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Tungsten Disulfide Nanotubes Reinforced Biodegradable Polymers for Bone Tissue Engineering

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Abstract

In this study, we have investigated the efficacy of inorganic nanotubes as reinforcing agents to improve the mechanical properties of poly(propylene fumarate) (PPF) composites as a function of nanomaterial loading concentration (0.01-0.2 wt%). Tungsten disulfide nanotubes (WSNTs) were used as reinforcing agents in the experimental groups. Single- and multi- walled carbon nanotubes (SWCNTs and MWCNTs) were used as positive controls, and crosslinked PPF composites were used as baseline control. Mechanical testing (compression and three-point bending) shows a significant enhancement (up to 28-190%) in the mechanical properties (compressive modulus, compressive yield strength, flexural modulus, and flexural yield strength) of WSNT reinforced PPF nanocomposites compared to the baseline control. In comparison to positive controls, at various concentrations, significant improvements in the mechanical properties of WSNT nanocomposites were also observed. In general, the inorganic nanotubes (WSNTs) showed a better (up to 127%) or equivalent mechanical reinforcement compared to carbon nanotubes (SWCNTs and MWCNTs). Sol fraction analysis showed significant increases in the crosslinking density of PPF in the presence of WSNTs (0.01-0.2 wt%). Transmission electron microscopy (TEM) analysis on thin sections of crosslinked nanocomposites showed the presence of WSNTs as individual nanotubes in the PPF matrix, whereas SWCNTs and MWCNTs existed as micron sized aggregates. The trend in the surface area of nanostructures obtained by BET surface area analysis was SWCNTs > MWCNTs > WSNTs. The BET surface area analysis, TEM analysis, and sol fraction analysis results taken together suggest that chemical composition (inorganic vs. carbon nanomaterials), presence of functional groups (such as sulfide and oxysulfide), and individual dispersion of the nanomaterials in the polymer matrix (absence of aggregation of the reinforcing agent) are the key parameters affecting the mechanical properties of nanostructure-reinforced PPF composites, and the reason for the observed increases in the mechanical properties compared to the baseline and positive controls.

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Keywords

polymer nanocomposites; carbon nanotubes; tungsten nanotubes; mechanical properties; bone tissue engineering

Introduction

Synthetic biodegradable polymers, nanocomposites, and porous scaffolds have been widely investigated as scaffolds for bone tissue engineering applications [1-3]. In general, polymeric scaffolds possess poor mechanical properties and are unsuitable for the tissue engineering of critical sized load bearing bones (e.g. femur) [4]. Several strategies for improving the mechanical properties (compression and flexural) of polymeric bone implants have been reported with a focus towards developing nanoparticle reinforced-biodegradable polymeric composites. Carbon nanostructures such as fullerenes, single- and multi- walled carbon nanotubes, ultra-short carbon nanotubes, single- and multi-walled graphene oxide nanoribbons, and graphene oxide nanoplatelets have been investigated as reinforcing agents [5-10].

Reinforcing agents possessing high intrinsic mechanical property allow efficient load transfer, enhancing the load bearing ability of the nanocomposite. Theoretical studies show that although individual carbon nanotubes possess exceptionally high mechanical properties (Young's modulus in tera-Pascal range) [11], the effective Young's modulus of CNTs in polymeric composites is significantly lower (≈ 500 GPa) [12]. Furthermore, to achieve significant improvements in the mechanical properties of polymeric composites, the presence of reinforcing agent as individual particles in the polymer matrix is highly recommended [13]. However, due to strong van der Waals interactions and π - π stacking (0.5 eV/nm) [14, 15], pristine carbon nanotubes exist as micron sized aggregates in the polymeric matrix resulting in stress concentration and failure [7].

Recently, inorganic nanomaterials such as tungsten disulfide nanotubes (WSNTs) and molybdenum disulfide nanoplatelets (MSNPs) have been used as reinforcing agents to improve the mechanical and tribological properties of epoxy composites, electrospun poly(methyl methacrylate) fibers, and biodegradable PPF nanocomposites [10, 16, 17]. WSNTs possess high mechanical properties (Young's modulus ≈ 150 GPa, bending modulus ≈ 217 GPa) [18, 19], functional groups (such as sulfide and oxy-sulfide), and can be readily dispersed in organic solvents, polymers, epoxy and resins [16]. Due to these potential benefits, the efficacy of WSNTs as fillers to improve the mechanical properties of biodegradable polymers used for bone tissue engineering needs to be investigated. In this study, poly(propylene fumarate) (PPF), an injectable, cross-linkable, biocompatible and biodegradable polyester, widely investigated for bone tissue engineering applications was chosen as the polymeric matrix [9, 20, 21]. PPF composites (baseline control), SWCNT-, MWCNT- (positive controls), and WSNT- (experimental group) reinforced (0.01-0.2) weight% PPF nanocomposites were fabricated. Mechanical properties (compression and flexural), crosslinking density and dispersion state of these experimental and control groups were characterized, and analyzed.

