to experimental solutions, plates were subjected to

centrifugation at 4000 rpm to remove cellular debris. 50 µL of supernatant was transferred to a new 96-well plate and mixed with 100 µL of LDH working solution. After 45 minutes of incubation in dark, absorbance of each well was recorded using a Spectramax-M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) at an absorbance wavelength of 492 nm. To compare cytotoxicity among experimental groups, LDH release was calculated using a standard curve and normalized to the LDH release for six wells (n=6) each containing 5000 lysed MC3T3 cells. Wells containing lysed cells (after exposure to 20 µL of lysis buffer) served as a positive (dead) control, six wells (n=6) containing 5000 viable cells served as a negative (live) control and wells containing only cell culture media (without cells) served to correct the background effect. The released LDH enzyme for each experimental group was normalized to dead control and calculated using the following equation:

$$\text{LDH release (\%)} = \frac{A_s - A_b}{A_c - A_b} * 100$$

Where A_s is the absorbance of experimental sample, A_b is the back ground absorbance (for the blank media) and A_c is the absorbance of positive (lysed) control [42]. Six wells (n=6) were tested for each experimental group.

2.9.3. Calcein-AM staining—In order to stain viable cells for fluorescence microscopy, calcein-acetoxymethyl ester (calcein-AM) staining that has been widely used to selectively stain metabolically active living cells was performed [6, 43]. 5 µL of calcein-AM stock solution (40mM, Sigma Aldrich, St. Louis, MO, USA) was mixed with 10 mL DBPS to prepare a working concentration of 4 µM. 1 mL of calcein-AM working solution. Then, the working solution was added to each well containing nanocomposite discs after MC3T3 cell culture for 24-h) and incubated at 37°C for 25 minutes. Samples were rinsed with DPBS and placed in 35-mm glass bottom petri dishes (Mattek Corp., Ashland, MA, USA) for confocal fluorescence microscopy at 485 excitation wavelength and 530 nm emission wavelengths. TCPS wells with same surface area (compared to nanocomposite samples), after seeding at 5000 cells/well and 24-h incubation, served as positive (live) controls. PPF discs after seeding at cell density of 5000 cells/well and 24-h incubation were treated with lysis solution and served as negative controls. Two samples (n=2) were stained for each experimental group.

2.10. *In vitro* cytocompatibility studies

2.10.1. Cytocompatibility of nanomaterials—To evaluate the cytotoxicity of nanomaterials, a direct extraction method according to The International Organization for Standardization (ISO) standard 10993-5 was used as described previously [6, 44]. BNNTs and BNNPs were dispersed in MEM-α media and subjected to bath sonication for 30 minutes (FS30H, Fisher Scientific, Madison, CT, USA). Stock solution of nanomaterial dispersions at 100 µg/mL concentration were prepared and further diluted using blank media to prepare 10 µg/mL and 1 µg/mL dilutions. Next, the media with 100, 10 and 1 µg/mL nanomaterial concentrations was added to six wells (n=6) wells of containing MC3T3 cells at a density of 5000 cells/well inside a 96-well plate. Finally, after incubation for 24 hours at 37°C, cell viability was evaluated using Presto Blue® and cytotoxicity of nanomaterials for

MC3T3 cells was quantified using LDH assay. Six readings (n=6) were carried out for each experimental group.

2.10.2. Cytocompatibility of components prior to crosslinking—To evaluate the cytotoxicity of nanocomposite components before crosslinking, blends of PPF/NVP, PPF/NVP/BNNTs, and PPF/NVP/BNNPs were prepared by dispersing nanomaterials at 0.2 wt. % concentration in non-crosslinked PPF and NVP (50:50) blend. After UV-sterilization, cytotoxicity evaluation was performed according to the extract dilution testing method [44]. The blends were incubated with MEM- α media for 24 hours (0.33 mL media per cm² contact area). After incubation, the supernatant was extracted and diluted 10 and 100 folds to prepare 1X, 10X, and 100X experimental media. MC3T3 cells inside 6 wells (n=6) were exposed to 1X, 10X and 100X experimental media for 24 hours. Cell viability was evaluated using Presto Blue® whereas cytotoxicity of non-crosslinked nanocomposites for MC3T3 cells was quantified using LDH assay. Six readings (n=6) were performed for each experimental group.

2.10.3. Cytocompatibility of crosslinked nanocomposites—Cytotoxicity of crosslinked nanocomposites was assessed using the extract dilution method as previously described [44]. Number of 6 (n=6) crosslinked nanocomposite disc specimens (6 mm diameter, 0.5 mm thickness) were UV-sterilized for 3 hours, washed with DPBS, and incubated with MEM- α media for 24 hours (0.33 mL per cm² contact area). MC3T3 cells were treated with extracted media (1X) or their 10X and 100X dilutions for 24 hours. Cell viability was evaluated using Presto Blue® and cytotoxicity of crosslinked nanocomposites was quantified using LDH assay. Six readings (n=6) were performed for each experimental group and PPF served as a baseline control.

