Bionanohydroxyapatite/ Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Composites with Improved Particle Dispersion and Superior Mechanical Properties

Bina Rai,[†,‡] Wadcharawadee Noohom,[‡] Prakash H. Kithva,[†,‡] Lisbeth Grøndahl,[†] and Matt Trau*[,‡]

School of Molecular and Microbial Sciences and Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Queensland, Australia 4072

Received October 23, 2007. Revised Manuscript Received January 28, 2008

The present work was inspired by a persistent limitation in the use of composite biomaterials for orthopedics, namely, the tendency of ceramic reinforcements to agglomerate in these composites due to interparticle van der Waals interaction. As a result, the composites possess poor mechanical properties that are unsuited for load-bearing applications. We propose using nanohydroxyapatite particles preadsorbed with heparin (nHA-HEP) to circumvent this issue. The key feature was the dual role that HEP would play in enhancing particle dispersion and biological response. Turbidity and zeta potential measurements revealed that the addition of HEP significantly improved the colloidal stability (23 days with minimal particle sedimentation) of nHA in water, dimethylformamide (DMF), and dimethylsulfoxide (DMSO) but had negligible effect in acetic acid. Anti-FXa activity results showed that 95% of the HEP adsorbed onto nHA retained its bioactivity in DMSO and water, while 51% was preserved in DMF and acetic acid. Composite fabrication was ultimately done using poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), a biodegradable polymer, dissolved in DMF. A decrease in water contact angle of solvent cast films with increasing weight percent (wt %) of nHA-HEP/PHBV was observed as compared to nHA/PHBV films. Scanning electron micrographs illustrated that nHA/PHBV films had poor dispersion of particles within the matrix and that large agglomerates settled to the bottom of the films during casting. On the contrary, the nHA-HEP/PHBV matrices had effective dispersion of particles and enhanced tensile properties. We found that the tensile elastic modulus and strength of the films increased with increasing wt % of nHA-HEP. A value close to the human cortical bone was obtained at 30 wt % loading of nHA-HEP. In conclusion, understanding the relationship between process, morphology, and property led to the development of nHA-HEP/PHBV composites that show promise for load-bearing bone applications.

Introduction

The wide application of composite biomaterials in the orthopedic milieu has been limited as a result of persistent problems inherent in existing fabrication technologies. Researchers have yet to tackle the issue of poor dispersion of the nano- or micrometer-sized ceramic additives in the polymer matrices. This is of utmost importance as particle agglomeration negates any intended benefits associated with the nanophase dimensions or ceramic component, which includes improved mechanical properties and nucleation of a uniform apatite layer on the surface of the composites to provide interfacial bonding.[1] This has resulted in a large number of articles directed at potential solutions such as ultrasonication,[2] surface modifications,[3] physical blending,[4] use of surfactants[5] or alternative material combinations.[2,6] However, the issue remains a challenge, and a composite biomaterial that possesses good dispersion of the filler with ensuing mechanical properties comparable to human cortical bone is yet to be realized.

Composite biomaterials, on their own, are at most bioactive or osteoconductive. The incorporation of growth factors and/or other extracellular matrix components into composites can impart osteoinductive properties, which refers to the recruitment and stimulation of immature cells to develop into preosteoblasts that are essential for bone remodeling.[7] Presently, most of the biological factors are added after processing of the composites because of the aggressive conditions of fabrication, such as high temperatures and organic solvents that may cause protein aggregation or denaturation.[8] This is however not efficient in terms of cost, time, and delivery kinetics. The prototype tested for this study

* Corresponding author. E-mail: m.trau@uq.edu.au. Telephone: (617) 3346 4173. Fax: (617) 3346 3973.
† School of Molecular and Microbial Sciences.
‡ Australian Institute for Bioengineering and Nanotechnology.

(1) Christenson, E. M.; Anseth, K. S.; van den Beucken, J. J. P.; Chan, C. K.; Ercan, B.; Jansen, J. A.; Laurencin, C. T.; Li, W. J.; Murugan, R.; Nair, L. S.; Ramakrishna, S.; Tuan, R. S.; Webster, T. J.; Mikos, A. G. *J Orthop. Res.* **2007**, *25*, 11.
(2) Misra, A. K.; Nazhat, S. N.; Valappil, S. P.; Torbati, M. M.; Wood, R. J. K.; Roy, I.; Boccaccini, A. R. *Biomacromolecules* **2007**, *8*, 2112.
(3) Nichols, H. L.; Zhang, N.; Zhang, J.; Shi, D.; Bhaduri, S.; Wen, X. *J. Biomed. Mater. Res.* **2007**, *82A*, 373.
(4) Coskun, S.; Korkusuz, F; Hasirci, V. *J. Biomater. Sci. Polym.* **2005**, *16*, 1485.
(5) Kim, H. W. *J. Biomed. Mater. Res.* **2007**, *83A*, 169.
(6) Bhardwaj, R.; Mohanty, A. K.; Drzal, L. T.; Pourboghrat, F.; Misra, M. *Biomacromolecules* **2006**, *7*, 2044.
(7) Albrektsson, T.; Johansson, C. *Eur. Spine J.* **2001**, *10*, S96.
(8) Babensee, J. E.; McIntire, L. V.; Mikos, A. G. *Pharm. Res.* **2000**, *17*, 497.

