
In vitro osteogenic differentiation of human mesenchymal stem cells photoencapsulated in PEG hydrogels

Charles R. Nuttelman,¹ Margaret C. Tripodi,¹ Kristi S. Anseth^{1,2}

¹Department of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80309-0424

²The Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309-0424

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Abstract: Much research has focused on the differentiation of human mesenchymal stem cells (hMSCs) in monolayer culture; however, little is known about their differentiation potential in three-dimensional culture conditions. In this research, hMSCs were encapsulated in a photocrosslinkable, injectable scaffolding system based on poly(ethylene glycol) (PEG) hydrogels. To demonstrate the ability of hMSCs to differentiate in PEG hydrogels, cell/polymer constructs were cultured in osteogenic differentiation media to elicit an osteoblastic response. First, viability of encapsulated hMSCs up to 4 weeks in culture was investigated using a membrane integrity assay. Second, gene expression of encapsulated cells was determined with reverse transcription polymerase chain reaction (RT-PCR) as a function of media composition. After 1 week in osteogenic differentiation media, encapsulated hMSCs expressed osteonectin, osteopontin, and alkaline phosphatase, which are all characteristic of osteoblasts.

Finally, von Kossa staining was used to evaluate mineralization of the PEG gels. Results support the hypothesis that hMSCs photoencapsulated in PEG hydrogels and cultured in the presence of osteogenic differentiation media are able to differentiate to osteoblasts inside the gel and mineralize the matrix. These experiments demonstrate the feasibility of using a PEG-based, photocrosslinkable system to culture and deliver human mesenchymal stem cells for bone tissue regeneration and repair. © 2004 Wiley Periodicals, Inc. *J Biomed Mater Res* 68A: 773–782, 2004

Key words: human mesenchymal stem cells; bone tissue engineering; photoencapsulation; osteogenic differentiation; polymer scaffold

INTRODUCTION

Recent interest has emerged in the use of bone marrow-derived stem cells^{1–4} for tissue engineering applications. Mesenchymal stem cells (MSCs), also commonly referred to as bone marrow stromal cells, are a unique class of multipotent cells that are noncommitted and remain in an undifferentiated state. When treated with the right chemicals, hormones, and growth factors, MSCs can be transformed into the cells of bone, cartilage, fat, tendon, muscle, and others.^{4–7} Much research has focused on characterizing MSCs in two-dimensional tissue culture, and the specific chem-

icals and hormones that cause the transformation of MSCs to osteoblasts have been elucidated. Specifically, osteogenic differentiation occurs when MSCs are treated with dexamethasone, β -glycerophosphate, and ascorbic acid,⁸ and this differentiation to osteoblasts is characterized by gene expression of osteopontin and alkaline phosphatase. Minguell et al. (2001) and Jackson et al. (2002) present excellent reviews of mesenchymal stem cells.

Of utmost importance from a tissue engineering standpoint is the fact that stem cells that have been transformed to osteoblasts are much like bone cells of a developing organism, and are capable of secreting large amounts of extracellular matrix. Moreover, the ease of isolation of MSCs make them very attractive for tissue engineering applications; aspiration of bone marrow is only slightly more complicated than a blood donation, and MSCs can be enriched to obtain a relatively pure population of cells.⁹ Several groups are investigating tissue engineering of bone using mesenchymal stem cells, and identification of an appropriate three-dimensional scaffold for their delivery is the focus of much research. These approaches include encapsulation of MSCs in alginates¹⁰ and modified

Correspondence to: K. S. Anseth; e-mail: Kristi.anseth@colorado.edu

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oligo[poly(ethylene glycol) fumarate] hydrogels,¹¹ as well as seeding MSCs on scaffolds based on PLGA.¹²

Although gels provide many advantages for *in situ* forming constructs and cell delivery, important questions need to be addressed. In general, cells do not attach to highly hydrated gels because of limited protein adsorption, so MSCs encapsulated inside gels are presented with a "blank" environment. There will be little to no interaction of integrins and other cell surface receptors with the gel. Furthermore, the degradation kinetics of the gel will also influence cell morphology, spreading, and migration of encapsulated MSCs.

