Bioresponsive Phosphoester Hydrogels for Bone Tissue Engineering

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ABSTRACT

Bioresponsive and intelligent biomaterials are a vehicle for manipulating cell function to promote tissue development and/or tissue engineering. A photopolymerized hydrogel based on a phosphoester–poly(ethylene glycol) polymer (PhosPEG) was synthesized for application to marrow-derived mesenchymal stem cell (MSC) encapsulation and tissue engineering of bone. The phosphor-containing hydrogels were hydrolytically degradable and the rate of degradation increased in the presence of a bone-derived enzyme, alkaline phosphatase. Gene expression and protein analysis of encapsulated MSCs demonstrated that PhosPEG–PEG cogels containing an intermediate concentration of phosphorus promoted the gene expression of bone-specific markers including type I collagen, alkaline phosphatase, and osteonectin, without the addition of growth factors or other biological agents, compared with pure poly(ethylene glycol)-based gels. Secretion of alkaline phosphatase, osteocalcin, and osteonectin protein was also increased in the PhosPEG cogels. Mineralization of gels increased in the presence of phosphorus in both cellular and acellular constructs compared with PEG gels. In summary, phosphate-PEG-derived hydrogels increase gene expression of bone-specific markers, secretion of bone-related matrix, and mineralization and may have a potential impact on bone-engineering therapies.

INTRODUCTION

BIOMATERIALS ARE PLAYING an ever-increasing role in biomedical applications, ranging from devices to drug delivery and tissue-engineering matrices.[1] Researchers have been incorporating biological signals into biomaterials to control surrounding cell and tissue behavior to enhance tissue regeneration. Biomaterials can also be designed to adjust their degradation profile or release of biological agents in response to their environment. These materials, referred to as intelligent or bioresponsive materials, are capable of exerting influence on cells and tissues by modulating their gene expression, matrix organization, and proliferation. Biomaterials for tissue-engineering scaffolds would also benefit from incorporation of bioresponsive elements. Ideally, scaffolds placed in complex *in vitro* or *in vivo* environments with or without cells should provide an initial framework and appropriate signals to promote tissue development. As cells lay down newly synthesized matrix, the scaffold needs to degrade at a similar rate to make space for the developing tissue. If the degradation is too fast, the nascent tissue will not survive incubation or implantation. One possible solution for providing quality scaffold in tissue engineering is to design bioresponsive and intelligent material that will promote tissue development while matching its degradation rate with the deposition of new tissue.

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This work investigates the development of bioresponsive materials for bone regeneration. Studies by Hubbell and colleagues have demonstrated the ability to regenerate bone using intelligent, bioresponsive materials that degrade in response to cell migration and release growth factors to promote tissue growth.[2,3] Longaker and co-workers have also demonstrated the requirement for hydroxyapatite in the scaffold for bone formation using adipose-derived stem cells.[4,5] In this study we create a hydrogel with both bioresponsive degradation and enhanced mineralization by incorporation of a phosphoester in poly(ethylene glycol)-based hydrogels. Biomaterials containing phosphate derivatives have been synthesized to promote mineralization for applications in dentistry and for applications in drug delivery, where they provide highly regulated degradation and release profiles.[6,7]

Our previous studies investigated the use of hydrogels for cell encapsulation and tissue-engineered cartilage *in vitro* and *in vivo*.[8,9] In this study we have designed a poly(ethylene glycol)-phosphoester polymer, PhosPEG, that can form a hydrogel and encapsulate cells for tissue engineering of bone. Mesenchymal stem cells (bone marrow-derived stem cells, MSCs) were utilized as a bone precursor cell. MSCs are pluripotent progenitor cells capable of regenerating musculoskeletal tissues including cartilages, bones, muscles, tendons, and ligaments.[10] MSCs were first expanded in monolayer and then injected, as a suspension in a biomimetic hydrogel, into a defect. In this case, biomaterials have the dual roles of mechanically containing and creating an appropriate microenvironment for tissue development. Accordingly, the bioresponsive and intelligent material properties of the PhosPEG gels were evaluated by studying (1) gene expression of bone markers, (2) degradation response to enzymes, and (3) mineralization pattern.

MATERIALS AND METHODS

Cell isolation and osteogenic cultivation

Primary goat mesenchymal stem cells (gMSCs) were isolated from the femur bone marrow of adult male goats as previously described by Williams *et al.*[11] Cells were expanded in monolayer in MSCGM (MSC growth medium; Cambrex Bio Science Walkersville, Walkersville, MD) with 10% fetal bovine serum (FBS) and passage 4 cells were employed for three-dimensional encapsulation in hydrogels.

The osteogenic medium (OM)[10,12] is composed of high-glucose Dulbecco's modified Eagle's medium (DMEM) (without sodium pyruvate; GIBCO, Grand Island, NY), 100 nM dexamethasone (Sigma, St. Louis, MO), 50 μM ascorbic acid 2-phosphate (GIBCO), 10 mM β-glycerophosphate (Sigma), 10% FBS (BD Biosciences Discovery Labware, Bedford, MA), and peni-

cillin (100 units/mL) and streptomycin (100 μg/mL) (GIBCO). Hydrogel samples were cultured in osteogenic medium and placed on an orbital shaker (70 rpm) at 37°C in 5% CO_2 for up to 3 weeks. MSCGM (Poietics, Cambrex Bio Science, Walkersville) was used for control cultures.

Hydrogels and cell encapsulation

The newly synthesized degradable PhosPEG hydrogel was constructed from the macromer precursor of poly-(ethylene glycol)-di-[ethylphosphatidyl(ethylene glycol) methacrylate] (MW 3400 Da).[9] As the nondegradable control, commercially available poly(ethylene oxide) diacrylate gel (PEODA, MW 3400 Da; Nektar Therapeutics, Huntsville, AL) was directly utilized. Cells (gMSCs) were resuspended in macromer solutions (phosphate-buffered saline, PBS) together with photoinitiator Irgacure D-2959 (Ciba-Geigy, Tarrytown, NJ)[13] to make a final concentration of 20 million/mL. Ultraviolet (UV) irradiation (365 nm, 4 mW/cm^{2}) was applied for 5 min to gelate the cell–polymer mixture. Three cellular groups were prepared: a 20% (w/v) PhosPEG gel ("PhosPEG"), a cogel of 10% PhosPEG and 10% PEODA ("cogel"), and a 10% PEODA gel ("PEODA"). As acellular controls, three corresponding empty hydrogel groups were also prepared.

Mineralization and degradation

After equilibration in deionized water overnight, the acellular hydrogel constructs were incubated in OM and PBS (pH 7.4). The 3-week dry weight variation of each sample was kinetically monitored and recorded. As a control, the acellular gels were also incubated in PBS (pH 7.4) supplemented with alkaline phosphatase (ALP, 50 units/mL; Sigma) for 6 weeks.

After harvest, hydrogel constructs were lyophilized, homogenized in 0.5 M HCl, and vigorously vortexed for 16 h at 4°C. The supernatant was collected for calcium assay. A Sigma calcium determination kit (procedure 587; Sigma) was employed according to the manufacturer's protocol. Zonal mineralization in acellular gels was also quantitatively evaluated by measuring the calcium and phosphor contents via elemental analysis (QTI, Whitehouse, NJ).

