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Biocomposites of pHEMA with HA/ β -TCP (60/40) for bone tissue engineering: Swelling, hydrolytic degradation, and *in vitro* behavior

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Abstract

The field of bone and cartilage tissue engineering has a pressing need for novel, biocompatible, biodegradable biocomposites comprising polymers with bioceramics or bioglasses to meet numerous requirements for these applications. We created hydrolytically degradable hydrogel/bioceramic biocomposites, comprising poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels and 50 wt% biphasic hydroxyapatite/ β -tricalcium phosphate (60/40) through in situ polymerization. The hydrolytic degradation starts with hydrolysis of the cross-linker, *N*, *O*-dimethacryloyl hydroxylamine, which was synthesized in house. Swelling and degradation were examined in details at a phosphate buffered saline solution at 37 °C over a 12-week period of time. To vary degradability, a co-monomer, acrylic acid (AA) or 2-hydroxypropyl methacrylamide (HPMA), was introduced, coupled with altering the concentration of the cross-linker and of the bioceramic. The co-monomer HPMA was found to be more effective than AA in enhancing degradation, though AA led to greater swelling ratios. 33% of weight loss was achieved in some of the biocomposites containing HPMA. Porous structures were developed during swelling and degradation in biocomposites with AA but not in those containing HPMA, suggesting different degradation mechanisms: bulk erosion vs. bulk degradation. Good biocompatibility, as evidenced by attachment and proliferation of mouse-derived osteoblast precursor cells from the MC3T3-E1 lineage, was observed on these biomaterials, regardless of the type of the co-monomer. The

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rationale and approaches employed here open up new opportunities for creating novel, complex organic-inorganic biomaterials in orthopedic tissue engineering.

Keywords

pHEMA; hydrolytic degradation; *N,O*-dimethacryloyl hydroxylamine

1. Introduction

Synthetic biopolymer (both porous and dense) composites with bioactive fillers (such as hydroxyapatite and tricalcium phosphate) play a central role in bone tissue engineering [1–10]. Characteristics of the biocomposites, e.g., biocompatibility, biodegradability, surface chemistry and topography, and mechanical properties, largely determine bone repair and regeneration, because these characteristics have substantial influences on cells which in return govern overall bone regeneration [9]. In clinical trials of bone repair and regeneration, complete biodegradation in 4–6 months is required for the biocomposites. However, development of such biocomposites still remains a great challenge [11–15]. Part of recent efforts in the field focused on developing soft biomaterials that could be press-fitted in applications bearing low to medium loads [16–18]. To fabricate useful biomaterials for the applications, we recently took pains in synthesizing biocomposites based on poly(2-hydroxyethyl methacrylate) hydrogels and hydroxyapatite with various degradation times [19]. Our strategy was to use a hydrolytically macromer/cross-linker, dimethacrylated poly(lactide-*b*-ethylene glycol-*b*-lactide), which was synthesized in house, to trigger overall degradation of the biomaterials. This strategy allowed us to create a wide range of the biocomposites with remarkable degradation rates, particularly when we used a co-monomer, methacrylic acid, in the synthesis. However, the substantial reduction in pH during degradation, which resulted from the acidic by-products of the cross-linker [5, 6, 20, 21], posed a barrier for such biocomposites to be used in clinical trials.

To overcome the reduction in pH during degradation, which is common to most biocomposites used for bone tissue engineering [6, 20, 21], we recently concentrated our efforts to create biodegradable, flexible biomaterials comprising pHEMA hydrogels and biphasic bioceramic hydroxyapatite/ β -tricalcium phosphate (60/40) using a cross-linker, *N,O*-dimethacryloyl hydroxylamine (DMHA) which was synthesized in our laboratory. This cross-linker has been reported to degrade hydrolytically when the pH exceeds 6.5 [22–24]. While it was used as a cross-linker for synthesizing virgin pHEMA [22] and poly(2-hydroxypropyl methacrylamide) hydrogels [23, 24], respectively, interestingly it has not been reported for creating any biocomposites with bioceramics or bioactive glasses. In bone and cartilage tissue engineering, the biocomposites are more useful than virgin synthetic biopolymers, because bone regeneration would not occur without the supply of calcium and phosphate ions. The objective of this work is to report on development of useful, biodegradable, hybrid biomaterials, which are composed of pHEMA hydrogels and a bioceramic of HA/TCP(60/40). We describe the design rationale, and discuss the effects of key variables, such as the type of the co-monomer and the concentrations of the co-monomer, the cross-linker, and the bioceramics, on affecting swelling, hydrolytic degradation, morphology, mechanical properties during degradation [25–30]. We also depict biocompatibility as revealed by *in vitro* studies on selected compositions.

2. Experimental section

2.1 Materials

Chemicals were obtained mostly from Sigma-Aldrich and used without further purification unless otherwise noted.