2.10.4. Cytocompatibility of nanocomposite degradation products—Crosslinked nanocomposite specimens were treated with calcium hydroxide (Ca(OH)₂, Fisher Scientific, Madison, CT, USA) and phosphoric acid (H₃PO₄, Fisher Scientific, Madison, CT, USA) to accelerate the hydrolytic degradation of PPF [6]. The following protocol was followed. Number of 3 nanocomposite cylinders from each experimental group (total weight of 2.5 g) were crushed and added into a 20 mL glass vial containing 1N Ca(OH)₂ and placed on a shaker table at 100 rpm for 14 days. The degradation products were then neutralized (pH ~7.4) using H₃PO₄ and filtered. Due to the absence of ingredients and supplements for cell survival in the degradation product solution, the neutralized and filtered degradation products were mixed with cell culture media at 1:1 ratio to prepare 2X experimental media. 10X and 100X experimental media were prepared by 10 and 100 fold dilutions of degradation products using cell culture media. Number of six wells (n=6) containing MC3T3 cells were incubated with 2X, 10X, and 100X experimental solutions for 24 hours. The viability of the cells was evaluated using Presto Blue® assay and cytotoxicity of degradation products for MC3T3 cells was quantified using LDH assay. Degradation products of crosslinked PPF polymer were used as a baseline control. Six readings (n=6) were carried out for each experimental group.

2.11. Osmolarity of degradation products

The osmolarity of 2X, 10X and 100X experimental solutions was measured using a 3D3 osmometer (Advanced Instruments Inc., Norwood, MA, USA) for degradation of n=3 samples (total weight of 2.5 g). Extracts of PPF degradation were used as a baseline control. The osmolarity of MEM- α media, filtered 400 $\mu\text{g/ml}$ $\text{Ca}_3(\text{PO})_4$ suspension was also recorded for comparison purposes. Number of six (n=6) recordings were performed for each experimental group.

2.12. Cell attachment and spreading on crosslinked nanocomposites

2.12.1. Cell attachment—UV-sterilized crosslinked nanocomposite discs (n=3, three batches of PPF nanocomposites) were placed inside 6-well plates and autoclaved stainless steel rings were placed on top of each. Then MC3T3 cells were seeded at a density of 20,000 cell/specimen inside the steel rings gradually over a span of 30 minutes. After another 30 minutes of incubation, 100 μL MEM- α media was added inside each ring and plates were incubated for another 90 minutes. Next, the rings were removed and 1.8 mL MEM- α media was added to each well. After 24-h incubation, cells were trypsinized and counted using a hemocytometer (Fisher Scientific, Madison, CT, USA). Cell attachment (fraction of attached cells) was calculated for all of 3 experimental groups using the following equation by counting six times (n=6) for each disc:

$$\text{Cell attachment (\%)} = \left(\frac{N_s}{N_i} \right) * 100$$

Where N_s is the number of cells after incubation and N_i is the number of cells used for seeding. Fraction of cells attached to bottom of three wells (n=3) with the same surface area were measured for comparison and served as cross-control.

2.12.2. Cell spreading characterized by confocal fluorescence microscopy—MC3T3 cells were seeded onto nanocomposite disc specimens at a cell density of 5×10^4 cells/specimen for 5 days. After 5 days, nanocomposite discs were washed with DPBS, incubated with calcein-AM (4 μM) for 25 minutes (for selective staining of viable cells), and placed in a glass-bottom petri-dish (Mattek Corporation, Ashland, MA, USA) for fluorescence imaging using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Thornwood, NY, USA) equipped with a 10X objective lens at excitation and emission wavelengths of 488 and 515 nm, respectively. TCPS samples with same surface area after seeding and incubation for 24 hours were used as positive controls, and cells cultured on PPF that were treated for 15 minutes with lysis solution served as negative controls. PPF composites were used as a baseline control. Two samples (n=2) from three different PPF nanocomposite batches were imaged for each experimental group.

2.12.3. Cell attachment and spreading characterized by Scanning electron microscope—Cellular attachment and the spreading of MC3T3 cells on nanocomposite surfaces was also characterized by scanning electron microscopy (SEM). The samples for SEM analysis were prepared as follows. Cells on nanocomposite discs used for confocal microscopy were fixed using a 2.5 % glutaraldehyde (Electron Microscopy Sciences Inc.,

Hatfield, PA, USA), washed twice using DBPS, dehydrated in gradient series of ethanol solutions (70%, 80%, 90% and 100% ethanol), and vacuum dried overnight. The discs were sputter coated using silver (Ag, 3 nm coating) and imaged at 5 kV using a high resolution 7600F HRSEM (JEOL, Peabody, MA, USA) at the Center for Functional Nanomaterials (CFN) in Brookhaven National Laboratory (Upton, NY, USA).