Biochemical assays

Hydrogels were moved to serum-free DMEM culture medium 24 h before harvest to remove the components of serum absorbed inside the gel. The medium was then collected for quantitative osteocalcin (OCN) enzyme-linked immunosorbent assay (ELISA). Hydrogel constructs were homogenized in 0.75 M 2-amino-2-methyl propanol (AMP, pH 10.3; Sigma) solution for ALP assay. The OCN ELISA was performed with Metra Osteo-

calcin enzyme immunoassay (EIA) kits (Quidel, San Diego, CA) according to the manufacturer's instruction. The AMP supernatants were collected for ALP assay, using Sigma alkaline phosphatase determination kits (procedure 245; Sigma) according to the manufacturer's protocol.

To prepare for DNA assay and glycosaminoglycan (GAG) assay, lyophilized hydrogel constructs were digested in papain–phosphate-buffered EDTA (PBE) solution (papainase, 125 μg/mL; Worthington Biomedical, Lakewood, NJ) at 60°C for 16 h. The digested solution was collected for measuring the DNA and GAG contents. DNA content was determined by fluorophotometry with Hoechst 33258 dye (Aldrich, Milwaukee, WI)[14] and GAG content was quantified by spectrophotometry with dimethylmethylene blue (DMMB) dye (Aldrich).[15]

RT-PCR

Fresh cellular hydrogels were homogenized in TRIzol (Aldrich) solution. An RNeasy minikit (Qiagen, Valencia, CA) was used for RNA isolation. The RNA yield and purity were determined by spectrophotometry at 260 and 280 nm. The cDNA was synthesized by reverse transcription (RT) with the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was performed with *Taq* DNA polymerase (Invitrogen) at an annealing temperature of 59°C (for type II collagen, 60°C) via 35 cycles. The sequences of PCR primers are as follows: β-actin, 5′-TGGCACCA-CACCTTCTACAATGAGC-3′ and 5′-GCACAGCTT-CTCCTTAATGTCACGC-3′; type I collagen (Col I), 5′-TGACGAGACCAAGAACTG-3′ and 5′-CCATCC-AAACCACTGAAACC-3′; total type II collagen (Col II-t), 5′-GTGGAGCAGCAAGAGCAAGGA-3′ and 5′-CTTGCCCCACTTACCAGTGTG-3′; type II collagen IIA/IIB (Col II-A/B), 5′-GTGAGCCATGATTCGCCT-CGG-3′ and 5′-CACCAGGTTCACCAGGATTGCC-3′; type X collagen (Col X), 5′-CCCTTTTGCTGCTAG-TATCC-3′ and 5′-CTGTTGTCCAGGTTTTCCTGGC-AC-3′; ALP, 5′-ACGTGGCTAAGAATGTCATC-3′ and 5′-CTGGTAGGCGATGTCCTTA-3′; osteonectin (ON), 5′-ATGAGGGCCTGGATCTTCTT-3′ and 5′-GCTTCTGCTTCTGAGTCAGA-3′; and cbfa1, 5′-CC-ACCCGGCCGAACTGGTCC-3′ and 5′-CCTCGTCC-GCTCCGGCCCACA-3′. Two percent agarose gels were used for gel electrophoresis.

Histology and immunohistochemical staining

Fresh hydrogel constructs were fixed in 4% paraformaldehyde solution (Sigma) at 4°C overnight and then stored in 70% ethanol at 4°C. Samples were sectioned and stained with hematoxylin and eosin (H&E), safranin O, Masson's trichrome, and von Kossa. As acellular controls, empty hydrogels incubated in OM supplemented with ALP (50 units/L) for 3 weeks were also fixed and sectioned for Masson's trichrome staining.

Immunohistochemical staining was performed with Histostain-SP kits (Zymed, South San Francisco, CA) on fixed sample sections, according to the manufacturer's protocol. Rabbit polyclonal antibodies to type I collagen and osteonectin (Research Diagnostics, Flanders, NJ) were utilized as primary antibodies.

Data statistics

Statistical analysis was performed by unpaired Student t test with a confidence level of 0.05. All values stated in this article are reported as the mean and standard deviation.

RESULTS

Degradation and automineralization

The newly synthesized PhosPEG gels demonstrated both hydrolytic and enzymatic modes of degradation. For PhosPEG gels, a linear weight loss was recorded over the 3-week culture in PBS (Fig. 1).[9] PhosPEG–PEG cogels also demonstrated a linear weight loss in PBS at a slower rate compared with the pure PhosPEG gels, which had more cleavage sites available for degradation. The weight loss of PhosPEG gels in ALP-supplemented PBS (50 units/L, pH 7.4) increased more than 2-fold, demonstrating the enzymatic degradation of these gels. No weight change was observed for nondegradable PEODA gel in both PBS and the ALP-supplemented PBS culture system (Fig. 2).

A significant difference in mass change was observed when samples were cultured in OM medium compared with PBS (Fig. 1). An exponential decay of weight in PhosPEG gels was seen during the earlier periods of culture, whereas the trend was reversed to precede a significant mass increase for the later half-period. The Phos-PEG–PEG cogels had a similar trend of mass loss followed by mass increase, but the mass increase was much larger. The nondegradable PEODA gels did not decrease in mass while incubated under PBS culture conditions. In contrast, a significant mass increase in the PEODA gels was found in the OM culture, particularly during the second half of the culture period.

Mineralization is responsible for the increase in weight in both degradable and nondegradable hydrogels (without cells) incubated under osteogenic culture conditions. The calcium content of hydrogels increased significantly after the first week of culture in all hydrogel samples (Fig. 3). The PhosPEG–PEG cogels exhibited the greatest accumulation of calcium after 3 weeks, reaching 46.5 ± 2.67 μg/mg total dry weight (Table 1). The morphological and quantitative distribution of calcium in the