Materials and Methods

Materials

Single walled carbon nanotubes (SWCNTs, Cat. No. 519308) and multiwalled carbon nanotubes (MWCNTs, Cat. No. 636843) were purchased from Sigma Aldrich (New York, USA). Multiwalled tungsten disulfide nanotubes (WSNTs, INT 15-100) were donated by

NanoMaterials Ltd., Yavne, Israel. *N*-vinyl-pyrrolidone (NVP), hydroquinone, potassium permanganate, benzoyl peroxide (BP) and zinc chloride were purchased from Sigma Aldrich (New York, USA). Methylene chloride, propylene glycol, ethyl ether, sodium sulfate, chloroform (HPLC grade) and hydrogen peroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Diethyl fumarate was purchased from Acros Organics (Fair Lawn, NJ, USA).

Synthesis of Poly(propylene fumarate)

PPF was synthesized and characterized as reported previously [22]. Briefly, diethyl fumarate (196.6 g, 1.14 mol) and propylene glycol (259.4 g, 3.41 mol) were reacted in the presence of zinc chloride (1.55 g, catalyst) and hydroquinone (0.25 g, crosslinking inhibitor) under a nitrogen atmosphere with constant mechanical stirring. The temperature was increased from 110-130°C and the reaction stopped when 90% of the theoretical yield of ethanol was collected (≈ 95 g). The resulting intermediate [bis(hydroxypropyl fumarate)] was transesterified under high vacuum yielding PPF. Purification of PPF was performed by sequential washes with HCl (1.85 vol %), distilled water, and brine. The resulting organic polymer was dried using anhydrous sodium sulfate and the solvents were removed using rotary evaporator. The resulting PPF was characterized using ^1H -NMR (structural) and gel permeation chromatography (molecular weight distribution) using a Styragel HR2 column (7.8×300 mm, Waters, MA), and a differential refractive index detector. Polystyrene standards (Fluka, Switzerland) were used to generate a calibration curve with chloroform (1 ml/min) as the mobile phase. PPF of $M_n = 3570$ (PDI = 1.39) was used in the study.

Raman spectroscopy

A WITec alpha300R Micro-Imaging Raman Spectrometer equipped with a 532 nm Nd-YAG excitation laser was used for Raman measurements between 50-3750 cm^{-1} . Point spectra were recorded at room temperature.

Atomic force microscopy

A NanoSurf EasyScan 2 Flex AFM (NanoScience Instruments Inc, Phoenix) was used for AFM imaging. All scans were performed in tapping mode operation using a V-shaped cantilever (APP Nano ACL – 10, $L = 225 \mu\text{m}$, $W = 40 \mu\text{m}$, frequency $f_c = 145$ -230 kHz, spring constant $k = 20$ -95 N/m , tip radius < 10 nm). SWCNTs, MWCNTs and WSNTs were dispersed in 1:1 water:ethanol mixture by using a Ultrasonicator LPX 750 probe sonicator (Cole Parmer, USA), operating at 25% peak amplitude, following a 1 second 'on' and 2 second 'off' cycle. Freshly cleaved silicon wafers (Ted Pella, USA) were washed using isopropanol and 100 μl of homogenously dispersed nanomaterial solution was drop casted for 30 seconds (excess solution removed using a blotting paper). The samples were dried and used for AFM imaging. To improve nanomaterial density on the silicon wafer, 100 μl of the nanomaterial solution was dropped on the same silicon wafer and process repeated. All images were recorded at 25°C and 50% relative humidity (ambient conditions).

Aspect ratio calculation

Length and diameter of the nanotubes were determined by analyzing multiple ($n=10$) TEM and AFM images. Aspect ratio of SWCNTs, MWCNTs, and WSNTs were characterized by the formula:

$$\text{Aspect ratio} = (\text{Mean length of nanotube} / \text{Mean diameter of nanotube})$$

Surface area analysis

Brunauer-Emmett-Teller (BET) surface area was measured by ABET sorptometer at 77K using nitrogen as the adsorption gas for SWCNTs and MWCNTs, and krypton for WSNTs. Single point measurements were performed at Porous Materials Inc., Ithaca, NY.