10.1021/cm703045u CCC: $40.75
Published on Web 03/15/2008

consisted of a polysaccharide, heparin (HEP), adsorbed onto nanohydroxyapatite (nHA) particles (nHA-HEP). HEP, a member of the glycosaminoglycan family, was primarily chosen for the current study because of its high net negative surface charge that makes it ideal as an adsorbent and dispersing agent able to induce steric and/or charge repulsion between particles.[9,10] In addition, HEP has been reported to play a role in sequestering and delivery of a vast array of bioactive molecules, such as the bone morphogenetic protein-2[11] and fibroblast growth factor[12] that are involved in bone repair, and this can contribute favorably to the initial cell attachment events in vivo that are vital for implant success.[13] It was also recently demonstrated that polysaccharides, and not proteins, form the interface between the organic and the mineral components of bone.[14]

Our group recently described colloidally stable bionanohydroxyapatite (bio-nHA) particles that show potential as reinforcements for composite orthopedic materials by enhancing both particle dispersion and biological response.[9] The present study represents a progression from that work and serves to prove our hypothesis that the developed bio-nHA particles can indeed improve ceramic dispersion in composites and achieve mechanical properties suitable for orthopedic applications. This study also presents a detailed investigation into the fabrication of a composite biomaterial, encompassing two crucial features that are often overlooked, namely, the colloidal stability of the bio-nHA particles in both aqueous and commonly used organic solvents and preservation of the bioactivity of the adsorbed biological factor after contact with the respective solvents.

As a result of their natural origin and lack of cytotoxic effects upon implantation, the biodegradable polymer, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), is considered an attractive material for biomedical applications, as several previous reviews have summarized.[4,15] PHBV was selected as the model polymer for this work because it possesses better mechanical properties (ranging between cancelous and cortical bone) than many synthetic, biodegradable polyesters.[4,15,16] In addition, PHBV was shown to support osteogenic progression of preosteoblastic cells in vitro [17,18] and in vivo,[19] which was further enhanced in nHA-based composites.[20] It must be noted that the proposed approach can be easily applied to nHA-HEP blended with other types of biodegradable polymers as well.

Materials and Methods

Synthesis of nHA. The nHA particles were created as previously described.[9] Briefly, 1.81 g of $Ca(OH)_2$ (95%, Sigma, U.S.A.) was suspended in 1 L of DI water (MilliQ system, 18.2 Mega ohm) using a stirrer. The $Ca(OH)_2$ suspension was heated to 70 °C, and the pH of the suspension was adjusted to 11 with the addition of 90 mL of NH_3 (28%, Univar, Australia). One milliliter of H_3PO_4 (85%, Fluka, Switzerland) was added dropwise to the rapidly stirring suspension over a period of 5 min. The HA suspension was then covered with aluminum foil and kept stirring at 70 °C for 2 h prior to the addition of HEP or use for fabrication of composites. The synthesized nHA displayed an infrared spectrum consistent with carbonated hydroxyapatite and had an aspect ratio of 10, specific surface area of 81 m^2/g, and surface Ca/P of 1.5 ± 0.1 as formerly documented.[9]

Synthesis of nHA-HEP and Evaluation of Colloidal Stability. A total of 0.1 mL (1g/L) of heparin sodium salt (H4784, MW 9000–12000, 170 USP units/mg, Sigma) solution in DI water (pH 7.4) was added to 0.9 mL of nHA (0.01 g/mL) suspension in DI water (pH 7.4). The suspensions were placed on a rotating wheel and incubated for 2 h at 24 °C. The samples (nHA-HEP) and control (nHA) were centrifuged for 5 min at 4600 rpm and resuspended in 1 mL of either DI water, dimethylformamide (DMF) (99.8%, Laboratory-Scan, Thailand), dimethylsulfoxide (DMSO) (99.9%, Laboratory-Scan), or glacial acetic acid (99.7%, Laboratory-Scan) and observed (digital photographs were taken) for turbidity changes for 1 month or until no further change was observed. The time taken for nHA to sediment (time of sedimentation) and the volume of sedimentation (volume occupied by the nHA floc) were recorded at that point.

Zeta Potential Measurements. Electrokinetic or zeta potential measurements were conducted on a Malvern Instruments Nanoseries ZS Zetasizer (300HSA, Southborough, Massachusetts) using an aqueous dip cell. Samples (nHA-HEP) and control (nHA) were dispersed in DI water, DMF, DMSO, and acetic acid at a concentration such that a count rate of 1800–2000 kilocounts per second was maintained and the samples appeared to be slightly turbid. The dielectric constants of the solvents were taken as 37, 47, 6, and 78 for DMF, DMSO, acetic acid, and water, respectively.[21] Ten runs were performed and averaged for each sample.

Bioactivity of HEP. The nHA-HEP particles were resuspended in 1 mL of DI water or the above-mentioned organic solvents for 20 min and centrifuged. To ensure that any residual solvents did not affect the bioactivity results, the particles were washed in DI water once, before dissolving in 1 mL of 0.1 M HCL to release the adsorbed HEP. The supernatants were then tested for the total amount of HEP adsorbed as compared to time 0 (before immersion) using the modified dimethylene blue assay.[22] The bioactive amount of HEP adsorbed (as compared to time 0) was also measured according to the manufacturer's instructions based on their anti-FXa activity (Stachrom Heparin kit, Diagnostica Stago, France).