2.2 Synthesis of the cross-linker, *N,O*-dimethacryloyl hydroxylamine

Synthesis of *N,O*-dimethacryloyl hydroxylamine has been described previously [22–24, 31]. A revised process was employed. 10 g hydroxylamine hydrochloride (0.145 mol) was added in a 250 mL round-bottom reaction flask and stirred under vacuum overnight to minimize moisture. 50 mL anhydrous pyridine was transferred into the flask via a metallic tube to dissolve the hydroxylamine hydrochloride entirely under stirring. 23.52 g methacryloyl chloride (0.243 mol) was slowly added dropwise to the solution under stirring and N₂ gas protection (the addition took 20–26 min), while maintaining the reaction temperature below 45 °C using an ice bath. When the addition of the methacryloyl chloride was completed, the reaction was maintained for an additional 4 h at room temperature under stirring and N₂ protection, until the methacryloyl chloride had entirely reacted. Monitoring of the reaction was done via thin-layer chromatography with mobile chloroform and iodine stain. 100 mL chloroform was added to dissolve the DMHA, and 21 mL saturated HCl was slowly added dropwise under stirring to turn pyridine into its chloride salt while an ice bath was employed for cooling. The whole solution was transferred into a separatory funnel, and 100 mL de-ionized water was added, followed by violent shaking upside down and a set for 30 min for separation. The oil phase (i.e., chloroform phase at the lower level) was separated and subsequently washed with 100 mL de-ionized water by portion at least four times until the aqueous phase was clear. The oil phase was then dried over magnesium sulfate and filtered under low vacuum with a fine 60 mL Buchner filter funnel. The oil phase was then vacuumed in a Schlenk line to remove chloroform, and a yellow/brown translucent oil product was obtained. 10 mL dimethyl ether was added to the oil product, followed by the addition of 20–50 mL n-hexane. The whole mixture was vacuumed under stirring in a Schlenk line to obtain an off-white crystalline solid material after drying out the solvent. The crystalline solid was washed three times with n-hexane, and vacuum dried. The yield was approximately 34%. The chemical structure of DMHA was verified via a Bruker Biospin Avance II 500 MHz NMR spectrometer.

2.3 Fabrication of degradable pHEMA/HA/TCP(60/40) biocomposites

The synthesis and fabrication of the biocomposites were carried out as follows [16, 17, 31]. The main monomer, HEMA, a co-monomer of HPMA or AA, the cross-linker of DMHA (100 mg/mL dimethyl sulfoxide), ethylene glycol, de-ionized water, and HA/TCP(60/40) were first mixed thoroughly for a minute with a VWR VDI 25 homogenizer at 9500 rpm. We then added a redox pair of free radical initiators, ammonium persulfate (480 mg/mL water) and sodium metabisulfite (180 mg/mL water), and mixed again for 30 sec. The whole mixture was quickly pumped into a BD 5-mL plastic syringe for gelling 48 h at 25 °C. A typical formulation was composed of HEMA: DMHA: ethylene glycol: water: ammonium persulfate: sodium metabisulfite = 100: 17.92: 35: 20: 5.48: 5.48 by volume. When a co-monomer, HPMA or AA, was incorporated, 5, 10, 20, 30 mol% co-monomer relative to HEMA was added. The compositions also contained various concentrations of DMHA cross-linker: 1.286, 5, 10, 20 mol% relative to the total amount of the monomer and co-monomer. Either 30 wt% or 50 wt% HA/TCP(60/40) was incorporated relative to the total weight of monomer, co-monomer, cross-linker, and HA/TCP(60/40). Two series of the hydrogel/HA/TCP(60/40) biomaterials were fabricated: one had HPMA incorporated and the other had AA as the co-monomer. The detailed formulations are listed in Table 1.

2.4 Swelling and degradation of the pHEMA/HA/TCP(60/40) biomaterials

To assess swelling and degradation behavior, the hydrogel/HA/TCP(60/40) biomaterials were cut into disk shape (12.8 mm in diameter and 3 mm thick) with a razor blade. The disk specimens (24 specimens for each composition) were put into a FreeZone® 4.5 liter freeze-dry system (Labconco Corporation, Kansas City, USA) under vacuum to obtain initial dry weights (m_{id}) after nearly two weeks. These freeze-dried specimens were then soaked in a phosphate buffered saline (PBS) solution in 20 mL vials at 37°C for swelling and degradation [32–35]. The vials were shaken twice daily. After reaching equilibrium, the swelled specimens were taken out at different time intervals (i.e., first week, second week, ..., 12th week), weighed to obtain the swelled weight (m_s), and put into a freeze-drying machine under vacuum to obtain the final dried weight (m_{fd}) in the calculation of weight loss in mass [36]. For each composition at each time interval, two specimens were used for examining swelling and degradation behavior. For the specimens composed of the AA co-monomer, the pH was adjusted to 7.4 with 0.5 N NaOH in the very beginning of swelling. The volumetric swelling ratio, Q , was calculated via the mass swelling ratio, $q = m_s/m_{fd}$, according to the following equation:

$$Q = 1 + \frac{\rho_{\text{composite}}}{\rho_{\text{solvent}}}(q - 1) \quad (1)$$

where $\rho_{\text{composite}}$ is the density of the biocomposite of pHEMA/HA/TCP(60/40), and approximated to be 1.5516 g/cm³ for the composition containing 30 wt% bioceramics and 1.8152 g/cm³ for the compositions comprising 50 wt% bioceramics, respectively. Pure pHEMA hydrogel and the two copolymer hydrogels have a density of 1.274 g/cm³ [37, 38]. In these pure hydrogels and corresponding biocomposites, the potential variation in density, arising from the change of cross-link density, is assumed to be minimal and ignored. ρ_{solvent} is the density of the buffered saline solution and was approximated as 1.0 g/cm³. The percent mass loss of each specimen was calculated according to the following equation:

$$\% \text{mass loss} = \frac{(m_{id} - m_{fd})}{m_{id}} \times 100\% \quad (2)$$

2.5 Morphology of the pHEMA/HA/TCP(60/40) after degradation for eight weeks

Degradation of the selected compositions was further evaluated through analyzing bulk morphology. The morphology of selected compositions before and after degradation for eight weeks was evaluated with a Hitachi Model S-4300SE/N scanning electron microscope operated at 10 kV. The freeze-dried specimens were fractured in liquid nitrogen, and the fractured bulk specimens were coated with gold (~2 nm thick) prior to being loaded into the SEM. Bulk (not surface) morphology was examined.