Nanocomposite preparation, thermal crosslinking and specimen fabrication

Nanomaterials were dispersed in chloroform by bath sonication (Fisher Scientific FS30) for 15 minutes (100 W), followed by probe sonication using an Ultrasonicator LPX 750 probe sonicator (Cole Parmer, USA) operating at 25% peak amplitude using a 1 second 'on' and 2 second 'off' cycle. Nanocomposites were prepared by mixing PPF and NVP at a mass ratio of 1:1 in chloroform, followed by addition of homogeneously dispersed nanomaterials at 0.01-0.2 wt%. The PPF-NVP-nanomaterial mixture was further sonicated for 15 minutes using a bath sonicator, followed by the removal of chloroform using a rotary evaporator (Büchi, Switzerland).

Benzoyl peroxide (free radical initiator) was dissolved in diethyl fumarate (0.1 mg/ml) and mixed with the nanocomposite mixture. Prefabricated Teflon® (McMaster-Carr, Cleveland, OH) molds were used for specimen fabrication for compression and three-point bending tests. The nanocomposite-BP mixture was poured into the molds and cured at 60°C for 24 hours. Compression testing molds yielded cylindrical specimens of 6.5 mm diameter and 14 mm length, and flexural testing molds yielded strips of 70 mm length, 12.7 mm width and 3.2 mm thickness. The specimens were further cut according to ASTM (American Society for Testing and Materials) standards using a low-speed diamond saw (Model 650, South Bay Technology, San Clemente, CA, USA). Nanocomposite cylinders of 6.5 mm diameter and 12 mm length were used for compression testing and strips of 65 mm length, 12.7 mm width and 3.2 mm thickness were used for flexural testing.

Mechanical testing

A MTS material testing machine (858 Mini Bionix II, MTS Systems, Eden Prairie, MN, USA) equipped with a 5kN load cell was used for compressive and flexural mechanical testing for n=4 samples at room temperature. Compression testing was performed according to ASTM Standard D695-08. Force and displacement were recorded throughout the compression of cylindrical specimens along the longitudinal axis until failure, and stress and strain curves were determined based on the initial sample dimensions. Slope of the initial linear portion of the stress-strain curve gave the value of compressive modulus. Compressive yield strength was determined by drawing a line parallel to the compressive modulus at 1% strain offset.

ASTM standard D790-07 was used to perform flexural testing. Samples placed on two support spans at 55 mm distance were loaded midway until failure and the corresponding force and displacement values were recorded. Flexural modulus (E_B) and flexural strength (σ_{FM}) were calculated using the following equations:

$$E_B = L^2 m / 4bd^3$$

$$\sigma_{FM} = 3PL / 2bd^2$$

Where E_B = Bending or flexural elasticity modulus (MPa), σ_{FM} = flexural strength (MPa) a.k.a. stress in the outer fibers at the midpoint, P = Yield point load on the load-deflection curve (N), L = support span (mm), b = width of the beam tested (mm), d = depth of the beam tested (mm) and, m = slope of the tangent to the initial straight line portion of the load – deflection curve (N/mm).

Sol fraction analysis

Changes in the crosslinking density of PPF in the presence of nanomaterials were assessed by sol fraction analysis since PPF/NVP, uncross-linked PPF, and their oligomers are soluble in methylene chloride whereas cross-linked polymer is not. Approximately 0.1 g of crosslinked PPF nanocomposite (W_i , accuracy 0.0001g) was placed in a scintillation vial containing 20 ml methylene chloride, sealed, and kept on the shaker (80 rpm) for 7 days. The residual solid fraction was filtered using a weighed filter paper (W_p) followed by drying at room temperature for 1 hour and 60°C for an additional 1 hour. The filter paper was weighed again (W_{p+s}). Sol fraction was assessed using the following equation (n=4):

$$\text{Sol fraction} = \left(\frac{W_i - (W_{p+s} - W_p)}{W_i} \right) * 100$$

Transmission electron microscopy (TEM)

TEM imaging was performed using a FEI BioTwinG² Transmission Electron Microscope (TEM) operating at an accelerating voltage of 80 kV. Samples for TEM imaging were prepared by dispersing nanomaterials in 1:1 water:ethanol mixture by using a Ultrasonicator LPX 750 probe sonicator (Cole Parmer, USA), operating at 25% peak amplitude, following a 1 second 'on' and 2 second 'off' cycle. The homogenously dispersed nanomaterial solutions were then subjected to ultracentrifugation (5000 rpm, 5 minutes). 10 μ l of the supernatant was dropped onto TEM grids (300 mesh size, holey lacey carbon, Ted Pella, USA), dried overnight at room temperature, and imaged. For TEM imaging of crosslinked PPF nanocomposites, 50-100 nm thick sections were mounted on copper mesh grids (Ted Pella, USA) and imaged.