Production of Composite Powder. The composite powders were prepared using a slight modification to a method previously described.[20] Predetermined amounts of PHBV (8% HV, MW 350 000, Sigma) was dissolved in DMF by stirring at 80 °C for 20 min. When dissolved, sample (nHA-HEP) or control (nHA)

(9) Rai, B.; Grondahl, L.; Trau, M. *Langmuir* **2007**, submitted.
(10) Nurcombe, V.; Kumarasuriyar, A.; Cool, S. M. *Drug Dev. Res.* **2004**, *62*, 303.
(11) Ruppert, R.; Hoffmann, E.; Sebald, W. *Eur. J. Biochem.* **1996**, *237*, 295.
(12) Jackson, R. A.; Nurcombe, V.; Cool, S. M. *Gene* **2006**, *379*, 79.
(13) Jackson, R. A.; McDonald, M. M.; Nurcombe, V.; Little, D. G.; Cool, S. M. *J Orthop. Res.* **2006**, *24*, 636.
(14) Wise, E. R.; Maltsev, S.; Davies, M. E.; Duer, M. J.; Jaeger, C.; Loveridge, N.; Murray, R. C.; Reid, D. G. *Chem. Mater.* **2007**, *19*, 5055.
(15) Misra, S. K.; Valappil, S. P.; Roy, I.; Boccaccini, A. R. *Biomacromolecules* **2006**, *7*, 2249.
(16) Galego, N.; Rozsa, C.; Sanchez, R.; Fung, J.; Vazquez, A.; Tomas, J. S. *Polym Test.* **2000**, *19*, 485.
(17) Kumarasuriyar, A.; Jackson, R. A.; Grondahl, L.; Trau, M.; Nurcombe, V.; Cool, S. M. *Tissue Eng.* **2005**, *11*, 1281.
(18) Shishatskaya, E. I.; Khlusov, I. A.; Volova, T. G. *J. Biomater. Sci., Polym. Ed.* **2006**, *17*, 481.
(19) Kose, G. T.; Korkusuz, F.; Korkusuz, P.; Hasirci, V. *Tissue Eng.* **2004**, *10*, 1234.
(20) Cool, S. M.; Kenny, B.; Wu, A.; Nurcombe, V.; Trau, M.; Cassady, A. I.; Grondahl, L. *J Biomed. Mater. Res.* **2007**, *82A*, 599.
(21) Riddick, J. A.; Bunger, W. B. *Organic Solvents*; Techniques of Organic Chemistry; Wiley-Interscience: New York, 1970; Vol. II.
(22) Farndale, R. W.; Buttle, D. J.; Barrett, A. J. *Biochim. Biophys. Acta* **1986**, *883*, 173.

suspensions in DI water (preheated to 80 °C) were slowly added dropwise in appropriate quantities to yield composites with 5, 15, and 30 wt % of nHA. The mixture was stirred energetically for 20 min and cooled rapidly in an ice bath until gelation occurred. Methanol (99.8%, Univar) was added to the gel to obtain a white composite powder and left for 10 min on a stirrer, before isolation by vacuum-filtration, followed by washing 3 times with methanol. The resulting powder was dried in an oven at 60 °C and subsequently further purified by stirring in methanol once at room temperature for 2 h, followed by vacuum-filtration and drying in an oven at 60 °C.

Preparation of Composite Films. Composite films were prepared by solvent casting. The composite powder (0.3 g) was dissolved in 15 mL of chloroform (99.4%, Pronalys) at 50 °C. A covered glass Petri dish (70 mm i.d.) was used as casting substrate. The suspensions were left to stand at room temperature for 3–4 days to allow for full evaporation of the solvent. The resultant films had a thickness ranging from 30 to 50 μm.

Characterization of Composite Films. *Thermogravimetric Analyses (TGA).* TGA measurements were conducted using a Shimadzu DTG-60. Heating was performed in a N_2 atmosphere at a rate of 10 °C/min up to 600 °C. Samples were held at 600 °C for 2 min and then cooled down at the same rate (10 °C/min).

Contact Angle Analyses. Advancing water contact angles (θ_A) were measured using the sessile drop method[23] on a custom build device.[17] Advancing contact angles were obtained using drops of DI water on the film surfaces. The contact angles were measured on a 5 μL drop and subsequently after each addition of 5 μL until a total 20 μL volume was added. Using the equation $2h/\Delta = \tan \theta_A/2$, contact angles ($\theta_A$) were calculated ($\Delta$ is the base diameter of the drop and h is the height of the drop).

Scanning Electron Microscopy (SEM). Specimens were placed onto aluminum grids coated with carbon adhesives. Platinum coating was performed using an EIKO IB-5 Sputter Coater for 3 min resulting in a coat thickness of about 10 nm. Images were obtained using a JEOL JSM 6300 at an aperture of 4 and an accelerating voltage of 6 kV.