A

B

C
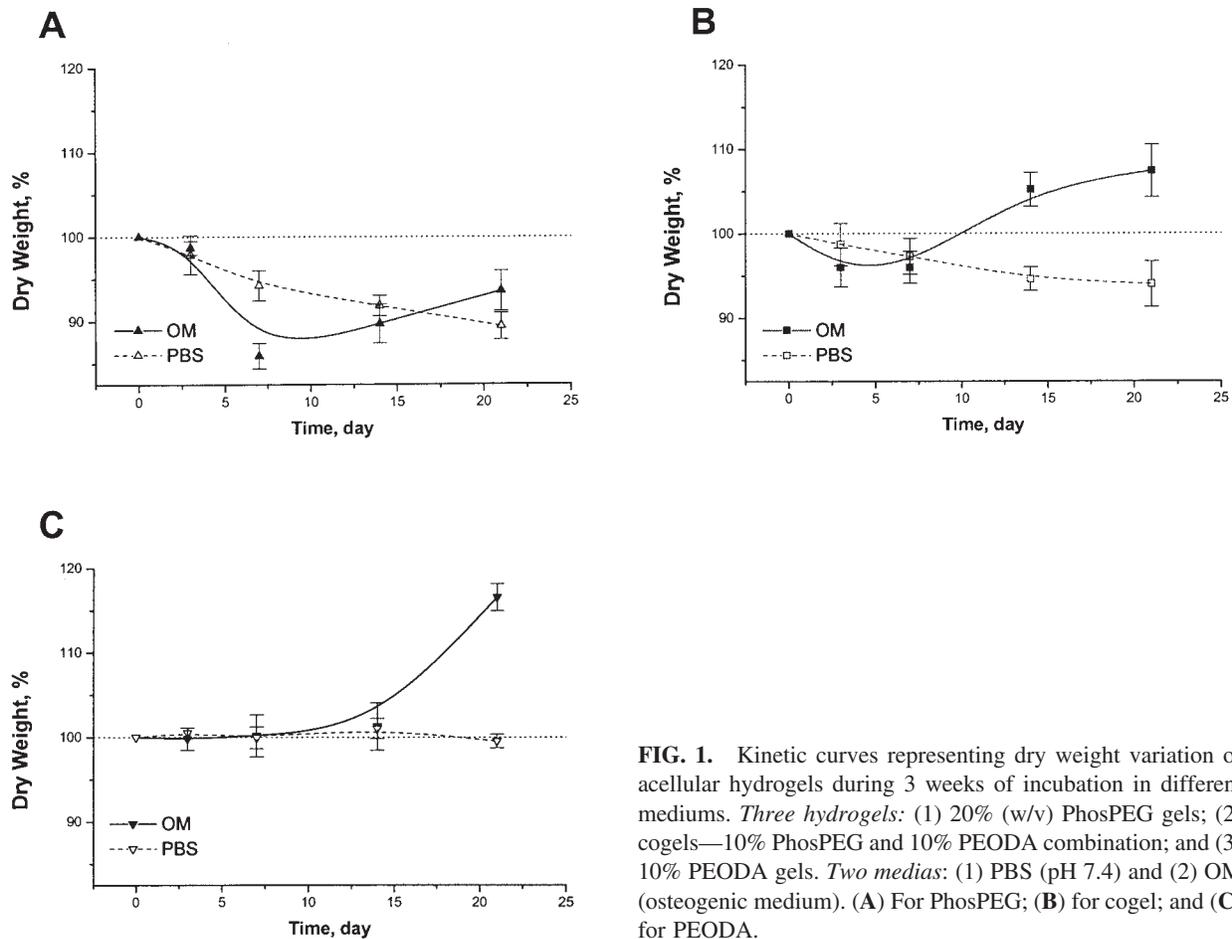
FIG. 1. Kinetic curves representing dry weight variation of acellular hydrogels during 3 weeks of incubation in different mediums. *Three hydrogels:* (1) 20% (w/v) PhosPEG gels; (2) cogels—10% PhosPEG and 10% PEODA combination; and (3) 10% PEODA gels. *Two medias*: (1) PBS (pH 7.4) and (2) OM (osteogenic medium). (**A**) For PhosPEG; (**B**) for cogel; and (**C**) for PEODA.

gels varied depending on the hydrogel chemistry (Fig. 3). In PhosPEG gels, mineralization occurred primarily in the core of the construct (core: Ca, 6.97%; and P, 5.11%; perimeter: Ca, 0.95%; and P, 2.59% [w/w]). On the other hand, the mineralization zone in cogels occurred in the perimeter of the construct (core: Ca, 1.63%; and P, 1.88%; perimeter: Ca, 6.16%; and P, 3.55% [w/w]); and in PEODA gels, the distribution of mineralization was generally homogeneous throughout the construct (Ca, 7.41%; and P, 2.60%).

Gene expression

Gene expression of bone-relevant markers in encapsulated bone marrow-derived stem cells determined by RT-PCR depended on the chemistry of the hydrogel (Fig. 4). Expression of osteogenesis-relevant markers osteonectin and alkaline phosphatase was found only in the PhosPEG–PEG cogels. Col X and cbfa1 were found in all of the gel formulations whereas types I and II collagen expression varied depending on the gel. Cells encapsulated in PhosPEG and PhosPEG–PEG cogels expressed cartilage-specific type II collagen, although

biochemical and histological analysis indicated no cartilage matrix was present in the gels. Type I collagen was expressed in PhosPEG–PEG cogel and PEODA gels.

Biochemistry and cell-related calcification

Bone marrow-derived mesenchymal stem cells survived encapsulation in all of the hydrogels and proliferated to various degrees. The cell number was used to normalize the protein contents determined in the gels (Table 2). Alkaline phosphatase (ALP) contents in the hydrogels correlated with the gene expression data from RT-PCR. The PhosPEG–PEG cogels accumulated the greatest amount of ALP after 3 weeks of culture under osteogenic conditions, whereas the PhosPEG and PEG gels contained significantly lower amounts of ALP (Fig. 5A). The PhosPEG–PEG cogels also accumulated the greatest amount of another bone specific marker, osteocalcin (OCN). The amounts of both ALP and OCN produced from MSC-encapsulated hydrogels that were cultivated in OM for 3 weeks are listed in Table 2.

The hydrogels accumulated calcium when cultured under osteogenic conditions, and the acellular calcium de-
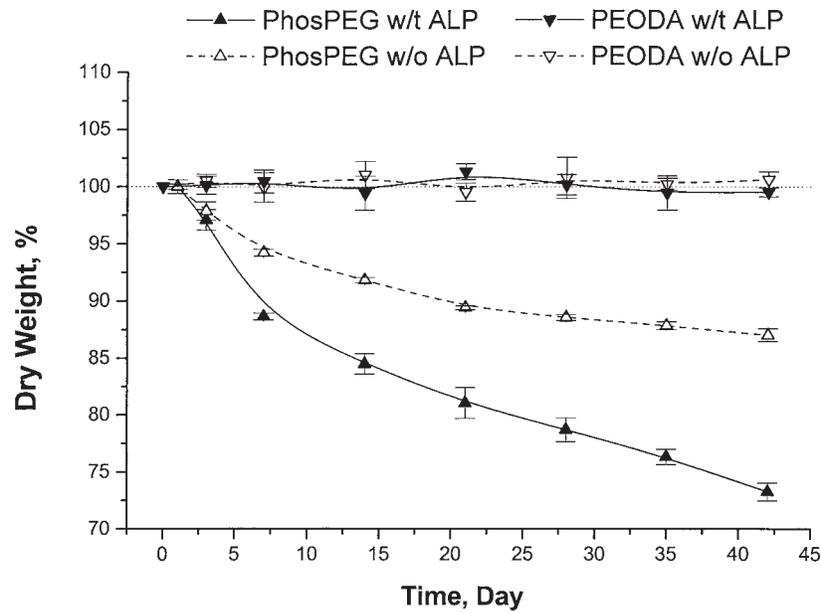
FIG. 2. Kinetic curves representing degradation-related dry weight variation of acellular hydrogels during 6 weeks of incubation in PBS (pH 7.4) with/without the presence of ALP (50 units/L). *Two hydrogels*: (1) 20% (w/v) PhosPEG gels and (2) 10% PEODA gels.

position can be deduced in the cellular constructs. Again, the PhosPEG–PEG cogels accumulated the most calcium, when normalized by dry weight or cell number, compared with the PhosPEG and PEG gels. The quantitative results