2.13. Statistical analysis

Statistical analysis was performed using a single-factor analysis of variance (one-way ANOVA) followed by Tukey-Kramer post-hoc test to identify significant differences between experimental groups using a 95% confidence interval ($p < 0.5$). All results are reported as mean $\pm$ standard deviation.

3. Results

3.1. Characterization of nanomaterials

Figure 1 displays representative TEM images of BNNTs and BNNPs. BNNTs (Figure 1a) showed characteristic tubular morphology with ~ 100 nm diameter and 1-2 μm length. BNNPs (Figure 1b) were stacks of polygonal-shaped platelets with smooth planar structure. BNNPs had diameters in 200-1800 nm range. The Raman spectra of BNNTs and BNNPs are presented in Figure 1(c). BNNTs samples showed peaks at 474 cm^{-1} , 1366 cm^{-1} and 2438 cm^{-1} . BNNPs samples showed peaks at 1370 cm^{-1} and 2444 cm^{-1} .

3.2. Compressive mechanical properties of nanocomposites

Figures 2(a) and 2(b) display the compressive modulus and compressive yield strength of BNNT and BNNP nanocomposites (nanomaterial loading concentration = 0.2 wt. %). The addition of BNNTs and BNNPs to PPF polymer resulted in $\sim 15\%$ and $\sim 38\%$ increase in compressive modulus, respectively, compared to PPF baseline control (Figure 2(a) and Table 1). Furthermore, up to 6% and 31% increase in compressive yield strength were observed for BNNT and BNNP nanocomposites, respectively (Figure 2(b) and Table 1). While both BNNP and BNNT nanocomposites showed increased compressive mechanical modulus and compressive mechanical strength compared to PPF baseline control, the increase was statistically significant only for compressive modulus in BNNP nanocomposites (Figure 2a).

3.3. Sol fraction analysis

Figure 2(c) shows the crosslinking density of nanocomposites and PPF control. The crosslinking density was determined from sol-fraction analysis which is based on the rationale that uncrosslinked PPF and NVP are soluble in methylene chloride and crosslinked polymer is insoluble [45]. The crosslinking density of PPF composites, BNNT nanocomposites, and BNNP nanocomposites were $\sim 85\%$, $\sim 90\%$ and $\sim 86\%$ crosslinking density, respectively. No statistically significant difference in the crosslinking density was observed between the three groups.

3.4 Transmission electron microscopy (TEM) of nanocomposites

TEM was used to study the BNNTs and BNNPs interface with PPF polymer in the crosslinked nanocomposites (n=20 images). Figure 2(d) displays representative TEM images of crosslinked BNNT and BNNP nanocomposites. BNNTs and BNNPs appear to have a defect-free interface and embedded in the polymer matrix; present as individual nanoparticles or aggregates of a few nanoparticles. BNNTs are tubular nanomaterials with ~100 nm diameter and 1-2 μ m length. BNNPs are nanoplatelets with 200-1800 nm diameter. TEM images of crosslinked nanocomposites (Figure 2d) clearly show the presence of BNNTs and BNNPs embedded in the PPF matrix. As shown in Figure 2(d), BNNTs were present as aggregates of a few nanotubes (the aggregate diameter is ~250 nm which corresponds to ~2-3 nanotubes) and BNNPs were present as individual or aggregates of few nanoplatelets (diameter of embedded BNNPs ~100nm-0.5 μ m).

3.5 Protein adsorption on crosslinked nanocomposites

Fig 5a-c show the adsorption of collagen-I, fibrin, and fibronectin after 1, 5, and 9 days of incubation with BNNT, BNNP and PPF discs quantified by bicinchoninic acid assay (BCA). The adsorption of collagen-I on the nanocomposites was similar or higher compared to the PPF baseline control in a time-dependent manner (Figure 3a), specifically after 9 days of incubation BNNT and BNNP nanocomposites showed $41\pm5\%$ and $18\pm9\%$ collagen-I adsorption, respectively; significantly higher than PPF control which showed $5\pm3\%$ collagen-I adsorption. The adsorption of fibrin and fibronectin on the nanocomposites were similar or lower compared to PPF adsorption at all-time points (Figure 3b and 3c). BNNT, BNNP and PPF discs showed $47\pm1\%$, $49\pm1\%$, and $52\pm2\%$ fibrin adsorption, respectively, after incubation for 5 days. After 9 days of incubation, BNNT and BNNP nanocomposites showed $39\pm1\%$ and $45\pm1\%$ fibrin adsorption, respectively; significantly lower than $50\pm2\%$ adsorption observed for PPF control. BNNT and BNNP and PPF samples showed $6\pm1\%$, $5\pm1\%$ and $7\pm2\%$ fibronectin adsorption, respectively, after incubation for 5 days. BNNT and BNNP nanocomposites and PPF composites showed $5\pm1\%$, $4\pm2\%$ and $6\pm2\%$ fibronectin adsorption, after incubation for 9 days, respectively. There were no significant differences in the measured fibronectin adsorption across all time points.