Mechanical Testing. Specimens for tensile testing (gauge length, 14 mm; width, 4 mm; and thickness, 30–50 μm) were cut from the composite films using a standard specimen cutter. The tensile tests were carried out on an Instron 5543 universal testing instrument under constant cross-head speed (1 mm/min). For each sample, a minimum of three specimens were tested, and the averages of the tensile properties (elastic modulus and tensile strength) were calculated from the stress versus strain curves.

Statistical analyses. All experiments were carried out in triplicates unless otherwise stated and expressed as mean ± standard deviation. Statistical significance was determined by Student's t test using Instat Software (GraphPad Software Inc., U.S.A.). A value of $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Characterization of nHA and nHA-HEP Particles. The first stage of this study compared the colloidal stability and surface charges of nHA and nHA-HEP particles immersed in various solvents. The motivation was to find the best solvent for composite fabrication and to improve our understanding of the complex behavior of the nHA particles in response to different solvents. Obtaining a homogeneous and stable dispersion of nHA particles in solvents is critical to produce composites with enhanced mechanical properties.[24]

Table 1. Colloidal Stability of the Reinforcing Phase (nHA and nHA-HEP Particles) in Various Solvents

particle	solvent	time to sediment	sedimentation volume (mm^3)	zeta potential (mV)
nHA	DMF	7 days	864 ± 10	−27 ± 7
nHA-HEP	DMF	23 days	157 ± 8	−68 ± 3
nHA	DMSO	7 days	785 ± 14	−42 ± 3
nHA-HEP	DMSO	23 days	157 ± 6	−65 ± 2
nHA	acetic acid	2 h	550 ± 20	−1 ± 4
nHA-HEP	acetic acid	7 days	550 ± 20	−13 ± 7
nHA	water	2 h	790 ± 20	−6 ± 3
nHA-HEP	water	23 days	79 ± 4	−37 ± 3

Colloidal Stability. The nHA particles suspended in water and acetic acid flocculated and settled out under gravity as early as 2 h, with sedimentation volumes of 790 ± 20 and 550 ± 20 mm^3, respectively. The poor dispersion in water has been previously reported[5] and attributed to the tendency of nHA to aggregate due to interparticle van der Waals interaction and hydrogen bonding between surface hydroxyl groups.[24] This was in contrast to the nHA particles immersed in DMSO and DMF that sedimented at day 7. Table 1 shows that the adsorption of HEP onto nHA enhanced its colloidal stability in DMF, DMSO, and water to 23 days. In addition, the sedimentation volumes were significantly reduced for nHA-HEP particles in DMF, DMSO, and water. No differences between nHA and nHA-HEP particles dispersed in acetic acid were observed, and particles flocculated at day 7 in this solvent.

Zeta Potential. Zeta potential measurements revealed that the overall surface charge of nHA particles differed significantly in the various solvents (Table 1). The nHA particles suspended in DMSO had the most negative potential (−42 ± 3 mV), followed by DMF (−27 ± 7 mV), water (−6 ± 3 mV), and acetic acid (−1 ± 4 mV). The adsorbed HEP significantly decreased the zeta potential for nHA-HEP particles in all solvents. The zeta potential measurements were in good accordance to the colloidal stability results, where nHA-HEP had very good stability in DMF and DMSO and moderate stability in water and was at the threshold of agglomeration in acetic acid in accordance to Riddick's definition of stability based on zeta potentials.[25] This indicated that charge–charge repulsion between the nHA-HEP particles was the predominant factor in the dispersion of particles in the four test solvents. It must also be noted that the dipole moments of the solvents do correlate with the colloidal stability and zeta potential measurements. Solvents displaying high dipole moments (DMF, 3.82, and DMSO, 3.96 debye) sufficed to be favorable for nHA and nHA-HEP dispersion as compared to the solvents with lower dipole moments (water, 1.85, and acetic acid, 1.74 debye).[26]

Bioactivity of HEP. The subsequent experiment investigated if HEP remained adsorbed onto nHA and if the

(23) Erbil, H. *Surface Tension of Polymers*; Handbook of Surface and Colloid Chemistry; CRC Press: New York, 1997.

(24) Lee, H. J.; Choi, H.W.5; Kim, K. J.; Lee, S. C. *Chem. Mater.* **2006**, *18*, 5111.

(25) Riddick, T. M. *Control of Colloid Stability through Zeta Potential*; Livingston Publishing Co.: New York, 1968; Vol. I, p 2.

(26) Lide, D. R. *CRC Handbook of Chemistry and Physics*, 86th ed.; CRC Press Inc.: New York, 2005.

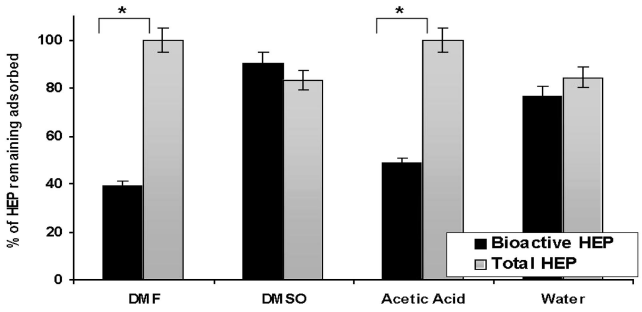

Figure 1. Percent of total and bioactive (anti-FXa activity) HEP that remained adsorbed onto nHA after immersion in DMF, DMSO, acetic acid, and DI water for 20 min. *$p < 0.05$ using Student's t test.