2.6 Dynamic mechanical properties of swelled pHEMA/HA/TCP(60/40) biomaterials

Dynamic mechanical properties were assessed before and after degradation for eight weeks on selected compositions. The swelled specimens were loaded onto a Triton 2000 dynamic mechanical analyzer (Triton Technology Ltd., UK) under tension in the viscoelastic regime. The dynamic mechanical properties were examined at 37°C and 0.1, 1, and 10 Hz, respectively. Storage modulus, loss modulus, and loss tangent were recorded.

2.7 Cell culture

Osteoblast precursor cells (MC3T3-E1) derived from mouse calvaria (purchased from ATCC, Cat# CRL-2593™) were cultured in T175 culture flasks (Corning) until passage 2. The cell culture was incubated at 37 °C and 5% CO₂ for the duration of the expansion. The

cells were cultured with a 30 mL growth medium—445 mL alpha MEM without nucleotides (Invitrogen, Cat# A10490-01), 50 mL 10% fetal bovine serum (JR Scientific Inc., Cat# 43640-500), 5 mL 1% penicillin/streptomycin (UCSF Cell Culture Facility, Cat# CCFGK004)—which was replaced every two days. Upon confluence, the growth medium was removed and the culture was washed twice with 15 mL calcium and magnesium free D-PBS (UCSF Cell Culture Facility, Cat# CCFAL003). Next, cells were released from the flask with 3 mL 0.05% trypsin solution for 5 min in incubation. The trypsinized cellular solution was neutralized with 12 mL growth medium to inhibit trypsin activity and transferred to a 50 mL Falcon tube (Fisher Scientific). Cells were collected by centrifugation at 800 rcf for 5 min and then re-suspended in 1 mL growth medium. To count cell numbers, a 10 μ L aliquot of suspended cells was added to 10 μ L of 0.4% Trypan blue stain (Gibco, Cat# 15250) and counted manually with cell-counting grids (Hycor, Cat# 87144); Trypan blue stain ensures only living cells were counted. The remaining suspension of cells was diluted into a solution at a concentration of 5×10^4 cells/50 μ L aliquot.

2.7.1 Preparation and seeding of degradable biocomposites with cells—The flexible biocomposites were synthesized as described above. For *in vitro* studies, we selected four compositions: (1) 20 mol% HPMA co-monomer and 1.3 mol% DMHA crosslinker, (2) 20 mol% AA co-monomer and 1.3 mol% DMHA, (3) 20 mol% HPMA co-monomer and 10 mol% DMHA, and (4) 20 mol% AA co-monomer and 10 mol% DMHA. These four compositions are highlighted and designated as # 6, 10, 14, and 18 in Table 1. The biocomposites (which were cut into disk shape) were freeze-dried at -50°C under vacuum for two weeks to remove and minimize the content of solvents and co-solvents such as water, ethylene glycol, and dimethyl sulfoxide (DMSO). The freeze-dried biomaterials were soaked in 20 mL de-ionized water for two days to dissolve unreacted monomers, cross-linker, and free radical initiator redox, followed by freeze-drying at -50°C under vacuum for one week. The freeze-dried specimens were sterilized for 30 min under ultraviolet light, hydrated with purified water for 4 h, and cut into uniform 4 mm $\times$ 4 mm sections. To seed the flexible specimens, a 5×10^4 cells/50 μ L aliquot of suspended cellular solution was pipetted onto each specimen in the well of a 24-well plate (Becton, Dickinson). For the positive control, a Thermanox plastic coverslip (Nalge Nunc) was seeded in the same method. Finally, 1 mL of growth medium was added to each well. Growth medium was changed every two days during culture.

2.7.2 Proliferation assay—Prior to the proliferation assay, the growth medium was changed to ensure a uniform environment across time points. 100 μ L MTT solution (Roche, Cat# 11465007001) was added to each well and incubated for 4 h. 1 mL of MTT solvent (0.1N HCl in anhydrous isopropanol) was added and incubated for another 4 h. At this time, the centrifuge tubes were vortexed for 5 sec. Two 100 μ L samples of solution per composition were transferred to separate wells of a 96-well plate and the absorbance was measured at 562 nm (BioRad Microplate Reader). Each composition type and positive control was done in triplicate on Day 1 and 4.

2.7.3 Attachment assay—Four hours after the initial seeding of specimens and positive control, the solution from each well was retrieved and transferred to a Falcon tube (Fisher Scientific). This was centrifuged at 800 rcf for 5 min and the supernatant was aspirated. The cells were re-suspended in 10 μ L of growth medium and manually counted with cell-counting grids (Hycor). Each composition type and positive control was done in triplicate.

2.7.4 Morphology of cell-covered specimens—To observe cell attachment, the specimens were collected on Day 4 and fixed in 2.5% glutaraldehyde (Sigma-Aldrich, #G5885) overnight at 4°C after being rinsed in PBS for 5 min. The specimens were rinsed

again in distilled water three times for 10 min each and dehydrated in graded series of ethanol (50%, 70%, 90%, 95%, and 100%) for two 5-min periods each. The specimens were then transferred in hexamethyldisilazane (Sigma-Aldrich, Cat# 52619) for 5 min before air drying under the hood. Cell attachment was examined on the cell-covered specimens, which were sputter-coated with gold under vacuum in advance, with a Hitachi Model S-4300SE/N scanning electron microscope operated at 15 kV.