are shown in Table 1. If the calcium accumulation determined in the acellular gels (Fig. 3) is subtracted from the content found in the cellularized gels, the net calcium production related to cell activity (Fig. 5C) follows sim-
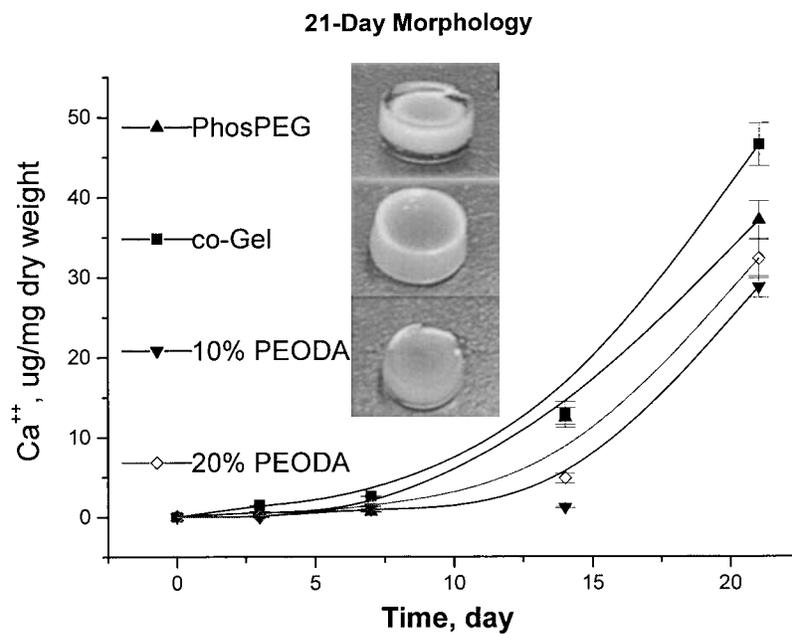
FIG. 3. Kinetic curves representing degradation-related autocalcification of acellular hydrogels during 3 weeks of incubation in osteogenic medium and the final morphologies of the hydrogel constructs (*inset*). *Four hydrogels*: (1) 20% (w/v) PhosPEG gels; (2) cogels—10% PhosPEG and 10% PEODA combination; (3) 10% PEODA gels; and (4) 20% PEODA gels.

TABLE 1. HYDROGEL PORE SIZE AND 3-WEEK CALCIFICATION

Sample	Day 0 M_c[a] (Da)	Acellular Ca^{2+} (μg/mg dry wt)	Total Ca^{2+}		Cellular Ca^{2+} (μg/million cells)
			μg/mg dry wt	μg/million cell	
PhosPEG gel	1297	37.0 ± 2.43	49.52 ± 11.7	399.4 ± 126.1	108.08 ± 26.1
PhosPEG–PEG cogel	1021	46.5 ± 2.67	76.13 ± 2.73	736.7 ± 32.3	400.82 ± 32.3
PEODA gel	1192	28.7 ± 1.34	66.98 ± 1.20	492.2 ± 17.0	285.19 ± 13.0
PEODA gel (20%)	1002	32.3 ± 2.50	—	—	—

[a]M_c, average molecular weight between two adjacent cross-linking functionalities (see Wang et al.[9]).

Histology

ilar trends. The calcification values are summarized in Table 1.

Histological staining supports the conclusion of differentiation toward osteogenesis. The general morphology of the constructs indicated that the cells encapsulated in phosphoester-containing hydrogels (PhosPEG and cogels) aggregated into clusters, whereas the cells in PEODA gels remained scattered in a homogeneous manner (Figs. 5A–C and 6). In parallel with the calcium biochemical content, von Kossa staining for calcium was positive in all groups, with the strongest deposition in the PhosPEG–PEG cogels and PEODA gels (Fig. 5C). The distribution of calcium in the gels was significantly different in the cogels, where the majority of staining was present in the intercellular space compared with larger crystals exclusively present in the cellular region in the PhosPEG and PEG gels. Immunohistochemistry (IHC) for type I collagen (Col I) and osteonectin (ON) supported the gene expression (RT-PCR) results (Fig. 6). Staining and gene expression for Col I and ON were negative in the PhosPEG gels, whereas Col I was present in the PEODA gels. The PhosPEG–PEG cogels were the only group that demonstrated gene expression and protein deposition by IHC of both Col I and ON. No chondrogenic matrix deposition was observed by safranin O staining (data not shown).

Hydrogels containing PhosPEG macromer demonstrated a background staining with Masson's trichrome that was not present in the PEG gels (Fig. 5A and B). Furthermore, it was noted that the pericellular region of cells in the PhosPEG and PhosPEG–PEG cogels under osteogenic conditions lost background staining after culture (Fig. 5A), and the reduction in pericellular background staining increased over time (Fig. 5B). Alkaline phosphatase (ALP) was directly involved in reduction of pericellular staining as determined with acellular control hydrogels (Fig. 5A). Acellular gels treated with ALP lost their background staining and appeared similar to the PEODA gels. The time dependence of ALP production
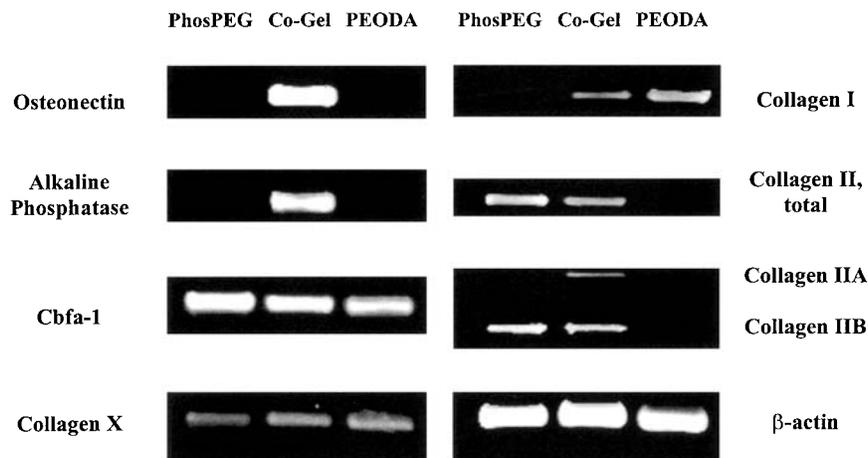

FIG. 4. Gene expression of gMSC encapsulated in hydrogels after 3 weeks of osteogenic cultivation. *Primers*: Osteonectin, alkaline phosphatase, cbfa-1, type X collagen (*left*); type I collagen, total type II collagen, type II collagen A/B, β-actin (*right*). *Three hydrogels*: (1) 20% (w/v) PhosPEG gels; (2) cogels—10% PhosPEG and 10% PEODA combination; and (3) 10% PEODA gels.

TABLE 2. THREE-WEEK BIOCHEMICAL ANALYSIS

Sample	Cell number (DNA assay)		ALP (units/L/construct)	OCN (ng/mL medium)
	Millions/construct	Cell proliferation (%)		
PhosPEG gel	1.06 ± 0.052	15	1.016 ± 0.24	0.804 ± 0.019
PhosPEG–PEG cogel	1.20 ± 0.025	5	2.936 ± 0.25	1.286 ± 0.016
PEODA gel	0.85 ± 0.025	26	1.227 ± 0.53	1.118 ± 0.021
PEODA in MSCGM	0.88 ± 0.034	27	0.329 ± 0.33	0.277 ± 0.019

also correlated with the decrease in pericellular background in staining only when constructs were cultured under osteogenic conditions (Fig. 5B), suggesting that the diffusion of ALP around the cells could be visualized in the PhosPEG-containing gels.

DISCUSSION

In this study, three important aspects of bone tissue engineering were investigated by the application of phosphoesters to bone repair, namely, (1) conditions that promote the gene expression and extracellular matrix (ECM) production of bone-related markers in marrow-derived MSCs, (2) materials that promote both cell-related and scaffold-related mineralization, and (3) production of scaffold degradation rates that respond to cellular activity and tissue development. All three of these factors, in combination, will influence the quality of engineered bone.