3.6 *In vitro* cytocompatibility study of nanocomposites

3.6.1 Cytocompatibility of BNNT and BNNP nanomaterials—Figure 4 displays the viability (assessed by Presto Blue® assay) of MC3T3 cells and their LDH release after exposure to nanomaterials dispersions, non-crosslinked nanocomposites, crosslinked nanocomposites and degraded nanocomposites. Figure 4(a) shows the viability of cells upon exposure to BNNT and BNNP dispersions. After incubation with 100 μ g/mL nanomaterial concentration for 24 hours, MC3T3 cells showed $99\pm13\%$ and $83\pm16\%$ viability for BNNTs and BNNPs, respectively (Figure 4a). Cells incubated with 10 μ g/mL nanomaterial concentration for 24 hours showed $77\pm14\%$ and $90\pm9\%$ viability for BNNTs and BNNPs, respectively. Finally, cells incubated with 1 μ g/mL nanomaterial concentration for 24 hours showed ~100% cell viability for both the nanomaterials.

Figure 4(b) shows the cytotoxicity inferred from LDH release (normalized to positive control), after exposure to BNNT and BNNP nanomaterials at 1-100 μ g/mL concentrations.

After 24 hours incubation with 100 µg/mL concentrations of BNNTs and BNNPs, the cells secreted 21±4% and 27±8% LDH, respectively. LDH release reduced upon incubation at lower nanoparticle concentrations. Cells incubated with 10 µg/mL dispersions of BNNTs and BNNPs showed 27±2% and 16±8% LDH release, respectively. Finally, the cells incubated with 1 µg/mL dispersions of BNNTs and BNNPs showed 19±4 and 28±5% LDH release, respectively.

3.6.2 Cytocompatibility of components prior to crosslinking—Figure 4(c) shows the viability of MC3T3 cells assessed by Presto Blue® assay after exposure to different concentrations of unreacted components extracted from nanocomposite blends. MC3T3 cells showed 4±6%, 6±7% and 3±4% viability after 24 hours of incubation with 1X experimental media of NVP/PPF, NVP/PPF/ BNNTs and NVP/PPF/BNNPs blends, respectively. Cell viability increased with further dilutions; 10X experimental media showed 46±5%, 48±6% and 42±3% viability for NVP/PPF, NVP/PPF/BNNTs and NVP/PPF/BNNPs, respectively. Finally, MC3T3 cells incubated with 100X extracted media showed ~100% viability for all three experimental groups.

Figure 4(d) displays the percentage of LDH release (normalized to positive control) upon exposure to unreacted components of BNNT and BNNP nanocomposite blends. After 24 hours incubation, 1X extracts of unreacted NVP/PPF, NVP/PPF/BNNTs and NVP/PPF/BNNPs blends showed 92±6%, 93±2% and 91±2% LDH release, respectively. Incubation with 10X experimental media showed 51±5%, 52±2% and 50±1% LDH release for NVP/PPF, NVP/PPF/BNNTs and NVP/PPF/BNNPs, respectively. Finally, 100X experimental media showed 24±2%, 25±1% and 23±1% LDH release for NVP/PPF, NVP/PPF/BNNTs and NVP/PPF/BNNPs.

3.6.3 Cytocompatibility of crosslinked nanocomposites—Figure 4(e) displays the results of Presto Blue® assay for viability of MC3T3 cells after 24-h exposure to extracts of crosslinked nanocomposites. Cells treated with 1X extracts of crosslinked BNNT and BNNP nanocomposites after 24 hours incubation showed 84±6% and 81±11% viability, respectively. Cells exposed to 1X extract of PPF controls showed 93±2% cell viability. Cells treated with 10X dilutions of extracts from crosslinked samples after 24 hours incubation showed 90±4%, 90±2% and 83±1% viability for BNNT, BNNP, and PPF samples, respectively. Cells treated with 100X dilutions of crosslinked nanocomposites or PPF control after 24 hours incubation showed ~92-96% viability for all three experimental groups. Overall, the results indicated that 80% MC3T3 cells were viable at each treatment concentration.

Figure 4(f) shows LDH release by MC3T3 detected using LDH assay after 24-h exposure to the extracts of crosslinked nanocomposites. 1X extracts of crosslinked BNNT and BNNP nanocomposites showed 10±6% and 21±6% LDH release, respectively. PPF controls showed 15±6% LDH release. For 10X dilutions, 12±8%, 22±10% and 17±5% LDH release was measured from extracts of crosslinked BNNT, BNNP and PPF samples, respectively. 100X dilutions of BNNT, BNNP and PPF experimental groups showed 18±5%, 25±4% and 24±6% LDH release, respectively.

