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Elastomeric high-mineral content hydrogel-hydroxyapatite composites for orthopedic applications

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Abstract

The design of synthetic bone grafts that mimic the structure and composition of bone and possess good surgical handling characteristics remains a major challenge. We report the development of poly (2-hydroxyethyl methacrylate) (pHEMA)-hydroxyapatite (HA) composites termed “FlexBone” that possess osteoconductive mineral content approximating that of human bone yet exhibit elastomeric properties enabling the press-fitting into a defect site. The approach involves crosslinking pHEMA hydrogel in the presence of HA using viscous ethylene glycol as a solvent. The composites exhibit excellent structural integration between the apatite mineral component and the hydroxylated hydrogel matrix. The stiffness of the composite and the ability to withstand compressive stress correlate with the microstructure and content of the mineral component. The incorporation of porous aggregates of HA nanocrystals rather than compact micrometer-sized calcined HA effectively improved the resistance of the composite to crack propagation under compression. Freeze-dried FlexBone containing 50 wt % porous HA nanocrystals could withstand hundreds-of-megapascals compressive stress and >80% compressive strain without exhibiting brittle fractures. Upon equilibration with water, FlexBone retained good structural integration and withstood repetitive moderate (megapascals) compressive stress at body temperature. When subcutaneously implanted in rats, FlexBone supported osteoblastic differentiation of the bone marrow stromal cells pre-seeded on FlexBone. Taken together, the combination of high osteoconductive mineral content, excellent organic-inorganic structural integration, elasticity, and the ability to support osteoblastic differentiation *in vivo* makes FlexBone a promising candidate for orthopedic applications.

Keywords

hydrogel; hydroxyapatite; elastomeric composites; osteoblastic differentiation; bone marrow stromal cells

INTRODUCTION

It is estimated that one seventh of the US population suffers from musculoskeletal impairment, costing the US economy >\$215 billion a year.^{1–4} Well over 600,000 orthopedic surgical procedures performed annually in the US would require bone grafts.⁵ With a growing and aging population, this number will continue to increase. Three types of bone grafts, autogenic, allogenic, and synthetic, are currently used clinically.⁶ Although considered as a gold standard, the limitations associated with the autografting procedure include donor site morbidity, the frequent needs for a second operation and an inadequate volume of transplant material.^{7,8} Allografts, on the other hand, suffer from significant failure rates, poor mechanical stability, and the inherent risks for disease transmission and immunological rejections.^{7,8} Synthetic bone grafts have the potential to become the primary materials of choice in the reconstructive repair of skeletal defects if they are properly engineered. Unfortunately, current synthetic bone substitutes (mostly gel foams or ceramics) have not yet achieved the level of sophistication structurally, mechanically, and biochemically to replace autografts or allografts. Consequently, their clinical use (<10%) has lagged far behind autografts (~50%) and allografts (>40%).⁶

To fundamentally improve the quality and performance of synthetic bone grafts, new generations of organic/inorganic composites that mimic the structure and composition of bone,⁹ and possess useful surgical handling characteristics (e.g. straightforward and stable fixation) are required. From a materials point of view, bone is a composite of collagen, a gel-like protein template that resists stretching forces, and the inorganic apatite crystals that resists compression. The bending and compression strength of human bone is known to positively correlate to bone mineral content.¹⁰ The quantity and quality of the deposited mineral (crystal size, maturity and structural integration with the organic matrices) influences the mechanical properties of bone.¹¹ Coupled with its osteoconductivity, hydroxyapatite (HA), the major inorganic component of bone, has long been recognized as an important design element for tissue-engineered bone substitutes.¹² Toward this end, methods for incorporating a high percentage of HA with synthetic hydrogels with good structural integration are highly desired.

Poly(2-hydroxyethyl methacrylate), or pHEMA, and its functionalized derivatives have been used in a wide range of biomedical applications. With physical properties similar to natural gel-like extracellular matrices, these hydrogel polymers have been explored as ophthalmic devices,¹³ soft tissue engineering scaffolds,¹⁴ carriers for drug or growth factor delivery,^{15,16} dental cements, and medical sealants.^{17,18} We previously developed a urea-mediated mineralization method that could lead to high-affinity integration of HA on the surface of pHEMA hydrogels.^{19–21} However, integration of HA with pHEMA at a high mineral-to-gel ratio throughout the 3D scaffold has not yet been achieved. Here we report the synthesis and characterization of well-integrated pHEMA-HA composites with mineral-to-organic matrix ratios approximating those of dehydrated human bone,^{22,23} yet exhibiting elastomeric properties. Termed as “FlexBone,” these composites are engineered to withstand moderate (megapascals) compressive stresses and exhibit elastomeric properties under physiological conditions to facilitate easy and stable surgical insertion to a site of skeletal defect. Compressive behavior of FlexBone as a function of its mineral content and microstructures is investigated, and the ability for FlexBone to support the osteogenic differentiation of bone marrow stromal cells (BMSC) *in vivo* is also examined.