### Acellular hydrogel response to an osteogenic microenvironment

Hydrogel degradation and mineralization can be changed by manipulating scaffold chemistry and porosity. The development and extent of both gel degradation and gel autocalcification fundamentally rely on two factors: (1) the chemical properties of the gel components, and (2) the pore size of gel constructs. The dry weight loss of phosphoester-containing hydrogels is due to a degrading reaction by hydrolysis cleavage occurring at the sites of phosphoester, which proceeds faster in OM (pH 7.4) compared with PBS (pH 7.4). Degradation of the hydrogels can be modulated by the content of cleavage site, the phosphoester.[6,7] The results demonstrate that the PEODA gels without phosphoester do not degrade at all; cogels, which contain 50% of the phosphoester content of pure PhosPEG gels, also degrade approximately 50% slower (Fig. 1).

The mass increase of acellular constructs is caused by deposition of insoluble minerals (mainly calcium phosphate and calcium carbonate) absorbed from the medium and entrapped within the gels, namely "hydrogel au-

tomineralization" (or "autocalcification").[16,17] The mineralization of the acellular constructs occurs only in OM with the calcium supplied from FBS. Results show that the PhosPEG–PBS system is degradable but free of autocalcification, whereas the PEODA–OM system autocalcifies without degradation, providing systems in which pure degradation or pure mineralization processes occur. The degradation without mineralization (PhosPEG–PBS) shows a linear dry weight loss with a brief initial logarithmic acceleration (Fig. 1). In contrast, autocalcification without degradation (PEODA–OM) demonstrates an exponential increase in dry weight, mostly in the second half of the culture period, which also agrees with the calcium accumulation (Fig. 3). By combining degradation and mineralization in phosphoester-containing gels incubated in OM, there is a combination and competition of the bulk degradation and autocalcification. In the early period of PhosPEG–OM hydrogel culture, bulk degradation dominates and leads to a mass loss, whereas in the later period, the exponential mass increase by autocalcification overcomes the degradation effects and reverses the tendency for overall mass loss (Fig. 1). Compared with PhosPEG gels, cogels demonstrate a much stronger autocalcification tendency.

Hydrogel degradation and mineralization rely on (1) the chemical properties of the gel and (2) the pore size of the hydrogel. The pore size of the hydrogel significantly impacts mineralization.[17,18] Pore size is determined by polymer concentration and cross-linking density, and the initial pore size of each sample is quantified by the corresponding values of M_c (average molecular weight between two adjacent cross-linking functionalities). For comparison with the number average molecular weight (M_n), of the macromers, M_c data are listed in Table 1.

Calcification of the acellular hydrogels is also modulated by the chemistry, or phosphoester content. Figure 3 indicates that phosphoester-containing gels autocalcify faster and to a greater extent than do PEODA gels. When comparing gels of similar chemistry and changing pore size, the gels with a smaller pore size calcify to a greater degree. However, the PhosPEG gels have larger pore size than the PEODA gels, yet still have greater autocalcifi-
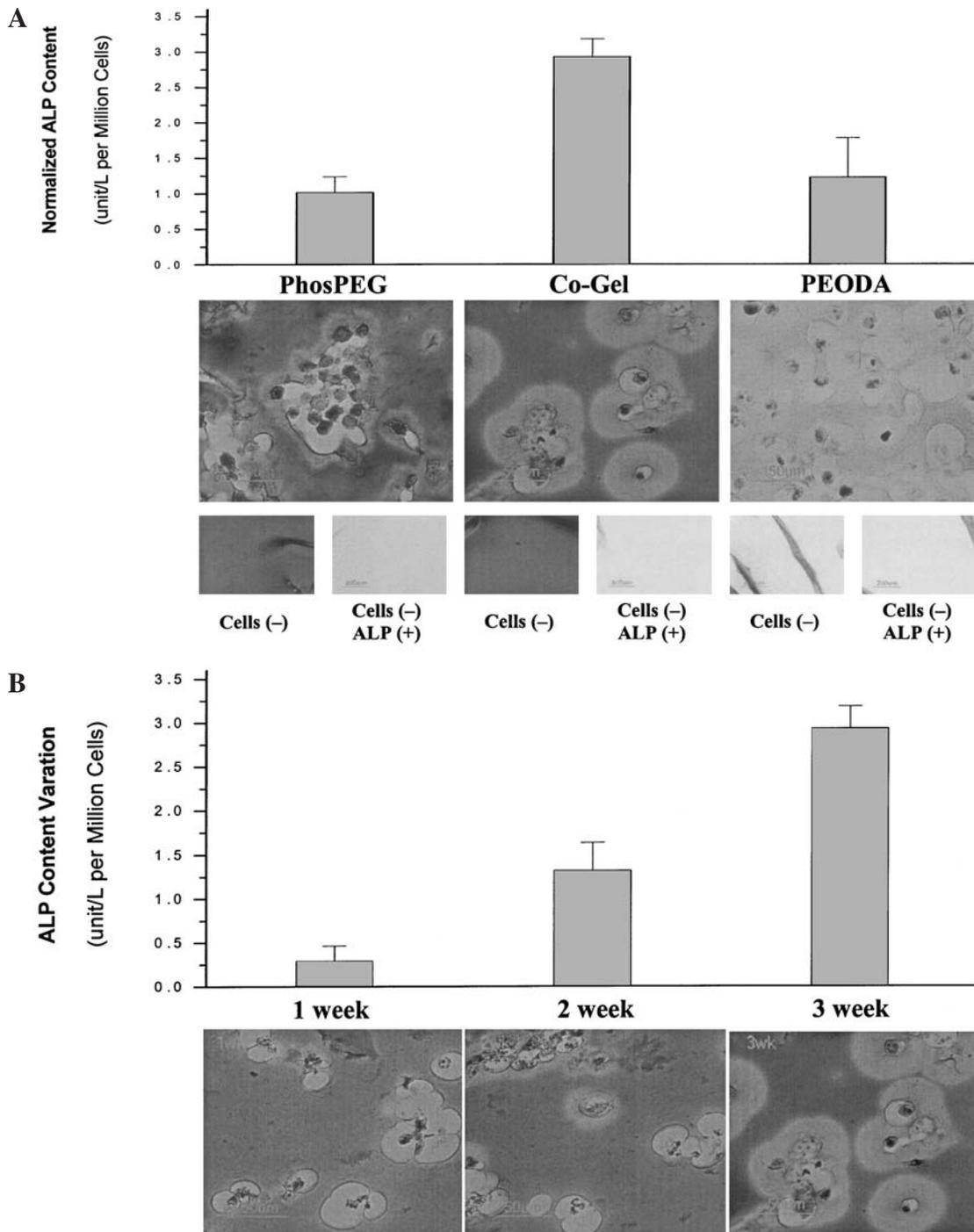
FIG. 5. Alkaline phosphatase (ALP) secretion and calcium modulation. (**A**) Normalized ALP content in gMSC-encapsulated hydrogels after 3 weeks of osteogenic cultivation (*top*); and final histological phenotype with Masson's trichrome staining for cellular hydrogels (*middle*) and acellular hydrogels [*bottom*, Cells (−)], as well as acellular controls cultivated in the presence of ALP at 50 units/L [*bottom*: Cells (−), ALP (+)] in osteogenic medium for 3 weeks. *Three hydrogels:* (1) 20% (w/v) Phos-PEG gels; (2) cogels—10% PhosPEG and 10% PEODA combination; and (3) 10% PEODA gels. (**B**) Variation of ALP content (*top*) and time-dependent expansion of "blank rings" (*bottom*) in gMSC-encapsulated cogels of 10% PhosPEG and 10% PEODA combination during 3 weeks of osteogenic cultivation. The histological phenotype was illustrated with Masson's trichrome staining. (**C**) Normalized cellular calcium content in gMSC-encapsulated hydrogels after 3 weeks of osteogenic cultivation (*top*); and final histological phenotype with von Kossa staining for cellular samples (*bottom*) cultivated in osteogenic medium for 3 weeks. *Three hydrogels*: (1) 20% (w/v) PhosPEG gels; (2) cogels—10% PhosPEG and 10% PEODA combination; and (3) 10% PEODA gels.
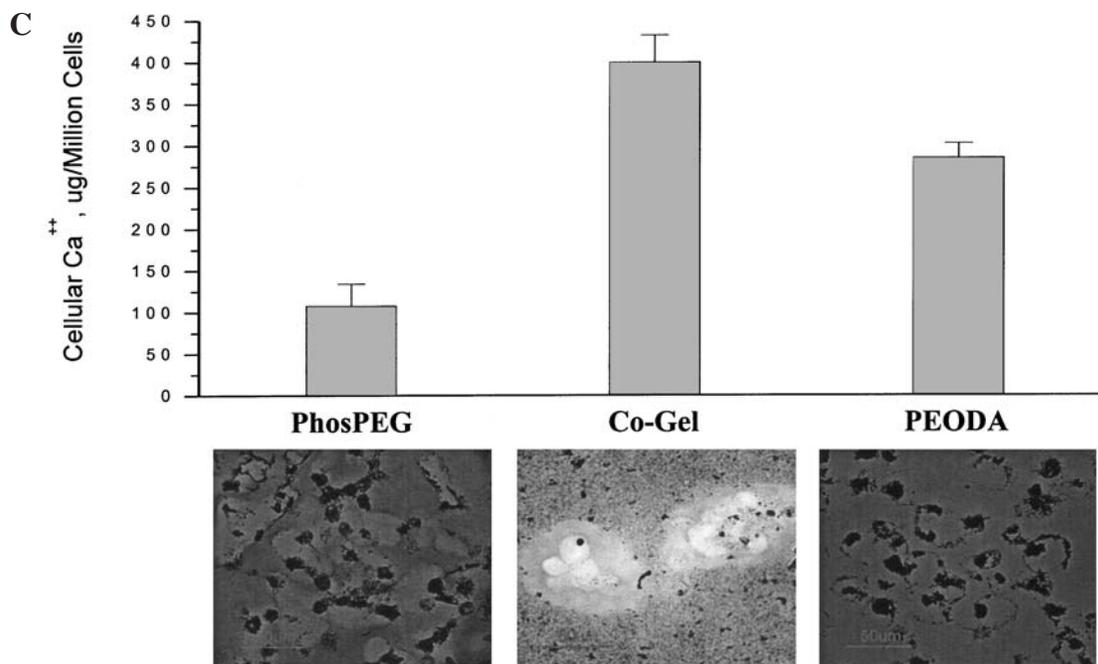
FIG. 5. *Continued*