3.6.4 Cytocompatibility of degradation products—Figure 4(g) shows the viability of cells assessed by Presto Blue® assay upon exposure to degradation products of BNNT, BNNP and PPF samples. After 24 hours incubation with 2X extracts of degraded nanocomposites, MC3T3 cells showed $40\pm16\%$, $30\pm3\%$ and $41\pm2\%$ viability for BNNT, BNNP and PPF samples, respectively. Incubation with 10X experimental media showed up to $80\pm5\%$, $93\pm8\%$ and $81\pm11\%$ for BNNT, BNNP and PPF samples, respectively. Finally, incubation with 100X extracts of degradation products, showed $\sim 100\%$ cell viability for all experimental groups.

Figure (4)h displays the normalized LDH release by MC3T3 cells after exposure to the extracts of nanocomposite degradation products. After 24 hours of exposure to 2X degradation extracts, MC3T3 cells showed $82\pm18\%$ and $82\pm14\%$ LDH release for BNNT and BNNP nanocomposites, respectively. PPF controls showed $78\pm19\%$ LDH release. For 10X dilutions, $30\pm3\%$, $10\pm10\%$ and $30\pm4\%$ LDH release was measured for BNNT, BNNP and PPF samples, respectively. 100X dilutions showed $13\pm4\%$, $27\pm5\%$ and $7\pm2\%$ LDH release for BNNT, BNNP and PPF experimental groups, respectively.

3.7 Osmolarity of the nanocomposite degradation products

Figure S-2 (supplementary information) shows the osmolarity of the degradation products. The osmolarity of 2X experimental media of degraded BNNT and BNNP nanocomposites was 242 ± 4 mOsm and 210 ± 1 mOsm, respectively. The extracts of degraded BNNT nanocomposites, BNNP nanocomposites and PPF controls showed osmolarity values between 271-284 mOsm (for 10X extracts) and 298-304 mOsm (for 100X extracts), respectively. The osmolarity values of PPF control and $\text{Ca}_3(\text{PO})_4$ control solution ($400 \mu\text{g/ml}$) were ~ 230 mOsm and 110 mOsm, respectively; significantly lower than experimental groups. The osmolarity of blank MEM- α media was 304 ± 1 mOsm.

3.8 Cell attachment and spreading on crosslinked nanocomposites

Figure 5(a) shows the fraction of attached cells (counted using hemocytometer) on BNNT, BNNP, and PPF crosslinked discs, after 24 hours of incubation. BNNT, BNNP and PPF discs showed $65\pm4\%$, $60\pm3\%$ and $57\pm6\%$ cell attachment, respectively. TCPS control showed $87\pm7\%$ cell attachment. Although no significant difference in cell attachment was observed between BNNT, BNNP and PPF discs, all three groups exhibited a significantly lower cell attachment compared to TCPS control. Figure 5(b) shows representative confocal scanning laser microscopy images of MC3T3 cells evenly spread out covering the surface of all experimental groups. The cell spreading observed on nanocomposites was comparable to TCPS controls.

Figure 5(c) displays SEM images of the surface of BNNT, BNNP and PPF discs. All the groups including PPF controls were completely covered with MC3T3 cells (marked with black arrows). The deposition of extra cellular matrix (ECM; marked with white arrows) can also be observed. MC3T3 cells displayed their characteristic spindle shaped morphology with the formation of numerous cytoplasmic filopodia extensions (marked with circles).

4 Discussions

The objective of this study was to assess the compressive mechanical properties and *in vitro* cytocompatibility of BNNT and BNNP reinforced PPF nanocomposites. Prior to nanocomposite fabrication and testing their mechanical properties and cytocompatibility, the structural and chemical properties nanomaterials were characterized by TEM and Raman spectroscopy, respectively. TEM confirmed the characteristic tubular morphology of BNNTs (Figure 1a) and hexagonal structure of BNNPs (Figure 1b). In the Raman spectra (Figure 1c), the intense peak at $\sim 1370\text{ cm}^{-1}$ for both nanomaterials can be attributed to the E_{2g} vibration mode in the hexagonal structure of boron nitride (in opposite directions, parallel to x axis in x-y plane) [46, 47]. The other minor peak at $\sim 474\text{ cm}^{-1}$ could be attributed to presence of boric acid residues [46], whereas peaks at 2438 cm^{-1} and 2444 cm^{-1} (for BNNTs and BNNPs, respectively) may occur due to the presence of impurities, functional groups and/ or defects.