Statistical analysis

All statistical analysis were performed for n=4 samples using a 95% confidence interval ($p < 0.05$). Kruskal–Wallis one-way analysis of variance (non-parametric statistical analysis) followed by Dunn's test (post hoc analysis) were used for comparisons between multiple groups.

Results

Figure 1 displays the representative atomic force microscopy (AFM) and transmission electron microscopy (TEM) images of the nanotubes. SWCNTs (Figure 1 A & B) and MWCNTs (Figure 1 C & D) were present as individual and bundled nanotubes. AFM height profile (inset, Figure 1 A) indicates that SWCNTs existed as bundles of $\approx$ 2-5 nanotubes (considering the Z of SWCNTs $\approx$ 1 nm). Additionally, thick bundles of SWCNTs ($\approx$ 20-25 nanotubes) were also imaged (Figure 1 B). SWCNTs possess an average diameter of 1-2 nm and length of $\approx$ 2-5 μ m. MWCNTs were imaged as individual and bundles of nanotubes possessing diameter $\approx$ 40-70 nm and length $\approx$ 0.5-2 μ m (Figure 1 C & D). WSNTs (Figure E and F) existed as individually- dispersed sharp needle-like nanotubes possessing a mean outer diameter $\approx$ 100 nm and length $\approx$ 1-15 μ m. The nanotube structure of SWCNTs, MWCNTs, and WSNTs appears smooth without any edge defects.

Figure 2 displays representative Raman spectra of nanotubes. Characteristic peaks at 160 cm^{-1} , 1580 cm^{-1} and 2655 cm^{-1} were observed for SWCNTs, corresponding to the RBM, G and G' bands, respectively. Raman peaks at 1347 cm^{-1} , 1572 cm^{-1} and 2683 cm^{-1} corresponding to D, G and G' bands were observed for MWCNTs. The I_D/I_G ratio was 0.08 for SWCNTs, and 0.11 for MWCNTs. Raman peaks at 344 cm^{-1} , 410 cm^{-1} , 696 cm^{-1} , and

804 cm⁻¹ were observed for WSNTs, corresponding to the E_{2g}, A_{1g}, and 4xLA modes of vibration, as reported previously [23, 24].

Figures 3 A-D show the compressive (compressive modulus and compressive yield strength) and flexural (flexural modulus and flexural yield strength) mechanical properties of WSNTs-reinforced PPF nanocomposites at 0.01-0.2 wt%. Also shown are the mechanical properties of PPF composites (baseline control), as well as SWCNT and MWCNT reinforced PPF nanocomposites at 0.01-0.2 wt% (positive controls). Significant increases in compressive and flexural mechanical properties were observed for all WSNT nanocomposites compared to the baseline or positive controls. Table 1 A-D tabulates the highest compressive modulus, compressive yield strength, flexural modulus and flexural yield strength for WSNT nanocomposites, corresponding loading concentration, and % increase compared to baseline and positive controls.

The compressive modulus (Figure 3 A) and compressive yield strength (Figure 3 B) for WSNT reinforced PPF composites at all loading concentrations were significantly greater than baseline control (PPF composites). WSNT nanocomposites at various loading concentrations also showed significant increase compared to positive controls. The highest compressive modulus for WSNT nanocomposites was $\approx 60\%$ greater compared to baseline control and $\approx 12-48\%$ greater than maximum and minimum values observed for positive controls, respectively (Table 1 A). The highest compressive yield strength values were $\approx 55\%$ greater than baseline control, and $\approx 5-48\%$ greater than maximum and minimum values observed for positive controls, respectively (Table 1 B).