3. Results

3.1 Effects of a co-monomer and the concentration of the cross-linker on swelling

To tune the swelling and degradability of biomaterials, we purposely incorporated a co-monomer. Our objective was to enhance the hydrophilicity of the backbone of the hydrogel chains to attract more water for swelling and degradation. We used HPMA and AA as the co-monomers, as they are known to be more hydrophilic than HEMA monomer [23, 24, 39]. We evaluated the role of a co-monomer in swelling and degradation while keeping the cross-linker content constant, i.e., 1.3 mol% (see Table 1). The effects of the co-monomer on swelling are shown in Figure 1.

As expected, incorporation of co-monomers results in significant swelling; a higher content of the co-monomer leads to a higher degree of swelling. The volumetric swelling ratio increases much more in biomaterials containing AA (max. 19.5) than those with HPMA (max. 3.3); in the composition with 5 mol% AA, the volumetric swelling ratio remains nearly unchanged after three weeks, while it monotonically increases in compositions with a greater amount of AA. However, for the biocomposites with HPMA, the volumetric swelling ratio remains nearly the same after three weeks regardless of the composition. These differences are attributed to the anionic nature of AA, allowing it to attract a significant amount of water during swelling. While the co-monomer HPMA is more hydrophilic than HEMA, it is far less hydrophilic than AA. Thus, it is not surprising that the compositions with HPMA have much lower volumetric swelling ratios.

The effects of cross-linker concentration on swelling are shown in Figure 2, where the concentration of the co-monomer AA or HPMA is fixed at 20 mol%. Different trends appear in these two series of biocomposites.

In the case of AA, the composition with 1.3 mol% DMHA exhibits a greater swelling ratio than do the other compositions with 5, 10, and 20 mol%, respectively, in the first seven weeks. After that, the composition containing 20 mol% DMHA shows the greatest swelling ratio, followed by the compositions with 1.3, 10, and 5 mol%, respectively. For the biomaterials containing HPMA as the co-monomer, the more DMHA is incorporated, the greater the volumetric swelling ratio is. These two series of biomaterials show unusual swelling behavior; for a hydrogel without incorporation of any bioceramics such as HA, a greater cross-linker content (presumably higher crosslink density) leads to a lower level of swelling as a result of a smaller mesh size in the 3-D network. Two major reasons might be responsible for the unusual swelling behavior. First, during polymerization, the amount of the solvents (DMSO, water, and ethylene glycol) can result in more cyclization of the cross-linker, in which the cross-linker is cycled back as a co-monomer to participate in copolymerization rather than crosslinking [40]. As a result, the crosslink density is lowered. A greater content of the cross-linker may still lead to a higher crosslink density, but the crosslink density does not vary very much. Second, when a higher content of the cross-linker (i.e., DMHA) is employed, the formed composite material is fragile under cutting with a razor blade compared with those with lower contents. The biomaterials were broken into small pieces after swelling for a few days. The significantly increased surface area-to-

volume ratios of the small pieces considerably enhance swelling. In addition, the differences in degradation mechanisms in these two series of biomaterials may influence the swelling.

3.2 Effects of the content of HA/TCP(60/40) on swelling of biocomposites

Apart from the roles of the co-monomer and cross-linker, we explored the effects of the concentration of HA/TCP(60/40) on the swelling of biocomposites. The concentration of HA/TCP(60/40) not only determines swelling and degradation of biomaterials, but also dictates mechanical properties and cell responses both *in vitro* and *in vivo*. Figure 3 shows the effects of HA/TCP(60/40) content on swelling. For the biocomposites with AA incorporated, the swelling ratio increases monotonically, while it remains nearly unchanged for the biomaterials with HPMA. Similar to typical rubbers with different concentrations of carbon black, a higher content of HA/TCP(60/40) leads to a lower swelling ratio, as might be expected. The HA/TCP(60/40), which is incorporated into the pHEMA hydrogel, acts as an expansion barrier for the 3-D network of the hydrogel. The effect of HA/TCP(60/40) is more significant for the system with AA than the one with HPMA.

3.3 Effects of the co-monomer on hydrolytic degradation of biocomposites

The effects of the co-monomer (AA or HPMA) on hydrolytic degradation are shown in Figure 4, in which the content of the cross-linker is fixed at 1.3 mol% in all compositions. The role of AA is observed to be minimal in affecting degradation. The biocomposites with AA lose about 16–18 wt% mass irrespective of AA content. When HPMA is the co-monomer, the more it is incorporated, the more the weight loss reaches. Mass loss remains nearly unchanged after three weeks for compositions with 5 and 10 mol% HPMA, while it progressively increases with time for the other two compositions with 20 and 30 mol% HPMA. As shown above, the biocomposites with AA exhibit much greater volumetric swelling ratios than do those with HPMA. Typically, for degradable hydrogels, greater swelling ratios lead to greater mass loss, which serves as the basis for us to incorporate AA. Here, an opposite trend emerges. This opposite trend may be understood through analyzing relative hydrophilicity of main monomer, the co-monomers, and the cross-linker. The hydrophilicity follows the order: AA $\gg$ HPMA > HEMA > DMHA. AA is far more hydrophilic than the other three organic molecules. The more hydrophilic the biomaterial, the more water the biomaterial attracts for swelling and degradation. Hydrolytic degradation starts with cleavage of the cross-linker. If the cross-linker molecules do not have enough water for continuous cleavage, the degradation of biocomposites would not occur significantly. While big swelling ratios are seen for the biocomposites with AA, more water molecules surround AA and thus relatively less water molecules might be available for the cross-linker in the 3-D networks. When AA content is high, e.g., 30 mol%, it may considerably pull back water molecules from DMHA. In addition, the strong ionic interaction of AA with HA/TCP(60/40) leads to lowered solubility of HA/TCP(60/40), which in part slows down degradation. For the biomaterials with HPMA incorporated, the differences in hydrophilicity between HEMA, HPMA, and DMHA are not great, and thus the increased swelling ratios, as a result of incorporation of HPMA, impart sufficient water for the cross-linker to continue cleavage. Hence, incorporation of HPMA considerably enhances mass loss.