BNNTs and BNNPs were dispersed at 0.2 wt.% in the polymer matrix to fabricate the nanocomposites due to the following reasons: a) it was observed that nanomaterials form micron sized aggregates in PPF matrix at concentrations higher than 0.2 wt. % and b) PPF with higher nanomaterials loading concentration shows changes in the viscoelastic behavior that may induce formation of air pockets during fabrication of nanocomposites [4]. The results of compressive mechanical testing (Figure 2 and Table 1) taken together indicated that BNNPs were more efficient in reinforcing PPF polymer than BNNTs. Sitharaman et al. investigated the effects of addition of 0.01-2 wt.% fullerenes, single-walled carbon nanotubes (SWCNTs) and ultra-short carbon nanotubes (US-tubes) on the mechanical properties of PPF. Results showed significant mechanical reinforcement, $\sim 13\%$ - 100% increase in compressive modulus and $\sim 20\%$ - 60% increase in compressive yield strength was observed with US-tubes nanocomposites exhibiting maximum mechanical reinforcement [48]. Lalwani et. al. have reported the mechanical properties of PPF nanocomposites upon addition of various one- and two-dimensional carbon and inorganic nanomaterials. PPF nanocomposites were fabricated using 0.01-0.2 wt.% of carbon (graphene oxide nanoribbons and nanoplatelets, single- and multi- walled carbon nanotubes) and inorganic (tungsten disulfide nanotubes and molybdenum disulfide nanoplatelets) nanomaterials. Results showed $\sim 35\%$ - 108% increase in compressive modulus and $\sim 26\%$ - 93% increase in compressive yield strength compared to pristine PPF polymer with PPF-molybdenum disulfide nanoplatelets showing the maximum mechanical reinforcement [4, 5]. The results of these studies cannot be directly compared due to variations in the molecular weight of the polymer, nanomaterial chemistry (organic vs. inorganic; functionalized vs. pristine) and morphology (one-dimensional tubes vs. two-dimensional platelets). However, these studies corroborate a salient feature of this study that addition of nanomaterials at very low loading concentrations can significantly improve the mechanical properties of PPF nanocomposites. These studies have identified some of the key factors responsible for good mechanical reinforcement. These factors include high mechanical properties of nanomaterials, presence of functional groups on nanomaterials surface, improved dispersion of the nanomaterial in the polymer matrix that prevents stress-concentration points which facilitate localized deformation under load and attenuate the mechanical properties of the nanocomposite, high specific surface

area and low aspect ratio of the nanomaterials that improve nanomaterials/polymer interactions and reduce stress concentration [4, 5, 49, 50]. Both BNNTs and BNNPs exhibit high compressive modulus in the range of 250-1200 GPa [20, 51]. Both nanomaterials possess similar functional groups on their surface, and these functional groups did not significantly increase the crosslinking density of nanocomposites compared to PPF control (Figure 3c). BNNPs have been reported to exhibit higher specific area compared to BNNTs ($1427 \text{ m}^2\text{g}^{-1}$ for BNNPs [52] compared to $254\text{-}789 \text{ m}^2\text{g}^{-1}$ for BNNTs [53]). BNNPs also possess lower aspect ratio compared to BNNTs [54]. Thus, these two factors may play a dominant role and may be responsible for observed variation in the mechanical properties.

Adsorption of proteins on nanocomposite surface can play an important role in regulating cell viability, attachment and proliferation [55, 56]. Adsorption of collagen-I, fibrin and fibronectin proteins on crosslinked nanocomposites was specifically studied due to their role in cell adhesion and viability thereby supporting tissue regeneration. Collagen-I is a key ECM protein responsible for providing structural support to new tissue growth [57]; fibrin is responsible for formation of blood clots and regulation of inflammatory response [58] (crucial for bone healing process); fibronectin is responsible for cell adhesion via integrin binding [59]. Previous studies show that increase in surface roughness can improve protein adsorption [60]. The increased time dependent collagen-I protein adsorption on the surface of nanocomposites (Figure 4) may be attributed that increase in surface roughness due to the presence of inorganic nanomaterials. However, similar differential increase in adsorption was not noted in case of fibrin or fibronectin. Thus, surface roughness certainly is not the central causal factor and additional studies are needed to obtain insights into the factors responsible for the differential protein adsorption results. It can implied from the results that the adsorption of the three proteins does not significantly go down compared to PPF controls.

The comprehensive evaluation of *in vitro* cytocompatibility of BNNT and BNNP nanocomposites included cytotoxicity assessment of non-crosslinked nanocomposite blends, crosslinked nanocomposite specimens, and their degradation products was performed. MC3T3 pre-osteoblasts cells were used for the cytotoxicity studies as they are widely accepted for *in vitro* cytotoxicity testing of polymeric nanocomposites designed for bone tissue engineering applications [61]. For all cytocompatibility experiments, a 24-hour time point was selected because majority of materials are excluded from implantation site by blood flow post *in situ* crosslinking and biodegradation. It has been reported that cytotoxicity assays such as MTT and XTT produce insoluble formazan crystals that interact with layered nanomaterials to produce erroneous results [62]. Previous studies show that Presto Blue® and LDH assays are not affected by the presence these type of nanomaterials [3], and thus were used in this study.

Prior to crosslinking and during nanocomposite degradation *in vivo*, cells will interact with released BNNTs and BNNPs from PPF nanocomposites. Therefore, cytotoxicity of BNNT and BNNP dispersions was analyzed. The hydrophobicity of boron nitride nanoparticles results in formation of aggregates in an aqueous media [63], however, due to the use of probe-ultrasonication to obtain final homogenous dispersions no surfactants were used. Viability and cytotoxicity results using Presto Blue® and LDH assays show that BNNT and

BNNP dispersions upto 100 µg/mL concentration and 24 hour incubation time do not adversely affect the cells (Figure 4a and 4b).