cation. Therefore, the presence of the phosphoester group significantly impacts mineralization to a greater degree than the change in pore size. The production of phosphoric acid when PhosPEG gels degrade promotes the immobilization of minerals via the *in situ* formation of insoluble calcium phosphate by phosphoric acid reaction with free calcium ion present in the medium. The degradation product of phosphoesters, phosphoric acid, can enhance the autocalcification in hydrogels by increasing the mobilization of free calcium in the medium to create insoluble calcium phosphate.[17] On the other hand, the smaller pore size helps physically trap the calcified mineralization and prevents the components from being extracted out of the gel. This also explains why the greatest autocalcification occurs in cogels. Further information comes from the zonal distribution of calcification in gels.

The zonal distribution of autocalcification in the gels is demonstrated in Fig. 3. In nondegradable PEODA gels, autocalcification develops via precipitation and accumulation of calcium phosphate/carbonate that is extracted from the medium. As a result of the self-adsorbing effect of the minerals, the procedure is exponentially self-accelerated. Given sufficient time, homogeneous calcification will gradually extend throughout the whole construct. In the phosphoester-containing gels, in addition to the calcium diffusion and precipitation, degradation enlarges the pore size and produces phosphoric acid.[7] The pore size increase leads to a release of entrapped minerals; on the other hand, the phosphoric acid is capable of immobilizing minerals via the *in situ* formation of insoluble calcium phosphate by reacting with free calcium ion present in the medium. Because the medium is more accessible at the outskirts of the gels, calcium is prone to be caught and precipitated in the outskirt area by reacting with the phosphoric acid. The situation also depends on the degradation pace of individual gels. In cogels, calcium precipitations are held and enriched mostly at the superficial level of the gel constructs and apparently form a "mineral ring" as shown in Fig. 3. However, in PhosPEG gels, because the cross-linking density is inherently lower and the degradation also extends from the edge to the core, the superficial area always experiences intensive degradation earlier and thereby becomes too sparse and weak to hold the minerals. Consequently, the mineral ring moves to a deeper level.

Osteogenic differentiation of MSCs in microenvironment

Two developmental pathways are responsible for the transformation of mesenchymal tissues into bone. One pathway is intramembranous ossification, in which neural crest-derived MSCs directly differentiate into osteoblasts. The second pathway is endochondral ossification, in which bone marrow-derived MSCs first develop into cartilage precursors that eventually are replaced by calcified and blood vessel-invaded bone tissues. Initially, MSCs condense into compact nodules and differentiate into chondrocytes. The committed chondrocytes rapidly pro-
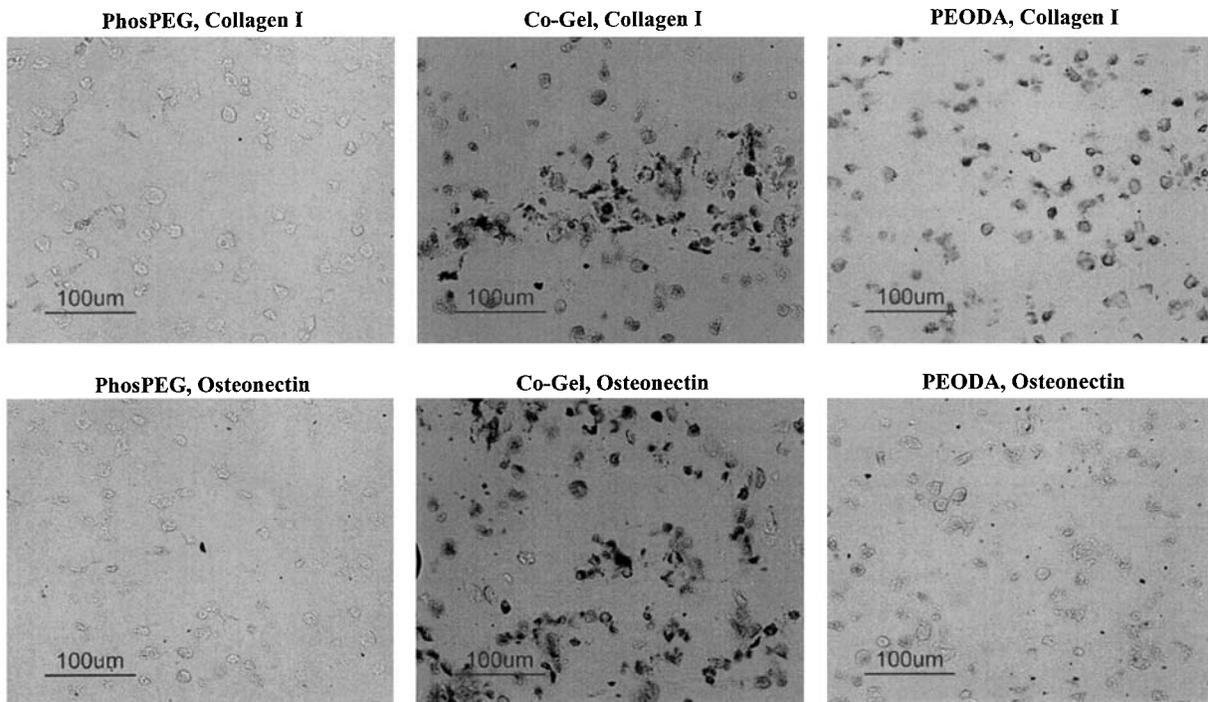
FIG. 6. Immunohistochemical staining of gMSC-encapsulated hydrogels for type I collagen (*top*) and osteonectin (*bottom*) after 3 weeks of osteogenic cultivation. *Three hydrogels:* (1) 20% (w/v) PhosPEG gels; (2) cogels—10% PhosPEG and 10% PEODA combination; and (3) 10% PEODA gels.