MATERIALS AND METHODS

Materials

The radical inhibitors in the commercial HEMA and ethylene glycol dimethacrylate (EGDMA) from Aldrich (Milwaukee, WI) were removed via distillation under reduced pressure and by passing through a 4 Å molecular sieve column prior to use, respectively. Polycrystalline

commercial HA powders (designated as ComHA) were purchased from Alfa Aesar (Ward Hill, MA) and used as received. The calcined HA powders (designated as CalHA) were obtained by treating ComHA at 1100°C for 1 h. Prior to use, the CalHA powders were ground in a planetary agate mill for 2 h and then passed through a 38 µm sieve to remove larger agglomerates. The microstructures and size distributions of these HA particles are shown in Figure 1. Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA) and the fetal bovine serum was purchased from HyClone (Logan, UT). All reagents for histochemistry were purchased from Sigma (St Louis, MO).

Preparation of FlexBone composites

The HA content of the FlexBone is defined as the weight percentage of the HA incorporated over the total weight of the HA, monomer HEMA, and crosslinker EGDMA used in any given preparation. In a typical procedure, freshly distilled HEMA was mixed with EGDMA along with ethylene glycol (EG), water and aqueous radical initiators ammonium persulfate (I-1, 480 mg/mL) and sodium metabisulfite (I-2, 180 mg/mL) at a volume ratio of HEMA:EGDMA:EG:I-1:I-2/100:2:35:20:5:5 (formulation 1). ComHA or CalHA powder was then added to the hydrogel mixture, thoroughly mixed by using a ceramic ball to break up the large agglomerates, and allowed to polymerize in a disposable syringe barrel or rigid PMMA tubing of a 7.0-mm or 4.7-mm inner diameter to afford composites with HA contents varying from 37 to 50%. The resulting elastic material was either used as it was (as-prepared), thoroughly exchanged with a large volume of water (fully hydrated), or freeze-dried. By altering the amount of EG and water relative to the HA, 70% of HA, a mineral content approximating that of human bone,^{22,23} can be incorporated. For instance, a volume ratio of HEMA:EGDMA:EG:I-1:I-2/100:2:60:40:5:5 (formulation 2) was used to prepare composites containing 70% CalHA with consistent properties. (In this article, however, only properties of composites containing up to 50% HA are discussed.) The resulting composites are denoted as ComHA-N-# or CalHA-N-#, where N stands for the type of hydrogel formulation and # denotes the weight percentage of HA content. For instance, ComHA-1-50 represents FlexBone composite containing 50% commercial HA that is formed using crosslinking formulation 1. Unmineralized pHEMA control was prepared using formulation 1 in the absence of HA particles.

Microstructural characterization

The microstructures of the composites were characterized using environmental scanning electron microscopy (ESEM) on a Hitachi S-4300SEB microscope (Hitachi, Japan). The chamber pressure was kept ~35 Pa to avoid complete sample dehydration and surface charging during the observation. The chemical composition was analyzed using energy dispersive spectroscopy (EDS) (Noran system SIX, ThermoFinnigan, USA) attached to the ESEM.

Mechanical testing

To assess the compressive behavior of FlexBone in as-prepared, fully hydrated and freeze-dried states as a function of the mineral microstructure and content, unconfined compression tests were performed on two different instruments, a Q800 Dynamic Mechanical Analyzer (DMA) and a high capacity MTS, to accommodate the needs for high sensitivities and high loading capacities, respectively. All samples were tested in accordance with ASTM D695 with the exception of sample size and slenderness ratio (recommended ratio: 1:4 diameter-to-length) due to sample height limitation of the DMA instrument (≤5 mm) and the concern over the significant error that preparing and testing extremely small-diameter samples may introduce. Shorter cylinders were also used for the high capacity MTS due to the concern over sample buckling under extremely high compressive strains. All stress-strain curves presented are

based on engineering stress and engineering strain recorded on each instruments, assuming a fixed cross-section of the material defined at the start of the test.

At least five specimens were tested for each sample. For as-prepared and water-equilibrated samples, cylindrical specimens with a diameter of 4.7 mm were transversely cut into 5.0-mm long cylinders using a custom-machined parallel cutter with adjustable spacing. Any visible roughness of the top and bottom surfaces of each specimen was reduced by sandpaper. An L-square was used to make sure that these surfaces were parallel prior to testing, and the final dimensions of each specimen were measured by a digital caliper. For freeze-dried samples, cylindrical specimens with the dimension of 7 mm × 6 mm (diameter × height) were used.