Compared to PPF composites, significant increase in the flexural modulus (Figure 3 C) was observed for WSNT nanocomposites at 0.02-0.2 wt% loading. Significant increase in the flexural modulus, compared to positive controls, was also observed at 0.05 wt% WSNTs loading. Additionally, WSNT nanocomposites showed significant increase in flexural yield strength at all concentrations compared to baseline control, and at various concentrations (0.05-0.2 wt%) compared to positive controls. The highest flexural modulus value for WSNT nanocomposites was $\approx 28\%$ greater than PPF composites, and $\approx 1-32\%$ greater than positive controls (Table 1 C). The highest flexural yield strength value was $\approx 191\%$ greater than baseline control, and $\approx 28-127\%$ greater than positive controls (Table 1 D).

The significant reinforcement in the mechanical properties of WSNT nanocomposites compared to positive or baseline controls can be attributed to the dependence and inter-dependence of surface area and aspect ratio of nanoparticles, and crosslinking density of the nanocomposites. The surface area of WSNTs was 8.43 m²/g, significantly lower than previously reported values for SWCNTs and MWCNTs [10]. The aspect ratio (length of nanotube/diameter of nanotube) values for SWCNTs, MWCNTs and WSNTs were calculated by measuring length and diameter of nanotubes from multiple (n=10) TEM and AFM images, and are reported in Table 2. The dimensions of SWCNTs were 1.2-1.5 nm $\times$ 2-5 μ m (D $\times$ L), MWCNTs were 40-70 nm $\times$ 5-40 nm $\times$ 0.5-2 μ m (O.D. $\times$ I.D. $\times$ L), and WSNTs were 30-150 nm $\times$ 1-13 (± 2) μ m (D $\times$ L). Thus, the aspect ratio value for SWCNTs was >1000 , and between 10-400 for MWCNTs and WSNTs.

Sol fraction analysis was performed to assess changes in the crosslinking density of PPF in the presence of nanostructures (Figure 4). The sol fraction values for PPF composites was 13.3%, SWCNT nanocomposites was 12.3-13.7%, MWCNT nanocomposites was 11.6-13.2% and WSNT nanocomposites was 7.7-9.8%. A higher crosslinking density was observed for all the concentrations of WSNT nanocomposites.

Transmission electron microscopy (TEM) was performed on 50-100 nm thick sections of crosslinked PPF nanocomposites to assess the dispersion of nanostructures in the polymer

matrix (Figure 5). No local heating and solvent dissipation were observed. TEM imaging of SWCNT and MWCNT nanocomposites showed the presence of SWCNTs and MWCNTs as micron-sized aggregates embedded in PPF matrix (Figure 5 A and B, red arrows). WSNTs were well dispersed, and existed as individual nanotubes (Figure 5 C).

Discussions

The objective of this study was to investigate the efficacy of one-dimensional inorganic nanomaterials (tungsten disulfide nanotubes) as reinforcing agents for the biodegradable polymer PPF, compared to one-dimensional carbon nanomaterials (single- and multi-walled carbon nanotubes). Towards this end, PPF nanocomposites were prepared by dispersing SWCNTs, MWCNTs, and WSNTs at various loading concentrations (0.01-0.2 wt%) via radical initiated thermal crosslinking. Compression and three-point bending test were performed according to ASTM standards to characterize the mechanical properties of the nanocomposites. Sol fraction analysis was performed to assess changes in the crosslinking density of the polymer in the presence of nanostructures. TEM analysis was performed on the crosslinked specimens to characterize the dispersion state of nanostructures in the polymer matrix.

Characterization of nanomaterials was performed using AFM, TEM and Raman spectroscopy. AFM and TEM imaging shows the characteristic tubular morphology of SWCNTs, MWCNTs and WSNTs. Raman spectrum for SWCNTs and MWCNTs show the characteristic D, G and G' bands, in addition to the RBM peak observed for SWCNTs. The radial breathing mode (RBM) corresponds to the coherent radial stretching of the carbon atoms, and is observed only for SWCNTs. The D band in the Raman spectra corresponds to the defects in the nanotube structure due to the disruption of the sp^2 domains whereas the G band corresponds to the intrinsic vibration of the graphitic carbon [25]. The I_D/I_G ratio for SWCNTs and MWCNTs is in the range observed for pristine carbon nanotubes suggesting a nearly defect free (pristine) structure of carbon nanotubes. Characteristic Raman peaks for WSNTs correspond to the de-agglomerated, pristine multiwalled tubular architecture [24]. Presence of defects in the reinforcing agents can act as handles for improved polymer interactions, thereby improving the mechanical properties of polymeric nanocomposites [26]. However, in this study, the observed mechanical reinforcement cannot be attributed to an increased polymer-nanomaterial interaction due to absence of structural defects (use of pristine SWCNTs, MWCNTs, and WSNTs).