3.4 Effects of cross-linker content on hydrolytic degradation

The effects of cross-linker content on degradation of biocomposites are shown in Figure 5. In all compositions, the content of the co-monomer is fixed at 20 mol%. The role of cross-linker content is negligible when its content is below 20 mol% in both series of biocomposites. The composition with 20 mol% co-monomer exhibits the slowest degradation, regardless of the co-monomer type, which is primarily ascribed to the highest

crosslink density. The mass loss remains nearly unchanged for all compositions with AA, while progressive weight loss emerges for the biomaterials containing HPMA.

3.5 Effects of HA/TCP(60/40) content on degradation of biocomposites

The effects of HA/TCP(60/40) content on degradation are shown in Figure 6. The co-monomer (AA or HPMA) content is fixed at 20 mol% in all cases. As expected, a higher content of HA/TCP(60/40) leads to less mass loss, irrespective of the type of a co-monomer. At the same contents of HA/TCP(60/40), the biocomposites containing HPMA always show greater weight loss than those with AA. There are at least two major roles for HA/TCP(60/40) to play in degradation. First, HA/TCP(60/40) restrains water uptake to the hydrogel and lowers the degradation rate. Second, the low solubility of HA/TCP(60/40) drags down the overall degradation of biocomposites. The more HA/TCP(60/40) is used, the more significant are these roles in slowing down the degradation.

3.6 Comparison of morphology of biocomposites before and after degradation for eight weeks

Examination of the morphology of the freeze-dried biocomposites before and after degradation for a certain period of time may provide insight into degradation mechanisms. During degradation, new micro-structures may emerge. Here, we compare the morphological evolution after eight-week degradation between two compositions with 50 wt % HA/TCP(60/40): poly(HEMA-*co*-AA) vs. poly(HEMA-*co*-HPMA) where the co-monomer (AA or HPMA) content is fixed at 20 mol%. The cross-section of a freeze-dried specimen is examined. Figure 7 shows comparison of the morphology between these two compositions. Prior to degradation, the morphology is rather similar: HA/TCP(60/40) is well dispersed in the hydrogel. The composition with HPMA appears to exhibit more uniform dispersion. After eight weeks of degradation for the biocomposite containing HPMA, its morphology appears similar to the one prior to degradation, except for a narrower distribution of HA/TCP(60/40) particles. For the biocomposite with AA, a highly porous structure is seen. Again, a narrower distribution of HA/TCP(60/40) particles emerges. The highly porous structure results dominantly from the huge amount of water uptake (volumetric swelling ratio of ~15.5) during degradation. The narrower particle distribution may be understood by the fact that small particles have greater diffusion coefficients and thus may be dissolved more easily than big ones. The differences in morphology between these two biocomposites imply distinct degradation mechanisms: bulk degradation for the biomaterial with HPMA vs. bulk erosion for the material with AA.

3.7 Comparison of dynamic mechanical properties before and after degradation for eight weeks

Mechanical properties are one of the key factors in the design of the biocomposites. It is always useful to know how mechanical properties of the biocomposites vary with degradation, because mechanical properties of tissue scaffolds directly influence the responses of cells and ingrowth of new bone tissues. We evaluated dynamic mechanical properties in selected compositions by examining swollen specimens at zero and eight weeks of swelling and degradation. Figure 8 shows storage moduli as a function of frequency. Storage moduli increase with frequency. The increase is more pronounced for pure pHEMA gel, pure poly(HEMA-*co*-HPMA)(80/20) gel, and the biocomposite composed of HEMA/HPMA=80/20 and 50 wt% HA/TCP(60/40). Eight-week degradation leads to a significant decrease in storage moduli. The decrease results from the overall effects of swelling and mass loss. Since the compositions with AA have much greater volumetric swelling ratios than the compositions with HPMA and pure gels, storage moduli are much more lowered.

3.8 Cell attachment and proliferation

Attachment and proliferation of the mouse-derived osteoblast precursor cells were assessed on the aforementioned four compositions, as shown in Figure 9. These four compositions show attachment ratio ranging from 0.85 to 0.90, while the attachment ratio reaches 0.975 for the positive control (Figure 9(a)). Here, the attachment ratio is defined as 1 minus the ratio of cells counted in solution versus total cells seeded. The data show that nearly all seeded cells (97.5%) attached to the positive control; however, only about 85–90% of seeded cells attached to the biocomposites, with 10–15% of the seeded cells remaining in the solution. This is not surprising because the positive control, i.e., polylysine, is known to increase the number of positively charged sites available for cell binding. Figure 9(b) compares proliferation of the attached cells among the biomaterials on Day 1 and Day 4. The cells proliferate significantly on Day 4 in all compositions. Interestingly, the proliferation of the cells is biggest for the composition with 20 mol% AA co-monomer and 10 mol% DMHA cross-linker, while it reaches the smallest for the composition with 20 mol% AA co-monomer and 1.3 mol% DMHA. Since these biocomposites have smaller attachment ratios (0.85–0.9) than does the positive control (0.975), the proliferation on these four compositions is underestimated to some degree; with the same attachment ratio as the positive control, the biocomposite, composed of 20 mol% AA and 10 mol% DMHA, perhaps exhibits greater proliferation than the positive control, based on what is observed on Day 4. Cell attachment is further confirmed and shown in Figure 10. These SEM photomicrographs show that the osteoblast precursor cells attach to the surface of the biomaterials regardless of the composition, although the extensive porous structures are seen in the biocomposite with 20 mol% AA as the co-monomer. All these *in vitro* results imply good biocompatibility of the biocomposites.