The compressive behavior of as-prepared and water-equilibrated FlexBone composites along with pHEMA control, particularly their elasticity, was evaluated on a Q800 DMA (TA Instruments) equipped with a submersion compression fixture. The instrument has an 18-N load cell, a force resolution of 10 µN and a displacement resolution of 1.0 nm. The as-prepared samples were compressed in a force-controlled mode in ambient air, ramping from 0.01 to 18.0 N at a rate of 3.0 N/min then back to 0.01 N at the same rate. The samples fully equilibrated with water were compressed in water at 37.5°C, ramping from 0.01 to 10.0 N at a rate of 3.0 N/min then back to 0.01 N at the same rate. To evaluate the reversibility of the compressive behavior, the controlled force cycle was repeated 10–40 times consecutively for each specimen unless the material failed (major cracks developed) during the force ramping, at which point the test would be terminated. In all cases, we observed little further shift of loading–unloading curves beyond 10 cycles. For clarity, in figures that compare the compressive behaviors among different samples [Fig. 2(A, C)], the first 10 cycles of the stress–strain curves from one representative specimen of each sample were plotted. To demonstrate the reliability of the measurements and the reproducibility of the compressive behavior of each sample, five specimens were examined for each sample. Because of the observed high reproducibility, however, only the first 10 cycles of the consecutive loading–unloading curves from three specimens for each sample are presented in overlaid fashion as Supplemental Information (Supplemental Fig. 1 and Supplemental Fig. 2).

The compressive behavior of freeze-dried FlexBone composites, particularly their ability to withstand high compressive loads without exhibiting brittle fractures, was evaluated in ambient air on a high-capacity MTS servo-hydraulic mechanical testing machine (MTS Systems Corporation) equipped with a 100 kN load cell and stiff, nondeforming platens. The samples were loaded under displacement control at a rate of ~0.015 mm/s up to 80–90% compressive strain, while the corresponding loads and displacements were continuously monitored using the built-in load cell and linear variable displacement transducer (LVDT).

Isolation and *in vitro* expansion of rat BMSC

All animal procedures were conducted in accordance with the principles and procedures approved by the University of Massachusetts Medical School Animal Care and Use Committee. BMSC were isolated from long bones of 4-week old male Charles River SD strain rats.²⁴ Briefly, marrow was flushed from femur with a syringe containing MEM. After lysing red blood cells with sterile water, the marrow cells were centrifuged and resuspended in minimum essential medium (MEM) supplemented with 20% FBS, 0.2% penicillin-streptomycin and 1% l-glutamine, and passed through a sterile metal filter. Cells were expanded on tissue culture plates (10 million cells per 100-mm plate initial seeding density) with media changes on day 4 and every other day thereafter before they were lifted off for plating on FlexBone.

Subcutaneous implantation of FlexBone composite in rats with and without preseeded BMSC

Thin half discs (7 mm in diameter, 1 mm in thickness) of FlexBone containing 40% ComHA (ComHA-1-40) were sterilized in 70% ethanol, re-equilibrated with sterile water before being seeded with BMSC and used for subcutaneous implantation in rats. Fifty microliters of BMSC suspension (in culture media described above) was loaded on the surface of thin disks of FlexBone to reach 5000-cells/cm² or 20,000-cells/cm² seeding density. The cell-seeded FlexBone were incubated at 37°C in humidified environment with 5% CO₂ without additional media for 6 h to allow cell attachment to the FlexBone substrate. Additional media were then added and the cells were cultured on the substrates for 2 days before being used for implantation. Four sets of samples were used for each cell seeding treatment. Thin discs of FlexBone without preseeded BMSC were also used for implantation as controls.

Rats were anesthetized by intraperitoneal (IP) injection of ketamine/xylazine (50 mg/5 mg per kg). They were shaved and swabbed with betadine before two 1/4 in bilateral skin incisions were made over the rib cage for insertion of the FlexBone discs with and without pre-seeded BMSC. The skin was closed with surgical staples and buprenorphine (0.02 mg/kg) was given subcutaneously. The rats were sacrificed by CO₂ inhalation and cervical dislocation at day 14 and day 28 for the retrieval of FlexBone. After removing the fibrous tissue encapsulation, the retrieved FlexBone was fixed in 4% paraformaldehyde (0.1M phosphate buffer, pH 7.4) for 5 h at 4°C before being analyzed by SEM, XRD, and histology.

X-ray powder diffraction

The crystalline phases of the mineral in the FlexBone composites before and after subcutaneous implantation in rats were evaluated by XRD with a Siemens D500 instrument using Cu K_α radiation. Phases were identified by matching the diffraction peaks to the JCPDS files.

Histochemical staining of explanted FlexBone for alkaline phosphatase activity

The 4% paraformaldehyde-fixed FlexBone explants were equilibrated in cacodylic buffer overnight, then in 30% sucrose solution (pH 7.3) for 2 days before being frozen-sectioned on a Bright Cryostat (Model OTF; Bright Instrument Ltd., Huntingdon, UK). Frozen-sectioning was repeated until reaching the depth of 100–200 μm away from the surface where the BMSC were initially seeded. The 12-μm frozen sections were held on adhesive slides using frozen sectioning tape. Histological staining for ALP activity, a marker of osteogenic differentiation, was performed as described in literature.²⁵ Briefly, the frozen sections of FlexBone explants were incubated with 1.5 mM naphthol-As-Mx phosphate disodium salt, 0.1% Fast Red and 2.7% DMF (v/v) in 0.1M Tris acid maleate buffer (pH 8.4) for 30 min, and the positive stains (in red) were detected by optical microscopy.