Table 2. Intended and Experimental wt % of nHA in Prepared Composites As Determined by TGA Analyses[a]

	nHA/HEP			nHA-HEP/PHBV		
intended wt % of nHA	5	15	30	5	15	30
intended vol % of nHA	2	7	15	2	7	15
experimental wt % of nHA	3	14	33	3	13	29
experimental vol % of nHA	1	7	16	1	6	14

[a] Vol % was calculated based on densities of 3.16 and 1.25 g/mL for nHA[27] and PHBV,[26] respectively.

biological function of HEP was preserved upon contact with the respective solvents. After a 20 min immersion into the solvents, 92 ± 9% of HEP remained adsorbed onto the nHA particles in all solvents (Figure 1). The anti-FXa analyses demonstrated that 95 ± 6% of the adsorbed HEP preserved its bioactivity in DMSO and water. The bioactivity of HEP was however significantly reduced by half in both DMF and acetic acid. It was apparent that DMSO was the ideal solvent for nHA-HEP particles. However, DMF was selected as the solvent for composite fabrication in this study because it could dissolve the polymer of choice, PHBV (unlike DMSO),[27] is miscible with water, and allows PHBV to undergo a rapid temperature-controlled gelation process that prevents large scale phase separation and reduces the agglomeration of particles in the composites.[20]

Characterization of Composite Films. *TGA Analyses.* Table 2 shows the intended and experimentally determined (via TGA) wt % of nHA and nHA-HEP combined with the PHBV polymer. It was evident that the intended weight compositions had been achieved for all the composites ($p > 0.05$). It must be noted that each sample in this paper is referred to by the intended wt % of nHA or nHA-HEP. The conversions into volume/volume % (vol %) were tabulated as well for better understanding of the dispersion of the particles within the polymer matrix. It was apparent that the vol % for the composites were about half of their wt %.

Water Contact Angle. It was found that although θ_A values of the air interface for nHA/PHBV composites were reduced slightly as compared to pure PHBV films (81 ± 3°), they remained similar (75 ± 2°) despite increasing the content of hydrophilic[29] nHA from 5 to 30 wt % (Figure 2). The nHA-HEP/PHBV composites, on the other hand, showed a

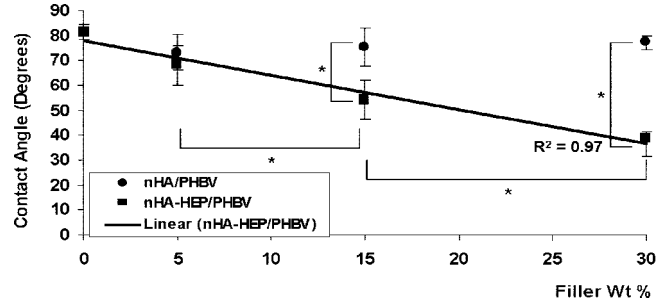

Figure 2. Contact angle analyses of the air interface of nHA/PHBV and nHA-HEP/PHBV composite films at 5, 15, and 30 wt % of nHA or nHA-HEP, respectively. *$p < 0.05$ using Student's t test.

significant linear decrease ($R^2 = 0.97$) in θ_A from 69 ± 8 to 39 ± 7° when the amount of nHA-HEP was increased from 5 to 30 wt %. This clearly indicated a great improvement in the hydrophilicity or wettability. This can be aptly explained by SEM micrographs (Figure 3) which showed that nHA-HEP particles were distributed throughout the cross-section of the polymer matrix, including being displayed on the surface. In contrast, the nHA particles (without HEP) tended to agglomerate and sediment to the bottom of the film, thus resulting in negligible change in the air interface (top) surface θ_A.

This change in surface properties of nHA-HEP/PPHBV films may result in better interaction of the composites with cells in vivo, as hydrophilic surfaces of most thermoplastic polymers have been reported since the early 1980s to be more conducive for cell adhesion, proliferation, and differentiation.[30,31]Consequently, this would make PHBV more attractive as a polymer for biomedical applications. An increase in hydrophilicity would also enhance the water adsorption capacity of the composites, followed by accelerated weight loss and a faster degradation rate as a consequence. This has been well-documented in literature for various ceramic/polymer composites.[4,32–35] PHBV, by itself, was reported to degrade in 53 weeks.[15] The addition of nHA-HEP is expected reduce this substantially, hence providing composite degradation rates that complement the rate of new bone regeneration, which is believed to be typically 6–9 months.[36]

SEM Images. The morphology of the nHA and nHA-HEP/PHBV films provided useful insights into the dispersion of the particles within the polymer matrix and can explain the mechanical properties and contact angle values obtained. Figure 3 demonstrates the scanning electron micrographs for the air interface (top) surface of the solvent cast composite films. The films had a range of pit sizes from 1 to 6 μm. The nHA/PHBV films showed that the nHA particles were sparsely present on this surface and agglomerated (~6 μm) in the polymer matrix. In contrast, the images indicated a homogeneous distribution of nHA-HEP particles within the