4. Discussion

Considering the key variables affecting the hydrolytic degradation of the biocomposites, we have synthesized and fabricated numerous biocomposites of pHEMA with HA/TCP(60/40). This approach allowed us to examine the roles of key parameters in determining swelling, degradation, morphological evolution, dynamic mechanical properties, and biocompatibility. We have demonstrated how our rationale and methodologies were applied for creating hydrolytically degradable, flexible biocomposites composed of HEMA, a co-monomer HPMA or AA, and 50 wt% HA/TCP(60/40). Our rationale works in all cases. The degradable cross-linker, DMHA, is shown to work well in breaking down the 3-D network of hydrogel for degradation. Since our ultimate goal is to apply these novel biomaterials *in vivo* and even in preclinical trials, a variety of factors would have to be carefully considered prior to such applications.

In the synthesis of these biocomposites, we used DMSO, ethylene glycol, and water as solvents and co-solvents. DMSO and ethylene glycol are toxic to cells to some degree. These solvents can be easily removed prior to implantation to minimize the effect of the solvents by freeze-drying and re-soaking in water. While we employed ethylene glycol as a co-solvent, it is not essential and may be replaced with ethanol and other solvents. Hydrolytic degradation occurs on the condition that the molecular weight of linear pHEMA chains is below 8–10k Da [41]. This requires a careful design of the concentration of the free radical initiator, $(\text{NH}_4)_2\text{S}_2\text{O}_8$, and the reducing agent, $\text{Na}_2\text{S}_2\text{O}_5$, and more importantly the free radical initiator/reducing agent ratio. Although the boundaries in the concentration of free radical initiator and reducing agent have not been established for the critical 8–10k Da molecular weight of pHEMA hydrogel, the reducing agent has been reported to play a dominant role in determining the molecular weight of hydrogels such as poly(N-isopropylacrylamide) and poly(acrylic acid) [42]. For the hydrogels synthesized with the initiator redox couple $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{Na}_2\text{S}_2\text{O}_5$, the molecular weight is found to decrease with

an increase in the concentration of $\text{Na}_2\text{S}_2\text{O}_5$. In our formulations, we purposely fixed the concentration of ammonium persulfate at 0.524 mol% relative to the monomers and $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{Na}_2\text{S}_2\text{O}_5 = 1/2$ by weight (1/2.4 by mole), since we knew that such concentration and ratio led to degradation. In addition, the concentration of $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{Na}_2\text{S}_2\text{O}_5$ are important to uniformly mix the components and transfer the mixture into a syringe for gelling. Depending on the concentration of the initiator redox couple $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{Na}_2\text{S}_2\text{O}_5$ and the ratio between them, the components may start gelling significantly during mixing even for 1–2 minutes at the homogenizer, which prevents uniform dispersion of HA/TCP(60/40) and subsequent transferring. Hence, at least a few minutes are required for mixing and transferring prior to gelling.

The method we employed in this work has been shown to be effective in creating biocompatible, hydrolytically degradable pHEMA-based biocomposites with 50 wt% HA/TCP(60/40). In the design of synthesizing these biocomposites, we do not intend to create biomaterials to replace PLA or PLGA/HA biocomposites for bone repair and regeneration [20, 21], for our biomaterials are designed to be flexible and thus have lower moduli and strengths than those based on PLA or PLGA. Rather, we designed the biomaterials to complement PLA or PLGA/HA biocomposites with applications that they fail to provide. Our flexible, bone-like biocomposites are useful for applications that do not require high moduli (e.g., 10 MPa) and strength. During synthesis, swelling, and degradation of the biocomposites, we found that the hydrogels had strong interaction with the bioceramic, HA/TCP(60/40). The bioceramic did not disintegrate from the hydrogel matrix even after being freeze-dried. The strong interaction between the hydrogel and bioceramic exerts significant impacts on swelling and degradation. The variations of the interaction as a result of introduction of the co-monomers, AA and HPMA, also influence swelling and degradation of biomaterials. Ca^{2+} ions are known to form complexation with hydroxyl and carboxylic groups of hydrogels [43]. More recently, Ca^{2+} ions have been proposed to form complexation with oxygen atoms of pHEMA [44]. Apparently, the interaction with carboxylic groups is much stronger than other interactions. We hypothesize that the strong interaction with carboxylic groups of the pHEMA hydrogel dominantly retards degradation of poly(HEMA-*co*-AA)-based biocomposites. In comparison with the biocomposites containing poly(HEMA-*co*-HPMA), the compositions with AA always exhibit less mass loss at the same concentrations of the co-monomer. This is unusual because significant swelling typically leads to great mass loss. While the less hydrophilic nature of the cross-linker (DMHA) than that of AA plays some role in slowing down degradation, we believe that the strong and favorable interaction of AA with Ca^{2+} ions plays a dominant role in deferring degradation.