RESULTS

Preparation and compressive behavior of as-prepared and fully hydrated FlexBone

FlexBone composites with varying mineral contents (37–70%) were prepared by crosslinking HEMA with 2% EGDMA in the presence of either porous aggregates of HA nanocrystals (ComHA) or compact micrometer-sized calcined HA (CalHA) particles (Fig. 1) using ethylene glycol as a solvent. Repetitive unconfined compressive tests performed on the as-prepared FlexBone with varying mineral contents revealed mineral content-dependent and mineral microstructure-dependent elastomeric compressive behavior. As indicated by the slopes of the compressive stress–strain curves shown in [Fig. 2(A)], FlexBone composites are stiffer (steeper slope) than the un-mineralized pHEMA hydrogel prepared with the same degree of crosslinking. In addition, Flex-Bone composites containing higher mineral contents are stiffer

than those containing less HA particles of the same type, showing a positive correlation between the stiffness and the mineral content of the polymer-mineral composite. Notable difference in compressive behavior as a function of the type of HA components incorporated was also observed, with FlexBone containing ComHA much stiffer than those containing the same percentages of CalHA. Finally, good overlaps of stress-strain curves were observed when 10 consecutive compressive loading/unloading cycles up to ~1 MPa (the maximal applicable loads of the DMA instrument with the chosen sample size) were applied to all as-prepared composites. Such good recovery under compressive strains up to 40% depending on the composition is expected to facilitate the press-fitting of these composites into a defect area. As a reference, the peak contact stresses in natural human joints during light to moderate activity typically range from 0.5–6 MPa by most *in vitro* measurements,^{26–29} and up to 18 MPa by some *in vivo* measurements.^{30,31} Overall, our data suggest that as-prepared FlexBone exhibit excellent shape recovery under repetitive, physiologically relevant compressive stress despite their high (37–50%) mineral contents.

The as-prepared composites can undergo solvent exchange with water to give fully hydrated Flex-Bone. The residue sulfur-containing radical initiators trapped in the as-prepared composites could be removed during the wash with water as indicated by the disappearance of the S signal detected from the energy dispersive spectroscopy (EDS) performed on the cross-section of the composite upon equilibration with water [Fig. 2(B)]. The compressive behavior of fully hydrated FlexBone was examined at body temperature in water using a DMA equipped with a submersion compression fixture. As shown in [Fig. 2(C)], mineral content-dependent and mineral microstructure-dependent compressive behavior similar to those exhibited by as-prepared FlexBone was observed with fully hydrated FlexBone. A noticeable difference, however, is that fully hydrated FlexBone containing CalHA failed (with major cracks formed) when >30% of compressive strain was applied. In contrast, FlexBone containing 37% and 50% ComHA could withstand repetitive megapascal compressive stress with excellent shape recovery in water. The difference observed with the hydrated composites containing same percentages of ComHA versus CalHA powders underscores the importance of the microstructures of the mineral component, and likely their differential behavior in interfacing with the polymer matrix, in determining the bulk mechanical properties of the polymer-mineral composites.

Compressive behavior and microstructures of freeze-dried FlexBone

To better understand how the microstructure of the mineral component and the organic–inorganic interface dictates the macroscopic compressive behavior of FlexBone, we examined the microstructural response of freeze-dried composites containing ComHA versus CalHA under very high compressive stress and strains (>80%). Freeze-drying the fully hydrated FlexBone composites did not lead to the delamination of the evenly distributed mineral components, either ComHA or CalHA, from the polymer matrix that they were embedded in [Representative SEM: Fig. 3(B, D)]. To apply high compressive strains to the freeze-dried composites, a high capacity MTS with 100 kN load cell was used to perform unconfined compression test. As expected, the freeze-dried composites were stiffer than their hydrated counterparts. Importantly, all tested freeze-dried FlexBone composites were able to withstand compressive stress in the order of hundreds of megapascals and compressive strains of >80% without exhibiting brittle fractures despite their high mineral contents [Fig. 3(A)]. In contrast, PMMA-based bone/dental cements or poly(lactic acid)-HA composites reported in literature typically exhibited brittle fracture at 50–150 MPa compressive loading.^{32–34}

A closer examination of the stress-strain curves revealed that freeze-dried composites containing ComHA tend to be stiffer than those containing same percentages of CalHA, as representatively shown in [Fig. 3(A)]. This is consistent with the trend observed with as-

prepared and hydrated FlexBone under lower compressive stresses. SEM analysis of freeze-dried CalHA-1-50 after compression tests resulting in >80% strains revealed the formation of cracks within the hydrogel phase whereas no distortion or fracture of the micrometer-sized compact CalHA particles was observed [Fig. 3(B) vs. 3(C)]. These cracks could affect the slope of the stress–strain curve. In contrast, the hydrogel-infiltrated aggregates of HA nanocrystals in freeze-dried ComHA-1-50 were flattened upon compression into plywood-like structures with no disruption of the continuity of the hydrogel matrix [Fig. 3(D) vs. 3(E)]. The rearrangement of the nanometersized HA crystallites can provide a mechanism for energy dissipation within the composite under high compressive stresses.