(27) Noda, I.; Marchessault, R. H.; Terada, M. *Polymer Data Handbook*; Oxford University Press Inc.: Oxford, U.K., 1999; p 586.
(28) Dorozhkin, S. V. *J. Mater. Sci.* **2007**, *42*, 1061.
(29) Xingyuan, G.; Gough, J. E.; Xiao, P.; Liu, J.; Shen, Z. *J. Biomed. Mater. Res.* **2007**, *82A*, 1022.
(30) Lydon, M. J.; Minett, T. W.; Tighe, B. G. *Biomaterials* **1985**, *6*, 396.
(31) van Wachem, P. B.; Beugeling, T.; Feijin, J.; Bentjes, A.; Detmers, J. P.; van Aken, W. G. *Biomaterials* **1985**, *6*, 403.
(32) Doyle, C.; Tanner, E. T.; Bonfield, W. *Biomaterials.* **1991**, *12*, 841.
(33) Wang, M.; Yue, C. Y.; Chua, B. *J. Mater. Sci.: Mater. Med.* **2001**, *12*, 821.
(34) Li, H.; Du, R.; Chang, J. *J. Biomater. Appl.* **2005**, *20*, 137.
(35) Yeo, A.; Rai, B.; Sju, E.; Cheong, J. J.; Teoh, S. H. *J. Biomed. Mater. Res.* **2007**, in press.
(36) von Arx, T.; Buser, D. *Clin. Oral Implants Res.* **2006**, *17*, 359.

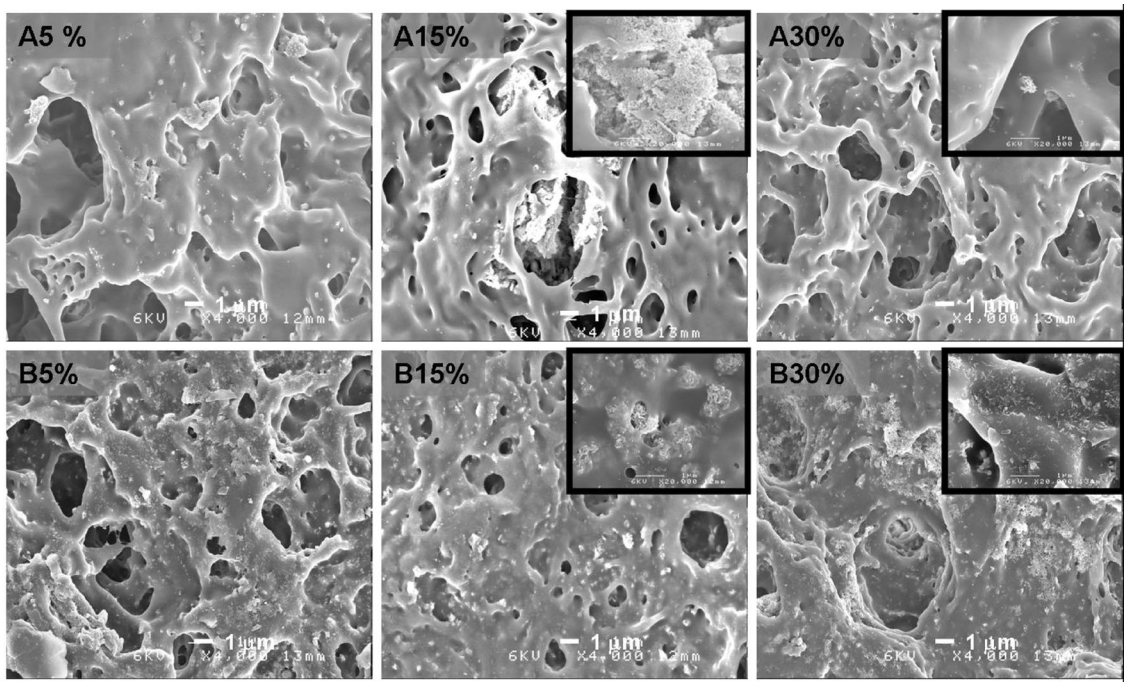

Figure 3. SEM images of the air interface (top) surfaces of the (A) nHA/PHBV and (B) nHA-HEP/PHBV composite films at 5, 15, and 30 wt % of nHA or nHA-HEP, respectively.