While AA, as a co-monomer, resulted in greater degree of swelling and less weight loss compared with its counterpart HPMA, it generated useful porous structures during swelling and degradation. The large swelling ratio is perhaps not desired for dense bone implants; however, it is useful for applications that require encapsulation of cells within the hydrogel. The porous structures, which were not originally created during formulation but solely generated during both later swelling and degradation, enable fast transport of nutrients, metabolites, gases, and cells throughout biomaterials. Although only dense biocomposites were synthesized in this work (in which every composition was dense prior to swelling and degradation, but the compositions with AA became macro-porous and compositions of HPMA were still dense after degradation as shown in Figure 7), macro-porous biocomposites prior to degradation may be created by modifying the current approach via incorporating a porogen such as NaCl or altering the free radical initiator redox couple [22, 45]. The additional porous structures, which may be obtained during swelling and degradation, are even more important to cell survival and new bone tissue regeneration,

since the macro-porous structures from a porogen would easily become smaller and pose limitations to new bone ingrowth as bone regeneration progresses.

The degradable cross-linker, DMHA, is effective in triggering degradation. About 33 wt% mass loss is observed for the composition comprising 30 mol% HPMA and 50 wt% HA/TCP(60/40) after 12-week degradation. However, only around 18% mass loss is obtained for the biocomposite with 20 mol% AA. The hydrolytic degradability may be tuned greatly to meet various applications requiring different degradation times. Based on the results of this work, we may vary the degradability by incorporating more HPMA co-monomer (e.g., 50 mol% and above) or less HA/TCP(60/40). Use of a bioceramic (e.g., tricalcium phosphate) or bioglass with greater solubility is of help in enhancing the degradability. In addition, a slight increase in pH value (e.g., pH = 8.0) should greatly boost the overall degradability though doing so would lower the solubility of the bioceramic to some extent [24].

For *in vivo* applications and preclinical trials, it is important to know the variation of pH of the solution during swelling and degradation of these biocomposites. We monitored the evolution of the pH of the PBS during the 12-week degradation time for each composition. For the composition without incorporation of a co-monomer (HPMA or AA) or HA/TCP(60/40) (i.e., Pure gel 1 (HEMA) in Table 1), the pH is only slightly lowered, ranging from 7.14–7.31, indicating that the cross-linker, DMHA, has a minimal effect on pH. Incorporation of the co-monomer of HPMA without the use of HA/TCP(60/40) (i.e., Pure Gel 2 (HPMA/HEMA) in Table 1) appears to further slightly lower the pH, ranging from 6.89–7.22, which is independent of degradation time. In these two cases without incorporation of the bioceramic HA/TCP(60/40), the reduction of pH is minimal. For the biocomposites containing the HPMA co-monomer (5–30 mol%) and 50 wt% HA/TCP(60/40), the pH is lowered from 7.4 to 6.24–6.91. The increase in HPMA concentration leads to a slight reduction in the pH. The alteration of the pH does not seem to depend on degradation time. For the biocomposites composed of 20 mol% HPMA but with various concentrations of the DMHA cross-linker, the pH is reduced to 6.13–6.92 from 7.4 and does not seem to depend on the concentration of DMHA, although the composition with 20 mol% DMHA has a little less reduction in pH than the other three compositions with 1.3, 5, and 10 mol% DMHA. For the biocomposites with the co-monomer AA, the final pH falls into the range of 6.74–8.44, which is greater than that with the HPMA co-monomer. This different trend largely results from the fact that the initial pH prior to swelling/degradation was adjusted a few times toward 7.4. Since the initial pH was adjusted to be a little higher than 7.4, it is likely that the starting pH for swelling and degradation after the adjustment was a little higher than 7.4. Based on the observation, the pH of the biocomposites with HPMA is a little lower than that for the biocomposites containing AA. Overall, the pH of the PBS solution is only slightly reduced.

5. Conclusions

Swelling and hydrolytic degradation of the bone-like biocomposites comprising pHEMA hydrogel and bioceramic HA/TCP(60/40) have been investigated, based on the rationale that a hydrolytically degradable cross-linker first decomposes by hydrolysis, which in return destroys the 3-D network of a hydrogel and simultaneously triggers the overall degradation of the biocomposites. A hydrolytically degradable cross-linker, *N,O*-dimethacryloyl hydroxylamine, has been synthesized and used in creating two series of degradable, bone-like biocomposites composed of pHEMA hydrogel and 50 wt% HA/TCP(60/40) for bone and cartilage tissue engineering through pre-dispersing the HA/TCP(60/40) and *in situ* polymerization. Swelling and hydrolytic degradation of the biomaterials in PBS solution were examined in a 12-week time frame. A variety of factors were investigated to determine their effects on swelling and degradation. These factors include the type of the co-monomer

(HPMA vs. AA), the concentration of the co-monomer, the concentration of the cross-linker DMHA, and the content of the bioceramic HA/TCP(60/40). In addition, the effects of swelling and degradation on dynamic mechanical properties were also explored.