***In vivo* osteogenic differentiation of BMSC supported by FlexBone**

To test the cytocompatibility and the *in vivo* resorption of FlexBone, we seeded hydrated composites ComHA-1-40 with BMSC isolated from rat femur, and implanted them subcutaneously (SC) in 4-week old male Charles River SD strain rats. The composites were retrieved at 14 and 28 days, with a degree of fibrous tissue encapsulation observed in all cases. After removing the fibrous tissue, the morphology and mineral phase of the retrieved implant were examined by SEM and X-ray powder diffraction (XRD). Little macroscopic change in shape or size of the retrieved FlexBone was observed, reflecting the non-degradable nature of the hydrogel scaffold that defines the overall shape of the composite. However, surface roughening was observed with both 14- and 28-day explants regardless whether they were pre-seeded with BMSC prior to implantation [Fig. 4(A, B)]. This is likely a combined outcome of slow dissolution of the mineral component and the extracellular matrix deposition from cells either pre-seeded on or newly attracted to the substrate *in vivo*. XRD analyses performed with the explanted composite [Fig. 4(C)] revealed a diffraction pattern matching with that of the ComHA powder, suggesting that the major mineral phase remained unchanged 4 weeks after the SC implantation.

To determine whether the composite can support the osteogenic differentiation of BMSC *in vivo*, the explanted composites with preseeded BMSC were stained histochemically for alkaline phosphatase (ALP) activity, a marker for osteogenic differentiation.³⁵ To avoid the harsh paraffin embedding conditions that may compromise ALP enzymatic activity,³⁶ frozen sectioning was performed on the explants prior to ALP staining. As shown in [Fig. 4(D)], ALP activity (indicated by red stains) was detected 14 days postimplantation on the periphery of the ComHA-1-40 preseeded with 5000-cells/cm² BMSC. More extensive ALP activity was also detected 28 days after the implantation on FlexBone preseeded with 20,000-cells/cm² BMSC. These data suggest that FlexBone was able to support the attachment and *in vivo* osteoblastic differentiation of osteoblast precursor cells.

DISCUSSION

We reported the preparation of a class of elastomeric pHEMA-HA composites, FlexBone, consisting of high percentages of osteoconductive HA using a straightforward protocol. The high viscosity of ethylene glycol, the solvent used during the fabrication of FlexBone, facilitated the easy dispersion of 50 wt % HA particles within the hydrogel formulation, thereby preventing the HA particles from settling by gravity during solidification. The intrinsic affinity of the hydroxyl side chains of the crosslinked pHEMA matrix to the surface of calcium apatite crystals led to the formation of strong interfaces between the organic and inorganic components. The good surface bonding of HA particles to the pHEMA matrix was maintained upon freeze-drying and contributed to the ability of the freeze-dried composites to withstand hundreds of megapascal compressive stress and >80% compressive strains without exhibiting brittle fractures.

Side-by-side comparisons of the compressive stress–strain curves obtained with FlexBone composites in as-prepared [Fig. 2(A)], hydrated [Fig. 2(C)] and freeze-dried [Fig. 3(A)] states revealed convincing correlations between the content/microstructures of the mineral component and the macroscopic compressive behavior of the composite. We have shown that the stiffness of FlexBone positively correlates with the content of a given microstructure of HA, with the slope of stress–strain curves of ComHA-1-50, for instance, steeper than that of ComHA-1-37 regardless of their solvent environment (ethylene glycol or water). The same trend was also observed with FlexBone containing CalHA. In natural bone, the bending, compression and tensile moduli of compact bone have been shown to exhibit a strong positive correlation with its mineral content.^{10,37,38} Our data have also demonstrated significant impact of the size and microscopic scale aggregation (structure) of HA minerals on the bulk compressive behavior of FlexBone. Whether in as-prepared, fully hydrated or freeze-dried state, FlexBone containing porous aggregates of HA nanocrystals (ComHA) are always significantly stiffer and stronger (“stronger” in terms of their resistance to crack formation and propagation under compression) than those containing the same percentage of compact micrometer-sized CalHA. The process of solvent exchange with water did not compromise the ability of as-prepared FlexBone containing ComHA to withstand repetitive physiological compressive stress and moderate (>10%) compressive strains, a feature highly desirable for the surgical insertion and use of FlexBone as synthetic bone grafts. In contrast, hydration significantly weakened the compressive strength of Flex-Bone containing CalHA (e.g. ultimate strength <0.6 MPa in water for CalHA-1-37 and CalHA-1-50), making them less suitable for moderate weight-bearing applications *in vivo*. Poor structural integration of polymer matrices with mineral components are also known to contribute to rapid and significant degradation of the mechanical integrity of other synthetic high mineral-content composites (e.g. PLA/HA composites) in aqueous environment.³⁹