A dose-dependent cytotoxicity (Figure 4c and 4d) of nanocomposite blends prior to crosslinking (1X>10X and 100X) was noted. PPF is a biocompatible polymer and BNNT/BNNP dispersions at the concentrations used to fabricate the blends do not show acute cytotoxicity (Figure 4a and 4b). Therefore, the acute cytotoxicity observed for 1X experimental solutions can be attributed to major contributions from unreacted crosslinker. A similar dose-dependent acute cytotoxicity response for non-crosslinked PPF macromers was observed by Timmer et. al. [64], Shi et. al. [6], and Farshid et. al. [3] wherein the observed cytotoxicity was primarily attributed to the leaching of crosslinking agent and radical initiator components. In an actual application, an injectable system, PPF nanocomposites will crosslink *in situ* within a few minutes and less non-reacted material will be release. The decrease in release non-reacted polymeric components and diluting role of blood flow will reduce *in vivo* exposure of cells to non-crosslinked polymeric blend would be minimal (simulated by low concentrations of experimental media). Additionally, small PPF oligomers, NVP crosslinker, BNNTs and BNNPs (components that will directly interact with cells *in vivo* post-injection) will leach out and clear from the injection site during the initial inflammatory response. The significant lower dose-dependent (1X > 10X > 100X) cytotoxicity of crosslinked PPF nanocomposites (Figure 4c and 4d) compared to non-crosslinked nanocomposite blends were similar to previous reports [3, 6, 64]. Crosslinking of PPF nanocomposites significantly slows down the leaching of toxic non-crosslinked components such as NVP crosslinker and residual BP radical initiator [3, 65].

After implantation PPF nanocomposites undergo gradual hydrolytic degradation into degradation products such as propylene glycol, and fumaric acid and release the nanomaterials [3, 6, 35] [66]. Crosslinked PPF degrades very slowly; therefore, an accelerated degradation process that leads to substantial degradation within a week is typically employed for these studies [64]. In this study, weaker degrading agents such as phosphoric acid (H₃PO₄) and calcium hydroxide (Ca(OH)₂) were used for accelerated degradation of PPF. Previous reports used a combination of HCl and Na(OH) for the acceleration degradation of crosslinked PPF nanocomposites [6]. This process forms NaCl as a by-product that increases the osmolarity (>1000 mOsm); cells in hypertonic medium shrink due to exosmosis of water resulting in damage to the cell membrane. Thus, it would be difficult to assess whether any observed cytotoxicity is due to high osmolarity or the PPF nanocomposite degradation products. H₃PO₄ and Ca(OH)₂ form insoluble calcium phosphate (Ca₃(PO₄)₂) crystals that can be easily removed. Furthermore, Ca₃(PO₄)₂ undergoes partial ionization resulting in lower osmolarity values (Figure S-2; 210-242 mOsm for 2X degradation extracts). Since BNNTs and BNNPs do not induce cytotoxicity response up to 100 µg/ml (Figure 4a and 4b), the dose-dependent (2X > 10X > 100X) cytotoxicity of degradation products cytotoxicity in (Figure 4g and 6h) can be attributed primarily due to the interaction of cells with PPF degradation products mainly composed fumaric- and acrylic- acids [67, 68]. These results are consistent with previous reports that show PPF degradation products elicit a dose-dependent response [6, 69, 70].

Attachment and spreading of MC3T3 cells on nanocomposite surfaces was analyzed by cell counting (Figure 5a), confocal fluorescence imaging (Figure 5b) and SEM (Figure 5c). The results indicated good cell viability, attachment, and spreading of MC3T3 cells on all experimental groups. Lower number of MC3T3 cells attached onto BNNT, BNNP and PPF discs compared to TCPS control after 24-hours of incubation might be a result of plasma treatment that adds negative charges on the surface of TCPS which improves cell adhesion [71]. The trend is similar to previous reports [3, 6]. We did not investigate time points after 5 days, since the cells were nearly confluent by day 5 for all the samples. We expect an insignificant difference in fraction of attached cells among BNNT, BNNP and PPF samples compared to TCPS control in later time points. Significant cell attachment, spreading, and proliferation observed on all nanocomposite surfaces after 5 days of culture (Figures 5b and 5c) also confirmed the cytocompatibility of the nanocomposites. SEM results also confirmed cell attachment and deposition of ECM components (Figure 5c) on nanocomposites. Deposition of ECM components promotes cell attachment and proliferation and reduces the interaction of cells with insoluble components such as poly(vinyl pyrrolidone) [64] and traces of non-crosslinked macromers (acting as cell attachment inhibitors [6]), leading to high density of MC3T3 cells on nanocomposite surfaces after incubation for 5 days.