The mechanical properties (i.e., compressive modulus, compressive yield strength, flexural modulus, and flexural yield strength) of WSNT reinforced crosslinked PPF nanocomposites were significantly higher than baseline controls at all loading concentrations. Significant increases in the mechanical properties, compared to positive controls, were also observed at various loading concentrations. The range of values for WSNT nanocomposites is comparable to the literature values of trabecular bone (Young's modulus $\approx$ 300-5000 MPa, compressive yield strength $\approx$ 0.1-13 MPa, flexural modulus $\approx$ 40-50 MPa, flexural yield strength $\approx$ 1.8-10 MPa) [27-29]. However, the values of WSNT nanocomposites are significantly lower than cortical bone (Young's modulus $\approx$ 12,000-20,000 MPa, compressive yield strength $\approx$ 170-190 MPa, flexural modulus $\approx$ 5000-23,000 MPa, flexural yield strength $\approx$ 130-295 MPa) [30-33]. Orthopedic implants possessing higher or lower mechanical properties compared to native bone tissue could lead to stress shielding and implant failure, respectively. Thus, the mechanical properties of WSNT nanocomposites permit their use towards tissue engineering strategies for trabecular bone.

Surface area and aspect ratio of the nanostructures, along with changes in the polymer crosslinking density are important parameters that can affect the mechanical properties of

nanoparticle reinforced polymeric composites [5]. Higher surface area of the nanoparticles would increase nanoparticle-polymer interface thereby allowing efficient load transfer from the polymeric matrix to the nanoparticles [26]. The measured BET surface area of WSNTs is significantly lower than previously reported values for SWCNTs and MWCNTs [10]. The aspect ratio of nano-fillers has been reported to affect the mechanical properties of polymeric nanocomposites [10, 34, 35]. For nanoparticle reinforced PPF nanocomposites, carbon nanostructures with lower aspect ratio lead to a better mechanical reinforcement compared to carbon nanostructures with higher aspect ratio [10]. The aspect ratio of WSNTs is comparable to MWCNTs but significantly lower than SWCNTs (Table 2). These results suggest that surface area and aspect ratio of nanostructures may not be the dominant factors responsible for the observed mechanical properties of WSNT reinforced PPF nanocomposites.

Sol fraction analysis was performed to assess the changes in the crosslinking density of PPF in the presence of WSNTs, since changes in the crosslinking density have been reported to alter the mechanical properties of nanoparticle-reinforced polymeric nanocomposites [8]. An increase in the crosslinking density of the polymeric nanocomposite (signified by a decrease in the sol fraction) results in significant increases in the mechanical properties. The sol fraction values measured for WSNT nanocomposites (at all loading concentrations) were significantly lower than PPF composites, and SWCNT and MWCNT nanocomposites, suggesting an increase in the crosslinking density of PPF in the presence of WSNTs. The presence of functional groups (such as sulfide and oxysulfide) formed during the synthesis of WSNTs could lead to chemical interactions between nanoparticle and the surrounding PPF polymer, resulting in the formation of strong nanoparticle-polymer interfaces. The results suggest that crosslinking density of polymeric nanocomposites may be a dominant factor over surface area and aspect ratio of nanoparticles, responsible for the observed differences in the mechanical properties of PPF nanocomposites.

Transmission electron microscopy was performed to assess the dispersion of nanostructures in PPF nanocomposites post thermal crosslinking. Individual dispersion of nanoparticles in the polymer matrix is recommended for efficient load transfer [8]. Presence of nanoparticles as aggregates in the polymer matrix can cause slippage between nanostructures leading to poor mechanical reinforcement. These aggregates can also act as sources of stress concentrators or crack initiators under external stress. The analysis of TEM images indicates that SWCNTs and MWCNTs are present as micron sized aggregates, whereas WSNTs are present as individually-dispersed nanotubes.