We hypothesize that the sub-micrometer scale aggregation of HA nanoparticles in the ComHA acted as “sponges”, absorbing the prepolymer hydrogel formulation and yielding larger surface contact areas between the hydrogel matrix and the ComHA crystals. The better structural integration of the organic and inorganic components has translated into a significantly reduced tendency for crack formation and propagation within the resulting composites under high compressive stress. SEM studies further elucidated that the hydrogel-infiltrated spherical aggregates of HA nanocrystals flattened into plywood-like structures upon compression, providing an important energy-dissipation mechanism for Flex-Bone under compressive stress. The enhancement of strength and ductility of ceramics and ceramic nano-composites as a function of decreasing grain sizes is a subject of intense recent investigation and debate.^{40,41} No simple extrapolation of earlier findings in either ceramic, metallic, or intermetallic systems can predict the behavior of FlexBone since the combination of the soft hydrogel with the hard apatite crystals is quite unique. However, the microstructure-compressive behavior correlation revealed in our system is reminiscent of that observed with the analogous composite in nature —bone. It is well-known that the quality of the structural integration of the hard apatite crystals with the soft collagen network on nanoscopic and microscopic levels directly impact the mechanical properties of bone.⁹ In fact, in aging bone, poorer structural integration of bone mineral with the collagen matrix is just as important as the decreasing mineral content in contributing to their weaker and more brittle mechanical properties. In the case of FlexBone, the impact of mineral microstructures on compressive behavior seems to have out-weighted that of the mineral content among the samples we examined. For instance, ComHA-1-37 is significantly stiffer than CalHA-1-50 and less likely to crack under megapascal-compressive stress in water [Fig. 2(A, C)].

Taken together, FlexBone containing ComHA exhibited tunable reversible compressive behavior in physiologically relevant environment (e.g. in water, at body temperature, and under megapascal compressive stress), making them appealing synthetic bone graft candidates.

Subcutaneous implantation of ComHA-1-40 preseeded with BMSC in rats showed that the osteoconductive composite provided a cytocompatible environment to support the attachment, penetration, and osteogenic differentiation of BMSC *in vivo*. An ideal synthetic bone graft is designed to fill an area of defect to provide immediate structural stabilization and to expedite the healing and repair of the skeletal lesion. Ideally, the synthetic grafts can be eventually remodeled and replaced by newly synthesized bone. From this perspective, biodegradability and osteoinductivity of the synthetic bone grafts are just as important as their osteoconductivity, mechanical strength, and material handling characteristics (e.g. elasticity facilitating surgical insertion). Future improvements include engineering the biodegradability of the organic matrix, enhancing the *in vivo* dissolution rate of the osteoconductive mineral component (e.g. by introducing the more soluble β -tricalcium phosphate, β -TCP,⁴² to the mineral phase), and locally retaining and releasing osteoinductive growth factors and cytokines on and from the synthetic scaffold.

CONCLUSIONS

In summary, lightweight FlexBone composites containing high percentages of HA were prepared by crosslinking HEMA in the presence of HA using ethylene glycol as a solvent. Despite their high mineral contents (37–50%), the as-prepared composites exhibited elastomeric properties and reversible compressive behavior under moderate (megapascals) compressive stress. Owing to the excellent structural integration between the apatite mineral and the pHEMA network, freeze-dried FlexBone could withstand hundreds-of-megapascals compressive stress and >80% compressive strain without exhibiting brittle fractures [Fig. 3 (A)]. We further showed that the incorporation of porous aggregates of HA nano-crystals, rather than compact micrometer-sized calcined HA, into the hydrogel matrix could effectively improve the overall stiffness of FlexBone and its resistance to crack formation and propagation under compression. Upon equilibration with water, these composites retained good structural integration, and were able to support the attachment and osteoblastic differentiation of BMSC *in vivo*. Combined with the elasticity that facilitates the easy and stable surgical insertion of FlexBone into an area of bony defect and enables better accommodation to the micro movement of bone, these osteoconductive composites can find important orthopedic applications.

More broadly, the strong organic/inorganic interface achieved with FlexBone demonstrates that non-covalent binding between apatite crystals and a highly hydroxylated organic matrix can be exploited in the rational design of bone-like composites. In addition, the mineral content-dependent and mineral microstructure-dependent compressive behavior exhibited by FlexBone underlines the importance of taking into account the combined effect of these parameters in the rational design of functional structural composites.

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References

1. Rice, DP.; Praemer, A.; Furner, S. Musculoskeletal Conditions in the United States. Chicago, IL: American Academy of Orthopedics; 1999.
2. Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville, MD: US Department of Health and Human Services, Public Health Service, Office of the Surgeon General; 2004.