Incorporation of a co-monomer HPMA or AA greatly increases the swelling and degradation of the biocomposites. While both co-monomers are found to elicit a considerably increased degree of swelling, AA is shown to be more effective than HPMA. The increased swelling is ascribed to the increased hydrophilicity of the hydrogel as a result of the introduction of the co-monomer, as HPMA and AA are more hydrophilic than the main monomer, HEMA. While the volumetric swelling ratio of the biomaterials containing AA is much greater than those with HPMA, more mass loss is observed in the latter than in the former at the same concentrations of the co-monomer. This unusual trend may be mainly attributed to strong interaction of anionic AA with the bioceramic HA/TCP(60/40), which may pull back this bioceramic from dissolution. In addition, the anionic nature of AA attracts more water for uptake than does the cross-linker DMHA in the 3-D network of the hydrogel; this is believed to be partly responsible for this unusual trend. The increase in co-monomer concentration leads to an increase in the swelling ratio regardless of the type of the co-monomer. The volumetric swelling ratio also depends on the concentration of the cross-linker DMHA and the content of HA/TCP(60/40). The effects of cross-linker concentration on swelling ratio are complex; for co-monomer HPMA, the increase in the concentration of the cross-linker results in an increase in swelling ratio. That is not the case for the biocomposites containing AA, where the trend is nearly the opposite during the first five-week degradation. The increase in the content of HA/TCP(60/40) leads to a reduction in the volumetric swelling ratio.

Degradation is found to depend strongly on several factors. The increase in the concentration of the HPMA co-monomer results in an increase in mass loss, while the mass loss is not generally affected by the concentration of the AA co-monomer. In general, the increase in the content of the cross-linker leads to a reduction in weight loss regardless of the type of the co-monomer. An increase in the concentration of bioceramic HA/TCP(60/40) results in a decrease in weight loss. In all cases, the biocomposites with HPMA exhibit greater mass loss than do those containing AA at the same concentrations of the co-monomer. This is attributed to the strong interaction of the co-monomer with the bioceramic HA/TCP(60/40), which is believed to restrain the bioceramic from dissolution. Among all biocomposites, the maximum weight loss of 33 wt% is achieved in the biocomposite with 30 mol% HPMA and 50 wt% of HA/TCP(60/40) or in biocomposites with 20 mol% HPMA and 30 wt% HA/TCP(60/40) after a 12-week degradation. The swelling and degradation of the biocomposites with AA generate unusual porous structures that do not emerge in the biocomposites containing HPMA. The generated porous structures imply different degradation mechanisms between them: bulk erosion for biocomposites with AA versus bulk degradation for those with HPMA. Good biocompatibility through cell attachment and proliferation is observed in four biocomposites with 50 wt% HA/TCP(60/40). The rationale and approaches employed in this work offer new paths in creating novel, complex organic-inorganic biocomposites for bone tissue engineering.

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Figure 1.
Effects of the concentration of co-monomers on swelling: (a) AA, and (b) HPMA

Figure 2.
Effects of cross-linker content on swelling: (a) co-monomer AA, and (b) co-monomer HPMA

Figure 3.
Effects of the concentration of HA/TCP(60/40) on swelling

Figure 4.
Effects of the concentration of co-monomers on mass loss: (a) AA, and (b) HPMA

Figure 5.
 Effects of cross-linker content on mass loss: (a) co-monomer AA, and (b) co-monomer HPMA

Figure 6.
Effects of HA/TCP(60/40) concentration on mass loss

Figure 7. Comparison of morphology before and eight degradation for 8 weeks: (a) 0 week, poly(HEMA-co-AA), (b) 0 week, poly(HEMA-co-HPMA), (3) 8 weeks, poly(HEMA-co-AA), and (d) poly(HEMA-co-HPMA)

Figure 8.
Comparison of storage moduli before and after degradation for eight weeks

Figure 9. Cell attachment ratio and proliferation on the four compositions: (a) attachment ratio, and (b) comparison of proliferation between Day 1 and Day 4

Figure 10.

SEM photomicrographs of mouse derived osteoblast precursor cells from the MC3T3-E1 attached biocomposites composed of: (a) 20 mol% HPMA and 1.3 mol% DMHA and, (b) 20 mol% AA and 1.3 mol% DMHA

Table 1

Formulations of biocompatible, hydrolytically degradable biocomposites based on pHEMA with 50 wt% HA/TCP(60/40)

No. of compositions	Designation of compositions	AA (mol%)	HPMA (mol%)	HEMA (mol%)	DMHA (mol%)
1	Pure gel 1 (HEMA)	0	0	100	1.3
2	Pure gel 2 (HPMA/HEMA)	0	20	80	1.3
3	Pure gel 3 (AA/HEMA)	20	0	80	1.3
4	AA0-HPMA5-HEMA95-DMHA1.3	0	5	95	1.3
5	AA0-HPMA10-HEMA90-DMHA1.3	0	10	90	1.3
6	AA0-HPMA20-HEMA80-DMHA1.3	0	20	80	1.3
7	AA0-HPMA30-HEMA70-DMHA1.3	0	30	70	1.3
8	AA5-HPMA0-HEMA95-DMHA1.3	5	0	95	1.3
9	AA10-HPMA0-HEMA90-DMHA1.3	10	0	90	1.3
10	AA20-HPMA0-HEMA80-DMHA1.3	20	0	80	1.3
11	AA30-HPMA0-HEMA70-DMHA1.3	30	0	70	1.3
12	AA0-HPMA20-HEMA80-DMHA1.3 (30 wt% HA/TCP(60/40))	0	20	80	1.3
13	AA0-HPMA20-HEMA80-DMHA5	0	20	80	5
14	AA0-HPMA20-HEMA80-DMHA10	0	20	80	10
15	AA0-HPMA20-HEMA80-DMHA20	0	20	80	20
16	AA20-HPMA0-HEMA80-DMHA1.3 (30 wt% HA/TCP(60/40))	20	0	80	1.3
17	AA20-HPMA0-HEMA80-DMHA5	20	0	80	5
18	AA20-HPMA0-HEMA80-DMHA10	20	0	80	10
19	AA20-HPMA0-HEMA80-DMHA20	20	0	80	20