To the best of our knowledge, this is the first systematic investigation comparing the efficacy of inorganic and carbon nanotubes as reinforcing agents for the fabrication of polymeric nanocomposites as biomedical implants for bone tissue engineering applications. Several reports have investigated the efficacy of carbon nanostructures as reinforcing agents for PPF nanocomposites. Fullerenes (C_{60}) reinforced PPF nanocomposites showed a marginal ($\approx 10\%$) increase in the mechanical properties compared to PPF composites. PPF nanocomposites containing 0.02-0.05 wt% pristine SWCNTs showed $\approx 65\%$ increase in the compressive modulus and $\approx 69\%$ increase in the flexural modulus [7]. In another study, PPF nanocomposites containing 0.2 wt% ultra-short carbon nanotubes (reduction in aspect ratio compared to SWCNTs) exhibited a two-fold increase in the compressive and flexural modulus compared to PPF [5]. Additionally, PPF nanocomposites fabricated with functionalized SWCNTs (to improve SWCNT dispersion in PPF matrix) show up to ≈ 2 fold increase in the compressive and flexural mechanical properties (compared to PPF composites) [8]. Although no direct comparisons between the results of previously reported studies with this study can be made due to differences in the curing temperature (60°C vs. 37°C), method of crosslinking (thermal vs. UV crosslinking), and the crosslinking monomer

(NVP vs. PPF-diacrylate) [7, 8]; these studies show that covalent functionalization (for uniform dispersion of nanoparticles) and reduction in the aspect ratio of nanoparticles can be used to further improve the mechanical properties of polymeric nanocomposites. Additionally, the use of pristine WSNTs would eliminate functionalization steps necessary to achieve a uniform dispersion of carbon nanotubes in the polymer matrix [8].

In comparison to carbon nanotubes [5, 6, 8, 10, 36-40], few reports have investigated the mechanical properties of WSNTs reinforced polymeric nanocomposites [16, 17]. However, none of these studies have focused on biomedical applications and have made direct comparisons between carbon and inorganic nanotubes as reinforcing agents. Zohar et al. reported $\approx 49\%$, $\approx 39\%$ and $\approx 85\%$ improvements in fracture toughness, shear strength, and peel strength of epoxy composites (compared to pristine epoxy controls) at 0.5 wt% loading of WSNTs [16]. Reddy et al. reported ≈ 22 fold improvement in the elastic modulus, and 30-35% improvements in tensile strength and toughness of electrospun PMMA fiber composites (compared to pristine PMMA fiber controls) at 2 wt% loading of WSNTs [17]. The results of these studies cannot be compared to each other due to variations in the polymeric matrix, WSNT loading concentrations, and the method of fabrication of polymeric nanocomposites. However, these studies show that the mechanical properties of polymeric nanocomposites can be significantly enhanced at very low loading concentrations of WSNTs, corroborating a salient feature of this study.

For WSNT nanocomposites, the consistently higher compressive and flexural mechanical properties may be due to two factors: (1) increase in the crosslinking density of WSNTs reinforced PPF nanocomposites and (2) uniform dispersion of WSNTs in PPF matrix. Increases in the crosslinking density of nanocomposites leads to significant improvements in the mechanical properties. Additionally, absence of nanomaterial aggregates (uniform dispersion of nanomaterials) in the polymer matrix reduces the risk of crack initiation and stress propagation under external load. Based on the above analysis, it can be inferred that chemical composition of nanostructures along with their dispersion state in the polymer matrix are the two most important factors for enhanced mechanical reinforcement, and inorganic nanotubes, in general are better reinforcing agents compared to carbon nanotubes.

In vitro and *in vivo* evaluation of biocompatibility is necessary to develop any new composite biomaterial for bone tissue engineering applications. Till date, cyto- and bio-compatibility studies using pristine WSNTs and WSNT-reinforced PPF nanocomposites have not been reported, and thus are currently in progress. There is now a wide body of published work on the cyto- and bio- compatibility of carbon nanotubes [41, 42]. Additionally, few studies also report the *in vitro* and *in vivo* bio-compatibility of SWCNTs-reinforced PPF nanocomposites [43] and porous scaffolds [9, 44]. Those studies show that unreacted components, degradation products and crosslinked components of PPF nanocomposites reinforced with SWCNTs (0.01-0.2 wt %) did not elicit cytotoxicity ($\approx 100\%$ cell viability) against rat fibroblasts *in vitro* [44]. Ultra-short-SWCNTs reinforced PPF scaffolds, implanted in the femoral condyles and subcutaneous pockets of New Zealand white rabbits exhibited reduced inflammatory cell density, and increased connective tissue formation and bone tissue ingrowth after 12 weeks of implantation [9].