3. Favus, M. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Vol. 5. Washington, DC: American Society for Bone and Mineral Research; 2003.
4. Cummings SR, Melton LJ. Epidemiology and outcomes of osteoporotic fractures. *Lancet* 2002;359:1761–1767. [PubMed: 12049882]
5. United States Census Bureau, US. Department of Commerce, US Census; 2000. http://www.allcountries.org/uscensus/201_organ_transplants_and_grafts.html
6. Bostrom MPG, Seigerman DA. The clinical use of allografts. Demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: A survey study. *HSS J* 2005;1:9–18. [PubMed: 18751803]
7. Goldberg VM, Stevenson S. Bone-graft options-Fact and fancy. *Orthopedics* 1994;17:809. [PubMed: 7800607]
8. Goldberg VM, Shaffer JW, Field G, Davy DT. Biology of vascularized bone-grafts. *Orthop Clin North Am* 1987;18:197–205. [PubMed: 3561973]
9. Weiner S, Wagner HD. The material bone: Structure-mechanical function relations. *Annu Rev Mater Sci* 1998;28:271–298.
10. Follet H, Boivin G, Rumelhart C, Meunier PJ. The degree of mineralization is a determinant of bone strength: A study on human calcanei. *Bone* 2004;34:783–789. [PubMed: 15121009]
11. Tong W, Glimcher MJ, Katz JL, Kuhn L, Eppell SJ. Size and shape of mineralites in young bovine bone measured by atomic force microscopy. *Calcif Tissue Int* 2003;72:592–598. [PubMed: 12724830]
12. El-Ghannam A. Bone reconstruction: From bioceramics to tissue engineering. *Expert Rev Med Devices* 2005;2:87–101. [PubMed: 16293032]
13. Kidane A, Szabocsik JM, Park K. Accelerated study on lysozyme deposition on poly(HEMA) contact lenses. *Biomaterials* 1998;19:2051–2055. [PubMed: 9870756]
14. Dalton PD, Flynn L, Shoichet MS. Manufacture of poly(2-hydroxyethyl methacrylate-co-methylmethacrylate) hydrogel tubes for use as nerve guidance channels. *Biomaterials* 2002;23:3843–3851. [PubMed: 12164188]
15. Lu S, Anseth KS. Photopolymerization of multilaminated poly (HEMA) hydrogels for controlled release. *J Control Release* 1999;57:291–300. [PubMed: 9895416]
16. Sefton MV, May MH, Lahooti S, Babensee JE. Making micro-encapsulation work: Conformal coating, immobilization gels and *in vivo* performance. *J Control Release* 2000;65:173–186. [PubMed: 10699279]
17. Yang JM, You JW, Chen HL, Shih CH. Calorimetric characterization of the formation of acrylic type bone cements. *J Biomed Mater Res* 1996;33:83–88. [PubMed: 8736026]
18. Prati C, Mongiorgio R, Valdrèb G, Montanaria G. Hydroxyethyl-methacrylate dentin bonding agents: Shear bond strength, marginal microleakage and SEM analysis. *Clin Mater* 1991;8:137–143.
19. Song J, Saiz E, Bertozzi CR. A new approach to mineralization of biocompatible hydrogel scaffolds: An efficient process towards 3-dimensional bonelike composites. *J Am Chem Soc* 2003;125:1236–1243. [PubMed: 12553825]
20. Song J, Saiz E, Bertozzi CR. Preparation of pHEMA-CP composites with high interfacial adhesion via template-driven mineralization. *J Eur Ceram Soc* 2003;23:2905–2919.
21. Song J, Malathong V, Bertozzi CR. Mineralization of synthetic polymer scaffolds: A bottom-up approach for the development of artificial bone. *J Am Chem Soc* 2005;127:3366–3372. [PubMed: 15755154]
22. An, YH. Mechanical properties of bone. In: An, YH.; Draughn, RA., editors. *Mechanical Testing of Bone and the Bone-Implant Interface*. Boca Raton: CRC Press; 2000. p. 41-63.
23. Phelps JB, Hubbard GB, Wang X, Agrawal CM. Microstructural heterogeneity and the fracture toughness of bone. *J Biomed Mater Res* 2000;51:735–741. [PubMed: 10880123]
24. Milne M, Kang M-I, Quail JM, Baran DT. Thyroid hormone excess increases insulin-like growth factor I transcripts in bone marrow cell cultures: Divergent effects on vertebral and femoral cell cultures. *Endocrinology* 1998;139:2527–2534. [PubMed: 9564868]