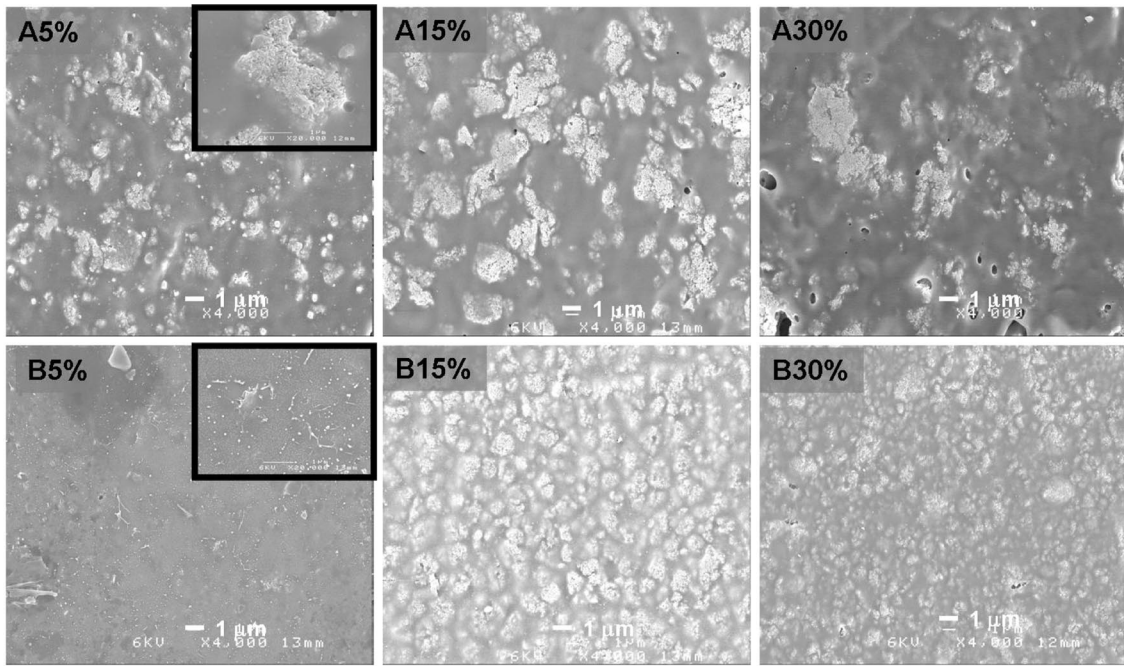

Figure 4. SEM images of the glass interface (bottom) surfaces of the (A) nHA/PHBV and (B) nHA-HEP/PHBV composite films at 5, 15, and 30 wt % of nHA or nHA-HEP respectively.

PHBV matrix, which was more evident at higher magnification. It was difficult to estimate the size of the nHA-HEP particles from the micrographs as they were less than 1 μm and existed as very fine crystallites that were well distributed. The agglomerations versus homogeneous distribution were consistent for the 5–30 wt % nHA and nHA-HEP/PHBV films.

SEM micrographs of the glass interface (bottom) surface of the nHA/PHBV composite films revealed large, irregular-shaped agglomerates that were randomly dispersed within the polymer matrix, indicating that the particles had sedimented to the bottom of the films during casting (Figure 4). The size of the agglomerates increased (~2–6 μm) when the wt % of nHA was increased from 5 to 30. This is commonly found for physically blended composites and has been shown to result in poor osteoblast adhesion and mineralization.[20,32] On the other hand, nHA-HEP particles appeared to be better dispersed with a size of less than 1 μm in the 5 wt % nHA-HEP/PHBV films. When the wt % was increased to 15 and 30, more particles were apparent and they were small (1 μm and less), regular shaped, and well dispersed. Higher magnification images showed that the

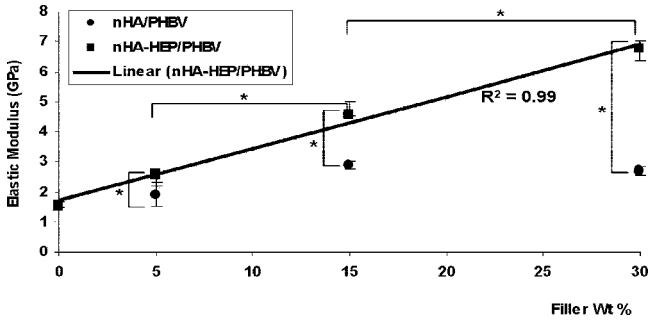

Figure 5. Tensile elastic moduli of nHA/PHBV and nHA-HEP/PHBV composite films at 5, 15, and 30 wt % of nHA or nHA-HEP, respectively. $*p < 0.05$ using Student's t test.

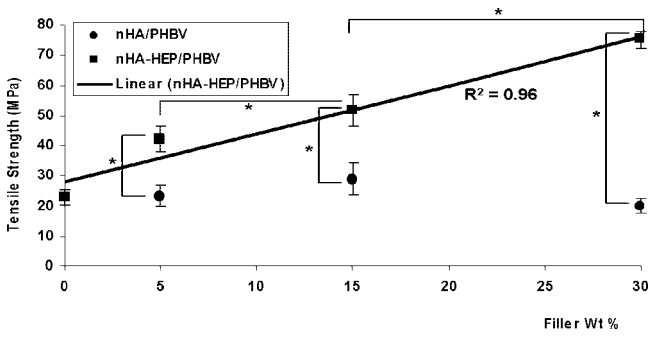

Figure 6. Tensile strengths of nHA/PHBV and nHA-HEP/PHBV composite films at 5, 15, and 30 wt % of nHA or nHA-HEP respectively. $*p < 0.05$ using Student's t test.

nHA-HEP particles were both embedded in the polymer matrix and displayed on the surface.