To the best of our knowledge, this is the first systematic investigation of mechanical properties and *in vitro* cytocompatibility of 1-D boron nitride nanotubes (BNNTs) and 2-D boron nitride nanoplatelets (BNNPs) reinforced biodegradable polymers for load bearing bone tissue engineering applications. Our results indicate that although both nanomaterials lead to an increase in compressive mechanical properties, the application of (2-D) nanoplatelets resulted in a further reinforcement compared to (1-D) nanotubes. Interestingly, in addition to cytocompatible nanomaterials, non-crosslinked nanocomposite components, crosslinked nanocomposites and degradation products of nanocomposites all showed excellent cytocompatibility similar to PPF control. Moreover, the presence of both BNNT and BNNP nanomaterials resulted in a better protein adsorption and cell spreading on the surface of PPF nanocomposite. In comparison to conventional metallic implants, the use of BNNT and BNNP nanocomposites for bone tissue engineering has several advantages. For instance, by varying the nanomaterial content, the mechanical properties of BNNT and BNNP nanocomposites can be tailored to match the demands of bone defect site to avoid complications such as stress shielding, implant-related osteopenia and re-fractures associated with metallic implants. Furthermore, due to increased protein adsorption, BNNT and BNNP implants may lead to increased biomineralization and better host-implant integration compared to metallic implants. Furthermore, the interesting thermal conductivity of boron nitride nanomaterials can be harnessed to improve *in situ* crosslinking of polymeric nanocomposite bone grafts. Considering the above mentioned benefits and the promising results of this study, the use of 1-D and 2-D boron nitride nanomaterials for fabrication of ultra-strong and cytocompatible synthetic bone grafts may overcome several challenges associated with metallic implant based bone treatment strategies and open avenues for their *in vivo* safety and efficacy studies.

5 Conclusions

In conclusion, biodegradable polymeric nanocomposites were fabricated by dispersing low loading concentrations (0.2 wt. %) of 1-D boron nitride nanotubes (BNNTs) and 2-D boron nitride nanoplatelets (BNNP) in poly(propylene fumarate) (PPF) polymer using N-vinyl pyrrolidone (NVP) as the crosslinking agent. After crosslinking, both, BNNT and BNNP nanocomposites exhibited a significant mechanical reinforcement and a higher adsorption of collagen-I protein -the building block of extracellular matrix (ECM)- compared to PPF control. Moreover, the cytotoxicity profiles for non-crosslinked components, crosslinked nanocomposites and their degradation products were similar to PPF baseline control. Furthermore, the crosslinked nanocomposites show an excellent cell attachment, spreading and ECM deposition. The results of this study demonstrate that BNNT and BNNP reinforced biodegradable polymeric nanocomposites are cytocompatible. These findings open new avenues towards the development of high-strength, nanomaterial-reinforced, lightweight polymeric nanocomposites as bone grafts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative TEM images (a) BNNTs and (b) BNNPs. (c) Representative Raman spectra of BNNTs and BNNPs

Figure 2.

(a) Compressive modulus, (b) compressive yield strength and (c) crosslinking density of PPF nanocomposites and (d) TEM images of BNNT and BBNP nanocomposites. Error bars represent standard deviations for $n=3$ samples. The symbol “*” indicates statistically significant difference in comparison to PPF baseline control ($p < 0.05$).

Figure 3.

Adsorption of (a) collagen I, (b) fibrin and (c) fibronectin after 1, 5 and 9 days of incubation with crosslinked PPF nanocomposites at 37°C. Error bars represent standard deviations for n=6 samples. The symbol “*” indicates statistically significant difference with respect to PPF baseline control ($p < 0.05$).

Figure 4. Cell viability and total LDH release for MC3T3 cells after 24-h exposure to aqueous dispersions of nanomaterials (a, b), extracts of unreacted components (c, d), extracts of crosslinked nanocomposites (e, f) and extracts of degradation products (g, h), respectively. Data is normalized with respect to live and dead controls and error bars represent standard deviations for n=6 samples.

Figure 5.

(a) Fraction of attached MC3T3 cells on the surface of nanocomposites and TCPS control after 24-h incubation (error bars represent standard deviations for $n=3$ samples and the symbol “*” indicates significant difference with respect to TCPS control ($p < 0.05$)), (b) confocal fluorescence images of MC3T3 cells spreading on crosslinked nanocomposites after 5 days of cell culture and (c) SEM images of the nanocomposites after 5 days of cell culture (cells are marked with black arrows. Cytoplasmic extensions and filopodia are marked with black circles. ECM is marked with white arrows).

Table 1

Mechanical properties of PPF nanocomposites.

Experimental group	Compressive modulus (MPa)	Increase in compressive modulus compared to PPF (%)	Compressive yield strength (MPa)	Increase in compressive strength compared to PPF (%)
PPF	308±19	-	16±1	-
BNNT	354±38	15%	17±2	6%
BNNP	426±55	38%	21±6	31%