25. Drissi H, Hushka D, Aslam F, Nguyen Q, Buffone E, Koff A, van Wijnen AJ, Lian JB, Stein JL, Stein GS. The cell cycle regulator p27(kip1) contributes to growth and differentiation of osteoblasts. *Cancer Res* 1999;59:3705–3711. [PubMed: 10446985]
26. Ahmed AM, Burke DL. *In vitro* measurement of static pressure distribution in synovial joints-part I: Tibial surface of the knee. *J Biomech Eng* 1983;105:216–225. [PubMed: 6688842]
27. Brown TD, Shaw DT. *In vitro* contact stress distributions in the natural human hip. *J Biomech* 1983;16:373–384. [PubMed: 6619156]
28. Whalen RT, Carter DR. Influence of physical activity on the regulation of bone density. *J Biomech* 1988;21:825–837. [PubMed: 3225269]
29. Brand RA. Joint contact stress: A reasonable surrogate for biological processes? *Iowa Orthop J* 2005;25:82–94. [PubMed: 16089079]
30. Hodge WA, Fijan RS, Carlson KL, Burgess RG, Harris WH, Mann RW. Contact pressures in the human hip joint measured *in vivo*. *Proc Natl Acad Sci USA* 1986;83:2879–2883. [PubMed: 3458248]
31. Hodge WA, Carlson KL, Fijan RS, Burgess RG, Riley PO, Harris WH, Mann RW. Contact pressures from an instrumented hip endoprosthesis. *J Bone Joint Surg Am* 1989;71:1378–1386. [PubMed: 2793891]
32. Saha S, Pal S. Mechanical properties of bone cement: A review. *J Biomed Mater Res* 1984;18:435–462. [PubMed: 6376513]
33. Shikinami Y, Okuno M. Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): Part I. Basic characteristics *Biomaterials* 1999;20:859–877.
34. Kühn, K-D. *Bone Cements*. New York: Springer; 2000.
35. Vanhoof VO, Debroe ME. Interpretation and clinical-significance of alkaline-phosphatase isoenzyme patterns. *Crit Rev Clin Lab Sci* 1994;31:197–293. [PubMed: 7818774]
36. Farley JR, Hall SL, Herring S, Libanati C, Wergedal JE. Reference-standards for quantification of skeletal alkaline-phosphatase activity in serum by heat inactivation and lectin precipitation. *Clin Chem* 1993;39:1878–1884. [PubMed: 8375064]
37. Currey JD. The effect of porosity and mineral content on the Young's modulus of elasticity of compact bone. *J Biomech* 1988;21:131–139. [PubMed: 3350827]
38. Currey JD. Physical characteristics affecting the tensile failure properties of compact bone. *J Biomech* 1990;23:837–844. [PubMed: 2384495]
39. Russias J, Saiz E, Nalla RK, Gryn K, Ritchie RO, Tomsia AP. Fabrication and mechanical properties of PLA/HA composites: A study of *in vitro* degradation. *Mater Sci Eng C* 2006;26:1289–1295.
40. Sternitzke M. Review: Structural ceramic nanocomposites. *J Eur Ceram Soc* 1997;17:1061–1082.
41. Dollár M, Dollár A. On the strength and ductility of nano-crystalline materials. *J Mater Proc Tech* 2004;157/158:491–495.
42. Kwon S-H, Jun Y-K, Hong S-H, Lee I-S, Kim H-E. Calcium phosphate bioceramics with various porosities and dissolution rates. *J Am Ceram Soc* 2002;85:3129–3131.

Figure 1.

Microstructures and size distribution of ComHA versus CalHA powders. (A) SEM micrograph of ComHA powders showing porous aggregates of polycrystalline HA. (B) Higher resolution SEM image of the circled area in (A) showing HA crystallites ~100 nm in size. (C) Grinded CalHA powders. (D) Particle size distribution of the CalHA as determined by sedimentation measurements for particles with diameters below 10 μm. Both SEM micrograph and the sedimentation measurement plot suggested a bimodal size distribution of CalHA powders with most particles sized 5 μm or below and the larger grains over 10 μm in size

Figure 2.

Compressive behavior and composition of as-prepared versus fully hydrated FlexBone. (A) Compressive behavior of as-prepared FlexBone and pHEMA control as a function of mineral microstructure and content. Ten consecutive load-controlled loading-unloading cycles (3.0 N/min, 0.01 N to 18.0 N to 0.01 N) were applied to each specimen in ambient air using a Q800 DMA equipped with a compression fixture. (B) EDS of the cross-sections of FlexBone showing the removal of residue S-containing radical initiators upon equilibrating the as-prepared sample with water. (C) Compressive behavior of fully hydrated FlexBone and pHEMA control at body temperature as a function of mineral microstructure and content. Ten consecutive load-controlled loading-unloading cycles (3.0 N/min, 0.01 N to 10.0 N to 0.01 N) were applied to each specimen in water using a Q800 DMA equipped with a submersion compression fixture. The hydrated FlexBone containing CalHA started to fail approaching >30% compressive strain during the first force ramping (denoted by *), thus did not continue with additional loading cycles.

Figure 3.

Microstructures and compressive behavior of freeze-dried FlexBone containing ComHA versus CalHA. (A) Stress–strain curves showing freeze-dried FlexBone containing 50% ComHA is stiffer than the one containing 50% CalHA. Unconfined displacement-controlled (~ 0.015 mm/s) compression test was performed on a high capacity MTS with a 100-kN load cell. (B) and (C): SEM of the cross-section of freeze-dried CalHA-1-50 before and after being compressed. (D) and (E): SEM of the cross-section of freeze-dried ComHA-1-50 before and after being compressed. The arrows in (C) and (E) indicate the direction of compression.

Figure 4. In vivo resorption and osteogenic differentiation of bone marrow cells supported by FlexBone ComHA-1-40. (A) SEM micrograph of a composite (pre-seeded with 20,000-cells/cm² BMSC) retrieved 28 days after SC implantation in rat; (B) SEM micrograph of a composite (without pre-seeded BMSC) retrieved 14 days after SC implantation in rat; (C) XRD of the explanted sample shown in (A), with diffraction patterns matching with that of the commercial HA powder; (D) ALP staining (red) of a 12-μm frozen section of an explanted composite (pre-seeded with 5,000-cells/cm² BMSC) on day 14. Magnification: 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]