Mechanical Testing Analyses. Tensile testing revealed that the nHA/PHBV films had an elastic modulus of 2.0 ± 0.1, 3.0 ± 0.4, and 3.0 ± 0.3 GPa for the 5, 15, and 30 wt % nHA, respectively (Figure 5). In addition, the films experienced an insignificant increase in tensile strength from 24 ± 4 to 29 ± 5 GPa when the wt % nHA was increased from 5 to 15 (Figure 6). When the wt % was further increased to 30, a decrease in the tensile strength to 20 ± 3 GPa was observed. The poor dispersion of nHA within the polymer matrix and the presence of numerous and large agglomerates that settled to the bottom of the films explains the poor tensile properties of nHA/PHBV films. Agglomerates are believed to act as weak points in the material that break easily when stress is applied. Broken agglomerates can then act as stress concentrators leading to microcracking, which effectively decreased the modulus of composites[2] and negated any beneficial effects of the inorganic component on the tensile properties of composites as observed for the nHA/PHBV films in the current study.

Remarkably, the nHA-HEP/PHBV films displayed a linear increase ($R^2 = 0.99$) in elastic modulus with increasing wt % of nHA-HEP (Figure 5). The films had a moduli of 3.0 ± 0.4, 5.0 ± 0.1, and 7.0 ± 0.1 GPa for the 5, 15, and 30 wt % nHA-HEP, respectively (Figure 5). These moduli were significantly greater than the nHA/PHBV films at each respective wt %, with a 133% increase for the 30 wt % films. Similarly, the nHA-HEP/PHBV films demonstrated a linear increase ($R^2 = 0.96$) in tensile strength with increasing wt % of nHA-HEP (Figure 6). The films had tensile strengths of 42 ± 4, 52 ± 5, and 75 ± 3 GPa for the 5, 15, and 30 wt % nHA-HEP, respectively. This was considerably higher than their counterparts at each respective wt %, with a 300% increase for the 30 wt % films. The substantial enhancement of the elastic modulus and tensile strengths in proportion to the increasing wt % of nHA-HEP can be attributed to the excellent dispersion of nHA-HEP throughout the PHBV matrices.

The superior tensile properties obtained for the nHA-HEP/PHBV films in this study are in contrast to results commonly reported in literature, which stated that HA inclusions have a profound detrimental effect on the stiffness and strength of the composites, especially at high wt %. Examples include Misra et al.,[2] who showed that the inclusion of Bioglass particles in PHB solvent-cast films reduced the elastic modulus of the composites from 1.1 to 0.84 GPa when Bioglass wt % was increased from 0 to 20%. The authors ascribed this partially to the agglomeration of the particles in the PHBV matrices. Chemically synthesized poly(lactic-coglycolic acid)/HA composites containing 10–30 wt % HA demonstrated a decrease in tensile strength (9 to 4 MPa) and modulus (5 to 2 MPa) with increasing HA wt %.[37] This was attributed to the presence of porosity at the interface of HA and the polymer. We also experienced similar trends in the tensile properties for the control (nHA/PHBV) films of this study. Hence, our strategy to use a biological colloidal dispersant, HEP, proved beneficial in avoiding the above issues of agglomeration and inadequate bonding between ceramic and polymer.

It was also appreciable that tensile values close to cortical bone (tensile modulus, 12 GPa; strength, 52 MPa)[38] were achieved for the 30 wt % composites in our study, and to the authors' best knowledge, this had not been documented previously for PHBV reinforced composites.[4,6,39] One reported reason for this was the weak binding strength between the nHA and the polymer matrix which compromised the mechanical strength of the composites due to phase separation.[3] Tensile strength is dependent on the matrix and the compatibility between the reinforcing phase and matrix, while tensile modulus is influenced by the ceramic impregnation and aspect ratio.[6] Thus, the high tensile values achieved for the nHA-HEP/PHBV films can be explained on the basis of effective (1) compatibility or interfacial adhesion of nHA-HEP with PHBV polymer and (2) dispersion of nHA-HEP in the PHBV matrices, leading to superior stress transfer ability in these films as compared to nHA/PHBV films.

Conclusions

To date, there is still no commercially available biodegradable orthopedic device with properties satisfactory to sustain fractured bone without help of metallic supports. This inspired our group to develop a novel method addressing the main issue faced by researchers in the field, namely, the agglomeration of ceramic reinforce-

(37) Petricca, S. E.; Marra, K. G.; Kumta, P. N. *Acta Biomater.* **2006**, *2*, 277.
(38) Currey, J. D. *The Mechanical Adaptations of Bones*; Princeton University Press: Princeton, NJ, 1984; p 294.
(39) Galego, N.; Rozsa, C.; Sanchez, R.; Fung, J.; Vazquez, A.; Tomas, J. S. *Polym. Test.* **2000**, *19*, 485.

ments in composites, which resulted in poor mechanical properties. We proposed the use of HEP adsorbed onto nHA, to serve as a colloidal dispersant and osteoinductive factor. It was evident that the nHA-HEP/PHBV matrices had effective dispersion of particles and significantly enhanced tensile properties, to a value that was close to the human cortical bone at 30 wt % of nHA-HEP. This study also emphasized the importance of understanding the relationship between process, morphology, and property for composite material fabrication.

Acknowledgment. This work was supported by the ARC Centre of Excellence for Functional Nanomaterials, Australia. M.T. acknowledges the support of the Australian Research Council Federation Fellowship (FF0455861).

Note Added after ASAP Publication. There was a change in the Acknowledgment in the version published ASAP March 15, 2008; the corrected version published ASAP April 1, 2008.

CM703045U