liferate and secret Col II and aggrecan (proteoglycan). Afterward, the cells stop dividing and hypertrophy, which is marked by the production of Col X. Accompanying the invasion of blood vessels, the hypertrophic chondrocytes undergo apoptosis and MSCs in bone marrow are employed and begin to differentiate into osteoblasts that secret Col I and OCN.[19] In the endochondral ossification pathway, cbfa1 is the earliest osteogenic marker that activates the gene expression of ALP, ON, OCN, and some other bone-specific proteins or enzymes.[20] Col II is a general marker for cartilage tissue (mature form, Col IIB), whereas the immature Col IIA may be found in osteoblasts. The secretion of Col X indicates the hypertrophy and apoptosis of committed chondrocytes, which is usually an indication for the differentiation and development through the endochondral ossification pathway. Investigations on the interaction between hypertrophic chondrocytes and Col II also suggest that the turnover of Col II accelerates the uptake of calcium and promotes ossification.[21] During the later period, essential bone proteins and enzymes are gradually produced for ossification.

RT-PCR demonstrated positive gene expression of cbfa1 and Col X in all three groups (PhosPEG, cogels, and PEODA), suggesting MSC osteogenic differentiation via the endochondral ossification pathway. This indication is confirmed by quantitative OCN assay. As a late

marker of osteogenesis, the observable content of OCN detected in all samples suggests that MSCs have undergone osteogenic differentiation. Cells encapsulated in cogels demonstrated ossification by the expression of endochondral ossification-specific gene markers as well as production and accumulation of bone-specific protein, including OCN, ALP, and ON. However, cells encapsulated in the higher phosphor-containing PhosPEG gels expressed abundant Col II that consisted primarily of mature Col II-B (Fig. 4), while they were negative for the expression of cellular mineralization-related proteins (ALP and ON) and Col I, indicating reduced osteogenesis. Reduced bone development with increasing concentrations of phosphates agrees with the results of Pogany *et al.*,[22] which demonstrated that high exogenous phosphate concentrations may hinder Col I fibrillogenesis and bone development. As the control without any exogenous phosphate, cells encapsulated in PEODA gels also positively expressed Col I, but cellular mineralization-related gene expression and protein production were weak.

Ninety percent of total bone protein is Col I, which is responsible for the tensile strength of the tissue. ALP, ON, and OCN are mineralization-related proteins. Glycosylated ALP is expressed mainly on cell surfaces or in matrix vesicles. It specifically degrades the organic phosphoesters in bone and cartilage, which inhibits cartilage mineralization and promotes the calcium deposi-

tion leading to mineralization in bone.[19,23] The gene expression of ALP increases before mineralization and decreases after the initiation of mineralization *in vitro*. ON is a phosphorylated and glycosylated SPARC (secreted protein acidic and rich in cysteine) protein that plays an important role in the initiation of active mineralization by linking the bone mineral and collagen phases with its several calcium-binding sites.[21,24] OCN is a small vitamin K-dependent BGP (bone gla protein) produced by osteoblasts that in the presence of calcium binds to phospholipid vesicles and hydroxyapatite. As a later marker for osteogenesis, OCN plays a regulatory role in balancing the maintenance or resorption of bone mineralization.[24–30]

In summary, the gene expression and production of collagens and mineralization-related proteins suggest enhanced ossification of MSCs encapsulated in PhosPEG–PEG cogels, with gene expression of markers relevant to multiple stages of endochondral osteogenesis. In PhosPEG gels, further differentiation and development were hindered by chondrocyte hypertrophy whereas in PEODA gels Col I was produced, but there was little protein production to modulate cellular calcification.

Bioresponsive materials: cell and scaffold interactions

The bioresponsive nature of biomaterials refers to the interplay between the functionalized synthetic scaffolds and the cell-related behavior or microenvironment. This study focused on bioresponsive scaffold behavior, that is, ALP-responsive scaffold degradation and calcification modulation. For the phosphoester-containing gels, the acceleration of degradation by exogenous ALP was confirmed in the acellular PhosPEG gels (Fig. 2). The cleavage of phosphoester groups in the PhosPEG network was accelerated by about 100% with the catalysis of ALP, even under pH-neutral conditions. Instead of directly cleaving the phosphotriesters on the backbone of PhosPEG scaffolds, the main targets for ALP cleavage are the less substituted phosphoesters, such as phosphodiesters and monophosphoesters, which are made by initial hydrolysis of phosphotriesters and incomplete methacrylate group (HEMA) conjugation during the synthesis of PhosPEG macromer.[9] As the hydrolysis of phosphotriester proceeds, the extra consumption of less substituted phosphoesters by ALP digestion promotes the initial hydrolysis reaction. The resulting change in charge with the destruction of the phosphotriester groups on the scaffold backbone dominates the degradation and also alters the general charging status, which subsequently affects the staining pattern seen in histology and allows visualization of the patterns of endogenous ALP pro-

duction. The effects of ALP secreted by encapsulated cells were visualized by Masson's trichrome staining and von Kossa staining (Fig. 5). ALP functions to cleave the covalent phosphoester bonds and ionize them into minerals, promoting the degradation of PhosPEG backbone, in addition to playing a significant role in calcification of hydrogels.[31] The net calcification contributed by cell-related activity was calculated by deducting the amount of gel autocalcification from total calcification, which was measured by calcium assay in acellular constructs. The greatest cellular calcification was detected from PhosPEG-PEG cogels. In addition to the quantitative calcium assay, von Kossa staining further demonstrated the pattern of calcification in the various gels (Fig. 5C). The mineralization in PhosPEG and PEODA gels occurred primarily inside the cell bodies or lacunae, and the minerals aggregated into big crystals. By contrast, small mineral crystals were extensively and uniformly distributed throughout the matrix of cogels and the cell clusters were surrounded by pericellular regions free of mineralization.

MSCs encapsulated in PhosPEG–PEG cogels were the only group with significant ON and ALP gene expression; they showed strongly positive immunohistochemical staining of ON and the greatest amount of ALP production. The action of ALP in enzymatically cleaving phosphoesters accelerated PhosPEG degradation and simultaneously promoted mineralization by converting the original organic phosphoesters into insoluble calcium phosphate. The role of ON is to link the minerals with Col I fibers, by which the format of mineralization is modulated and optimized. Because of the lack of both Col I and mineralization-related proteins (ALP and ON) in the PhosPEG gels, similar mineralization could not occur. In PEODA gels Col I is secreted, but without ON to bridge the connection with the minerals, calcium deposition resulted in the nonphysiological formation of large crystals (Fig. 5C).