In conclusion, PPF nanocomposites were fabricated at low loading concentrations (0.01-0.2 wt %) of WSNTs towards the fabrication of biodegradable polymeric implants possessing improved mechanical properties (i.e. compressive modulus, compressive yield strength, flexural modulus, and flexural yield strength) for bone tissue engineering applications. Compressive and flexural mechanical properties for all concentrations of WSNT loading were significantly higher than PPF composites (baseline control). Significant increases in the mechanical properties at various concentrations were also observed compared to

SWCNT and MWCNT nanocomposites (positive control). In comparison to SWCNTs and MWCNTs, significant increases in the crosslinking density of PPF was observed for all loading concentrations of WSNTs. Additionally, compared to SWCNTs and MWCNTs, which were present as micron sized aggregates in PPF matrix, WSNTs showed excellent dispersion and existed as individual nanotubes in PPF matrix after thermal crosslinking. The results of mechanical testing, sol fraction analysis and TEM analysis taken together suggest that WSNT nanocomposites are stronger than SWCNT and MWCNT nanocomposites, and in general inorganic nanotubes are better reinforcing agents than single- and multi-walled carbon nanotubes.

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Figure 1. Atomic force microscopy and transmission electron microscopy images of SWCNTs (A & B), MWCNTs (C & D) and WSNTs (E & F). Insets in (A), (C) and (D) corresponding height (Z) profiles.

Figure 2.
Representative Raman spectra of (a) SWCNTs, (b) MWCNTs, and (c) WSNTs, respectively.

A

B

C

D

Figure 3.

(A) compressive modulus, (B) compressive yield strength, (C) flexural modulus and (D) flexural yield strength of PPF nanocomposites as a function of nanoparticle loading concentration. Data is represented as mean $\pm$ standard deviation of $n=4$ samples. '*' corresponds to a significant difference compared to PPF composites (baseline control) and '**' corresponds to a significant difference compared to SWCNT and MWCNT composites (positive controls) for $p < 0.05$.

Figure 4.

Sol fraction analysis of thermally crosslinked PPF nanocomposites as a function of loading of SWCNTs, MWCNTs and WSNTs. Data is represented as mean $\pm$ standard deviation of $n=4$ samples. '*' corresponds to a significant difference compared to PPF composites and '**' corresponds to a significant difference compared to SWCNT and MWCNT composites (positive controls) for $p < 0.05$.

A

B

C

Figure 5. Representative transmission electron microscopy images of PPF nanocomposites at 0.1 wt% loading of (A) SWCNTs, (B) MWCNTs, and (C) WSNTs. Red arrows correspond to the presence of nanoparticles.

Table 1 A

Compressive modulus of PPF nanocomposites

Nanoparticle	Highest compressive modulus (MPa)	Concentration (wt%)	% increase compared to PPF	% increase compared SWCNTs	% increase compared to MWCNTs
SWCNT	1246.8 ± 105.9	0.02	26.9	-	-
MWCNT	1401.2 ± 121.1	0.1	42.6	-	-
WSNT	1578.2 ± 71.39	0.02	60.6	26.5-43	12.4-48.5

Table 1 B

Compressive yield strength of PPF nanocomposites

Nanoparticle	Highest compressive yield strength (MPa)	Concentration (wt%)	% increase compared to PPF	% increase compared to SWCNTs	% increase compared to MWCNTs
SWCNT	58.4 ± 2.2	0.02	40.3	-	-
MWCNT	61.8 ± 2.8	0.2	48.4	-	-
WSNT	64.7 ± 2.8	0.2	55.3	10.6-48.2	4.6-23.3

Table 1 C

Flexural modulus of PPF nanocomposites

Nanoparticle	Highest flexural modulus (MPa)	Concentration (wt%)	% increase compared to PPF	% increase compared to SWCNTs	% increase compared to MWCNTs
SWCNT	759.7 ± 45.9	0.1	15.5	-	-
MWCNT	838.1 ± 13.6	0.05	27.4	-	-
WSNT	845.5 ± 62.9	0.05	28.5	11.2-23.4	0.8-31.9

Table 1 D

Flexural yield strength of PPF nanocomposites

Nanoparticle	Highest flexural yield strength (MPa)	Concentration (wt%)	% increase compared to PPF	% increase compared to SWCNTs	% increase compared to MWCNTs
SWCNT	14.13 ± 1.7	0.1	107.1	-	-
MWCNT	15.5 ± 1.29	0.1	127.2	-	-
WSNT	19.83 ± 2.19	0.1	190.7	40.3-127.4	27.9-73.18

Table 2

Aspect ratio of various nanoparticles

Nanoparticle	Aspect ratio *
SWCNT	>1000
MWCNT	≈ 10-400
WSNT	≈ 10-400

* Aspect ratio = (length of nanotube/diameter of nanotube), n=10