In summary, photopolymerized phosphoester-containing hydrogels were used as the scaffold for MSC transplantation. The cross-linked hydrophilic polymer network of the hydrogel is capable of homogeneously suspending and encapsulating cells. Simultaneously, it allows for the free penetration of water, nutrients, and metabolic waste, and faciliates cell migration. The phosphate moieties localized on the backbone of a hydrogel scaffold demonstrated three functions enhancing bone tissue engineering: (1) they provided a site for scaffold degradation,[9,10] which produced functional groups to promote autocalcification; (2) they promoted MSC osteogenic differentiation based scaffold chemistry; and (3) they responded to osteogenesis-related enzymes, such as ALP, to accelerate degradation via ALP-catalyzed hydrolysis cleavage in response to tissue development.

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REFERENCES

1. Langer, R., and Vacanti, J.P. Tissue engineering. Science **260,** 920, 1993.

2. Cruise, G.M., Scharp, D.S., and Hubbell, J.A. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. Biomaterials **19,** 1287, 1998.

3. Halstenberg, S., Panitch, A., Rizzi, S., Hall, H., and Hubbell, J.A. Biologically engineered protein–graft–poly(ethylene glycol) hydrogels: A cell adhesive and plasmin-degradable biosynthetic material for tissue repair. Biomacromolecules **3,** 710, 2002.

4. Cowan, C.M., Shi, Y.Y., Aalami, O.O., Chou, Y.F., Mari, C., Thomas, R., Quarto, N., Contag, C.H., Wu, B., and Longaker, M.T. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat. Biotechnol. **22,** 560, 2004.

5. Warren, S.M., and Nacamuli, R.P. Tissue-engineered bone using mesenchymal stem cells and a biodegradable scaffold. J. Craniofac. Surg. **15,** 34, 2004.

6. Wang, J., Mao, H.Q., and Leong, K.W. A novel biodegradable gene carrier based on polyphosphoester. J. Am. Chem. Soc. **123,** 9480, 2001.

7. Wang, J., Zhang, P.C., Mao, H.Q., and Leong, K.W. Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier. Gene Ther. **9,** 1254, 2002.

8. Elisseeff, J., Anseth, K., Sims, D., McIntosh. W., Randolph, M., and Langer, R. Transdermal photopolymerization for minimally invasive implantation. Proc. Natl. Acad. Sci. U.S.A. **96,** 3104, 1999.

9. Wang, D.A., Williams, C.G., Li, Q., Sharma, B., and Elisseeff, J.H. Synthesis and characterization of a novel degradable phosphate-containing hydrogel. Biomaterials **24,** 3969, 2003.

10. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.

11. Williams, C.G., Kim, T.K., Taboas, A., Malik, A., Manson, P., and Elisseeff, J. *In vitro* chondrogenesis of marrow-derived mesnechymal stem cells in a photopolymerizing hydrogel. Tissue Eng. **9,** 679, 2003.

12. Peter, S.J., Liang, C.R., Kim, D.J., Widmer, M.S., and Mikos, A.G. Osteoblastic phenotype of rat marrow stromal cells cultured in the presence of dexamethasone, β-glycerolphosphate, and L-ascorbic acid. J. Cell. Biochem. **71,** 55, 1998.

13. Bryant, S.J., Nuttelman, C.R., and Anseth, K.S. Cytocompatibility of UV and visible light photoinitiating system on culture NIH/3T3 fibroblast *in vitro*. J. Biomater. Sci. Polym. Ed. **11,** 439, 2000.

14. Kim, Y.J., Sah, R.L.Y., Doong, J.Y.H., and Grodzinsky, A.J. Fluorometric assay of DNA in cartilage explants using Hoechst-33258. Anal. Biochem. **174,** 168, 1988.

15. Farndale, R., Buttle, D., and Barrett, A. Improved quantitation and discrimination of sulphated glycosaminoglycans by the use of dimethylmethylene blue. Biochim. Biophys. Acta **883,** 173, 1986.

16. Murphy, W.L., and Mooney, D.J. Bioinspired growth of crystalline carbonate apatite on biodegradable polymer substrata. J. Am. Chem. Soc. **124,** 1910, 2002.

17. Hossainy, S.F.A., and Hubbell, J.A. Molecular-weight dependence of calcification of polyethylene-glycol hydrogels. Biomaterials **15,** 921, 1994.

18. Behravesh, E., Jo, S., Zygourakis, K., and Mikos, A.G. Synthesis of *in situ* cross-linkable macroporous biodegradable poly(propylene fumarate-co-ethylene glycol) hydrogels. Biomacromolecules **3,** 374, 2002.

19. Gilbert, S.F. Developmental Biology, 6th ed. Sunderland, MA: Sinauer, 2000.

20. Sodek, J., and Cheifetz, S. Molecular regulation of osteogenesis. In: Davies, J.E., ed. Bone Engineering. Toronto, Canada: Em Squared, 1999, Chap. 4.

21. Zuscik, M.J., D'Souza, M., Ionescu, A.M., Gunter, K.K., Gunter, T.E., L'Keefe, R.J., Schwarz, E.M., Puzas, J.E., and Rosier, R.N. Growth plate chondrocyte maturation is regulated by basal intracellular calcium. Exp. Cell Res. **276,** 310, 2002.

22. Pogany, G., Hernandez, D.J., and Vogel, K.G. The *in vitro* interaction of proteoglycans with type I collagen is modulated by phosphate. Arch. Biochem. Biophys. **313,** 102, 1994.

23. Gomez, S., Lpoez-Cepero, J.M., Silvestrini, G., and Bonucci, E. Matrix vesicles and focal proteoglycan aggregates are the nucleation sites revealed by the lanthanum incubation method: A correlated study on the hypertrophic zone of the rat epiphyseal cartilage. Calcif. Tissue Int. **58,** 273, 1996.

24. Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., and Martin, G.R. Osteonectin, a bone-specific protein linking mineral to collagen. Cell 26, 99, 1981.

25. Price, P.A. Bone and Mineral Research, Vol. 1. Peck, W., ed. Amsterdam: Excerpta Medica, 1983, pp. 157–190.

26. Hauschka, P.V., Lian, J.B., and Gallop, P.M. Direct identification of calcium-binding amino-acid, γ-carboxyglutamate, in mineralized tissue. Proc. Natl. Acad. Sci. U.S.A. **72,** 3925, 1975.

27. Gundberg, C.M., Hauschka, P.V., Lian, J.B., and Gallop, P.M. Osteocalcin: Isolation, characterization, and detection. Methods Enzymol. **107,** 516, 1984.

28. Delmas, P.D., Stenner, D.D., Romberg, R.W., Riggs, B.L., and Mann, K.G. Immunochemical studies of conformational alterations in bone γ-carboxyglutamic acid containing protein. Biochemistry **23,** 4720, 1984.

29. Gendreau, M.A., Krishnaswamy, S., and Mann, K.G. The interaction of bone Gla protein (osteocalcin) with phospholipids: Vesicles. J. Biol. Chem. **264,** 6972, 1989.

30. Romberg, R.W., Werness, P.G., Giggs, B.L., and Mann, K.G. Inhibition of hydroxyapatite crystal-growth by bone-specific and other calcium-binding proteins. Biochemistry **25,** 1176, 1986.

31. Luppen, C.A., Smith, E.A., Spevak, L., Boskey, A.L., and Frenkel, B. Bone morphogenetic protein-2 restores mineralization in glucocorticoid-inhibited MC3T3-E1 osteoblast cultures. J. Bone Miner. Res. **18,** 1186, 2003.

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