We are particularly interested in developing a photopolymerizable, degradable hydrogel system in which hMSCs can be encapsulated three dimensionally. Our approach to tissue engineering utilizes gels; through rational design, important cell adhesion and cell signaling moieties can be linked onto the polymer backbone. The goal is to tune the scaffold chemistry and properties so encapsulated stem cells will differentiate into either osteoblasts or chondrocytes inside the gel and eventually form the tissues of bone or cartilage as degradation of the polymer scaffold occurs. In a clinical setting, photopolymerization has added benefits due to the spatial and temporal control of gelation; polymerization only occurs where ultraviolet light is directed and the polymerization can be stopped and started simply by shuttering the light source. Furthermore, a cell/polymer solution can be injected through a needle into a bone or cartilage defect and cured using a fiber optic light guide, thus minimizing surgical intervention. At this point, however, little is known about the behavior and function of hMSCs in photocrosslinked PEG gels.

In this article, we present approaches to photoencapsulate hMSCs in nondegrading poly(ethylene glycol)-based hydrogel scaffolds and demonstrate their subsequent *osteogenic* differentiation. The ability of hMSCs to transform into osteoblasts in a three-dimensional hydrogel scaffold is evaluated by analyzing gene expression of encapsulated cells and mineralization of the scaffold. Specifically, gene expression of osteonectin, osteopontin, and alkaline phosphatase (all characteristic of osteoblasts) is evaluated, as well as gene expression of chondrocyte-specific collagen type II to rule out chondrogenic differentiation. Based on expression of osteoblastic genes and mineralization of the hydrogel scaffold, it can be concluded that photoencapsulated hMSCs differentiate to osteoblasts inside the PEG hydrogel.

MATERIALS AND METHODS

Macromer synthesis

Poly(ethylene glycol) of average molecular weight 4600 daltons (PEG4600, Aldrich Chemical Co.) was purchased

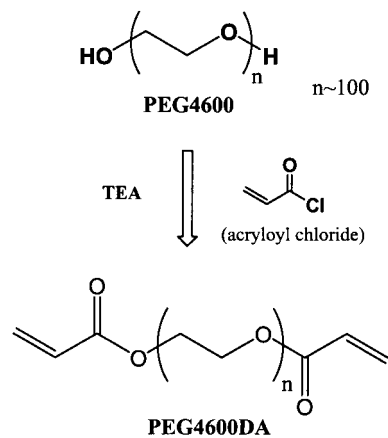


Figure 1. Synthesis of diacrylated poly(ethylene glycol) (PEG4600DA). PEG of average molecular weight 4600 is reacted with acryloyl chloride in the presence of triethylamine (TEA) to produce PEG4600DA.

and used as received. The terminal hydroxyl groups of PEG were modified with acrylate groups using acryloyl chloride (see Fig. 1). PEG4600 (30 g) was placed in a 500-mL, three-neck, round-bottom flask, and methylene chloride (300 mL) was added to dissolve the PEG4600. Triethylamine (TEA, Aldrich Chemical Co., 2.73 mL) was added to the flask and argon gas was bubbled through the solution for 5 min. Acryloyl chloride (Aldrich, 1.59 mL) was dissolved in 15 mL methylene chloride, and this solution was slowly (over 45–60 min) dripped into the round-bottom flask on ice. The flask was then capped and stirred for 24 h at 4°C. The diacrylated product (PEG4600DA) was obtained by precipitating the solution in cold ethyl ether. It was then filtered and dried in a vacuum oven at 50°C overnight. The dry solid was further purified by dialyzing against distilled water for 24 h (molecular weight cutoff of 300 Da) and was lyophilized to obtain a white solid. Toxicity of PEG4600DA was evaluated by dissolving a small amount of product in cell culture media, sterilizing using a 20- μ m syringe filter, and culturing NIH3T3 fibroblast cells in the presence of this solution. Cell death due to the polymer was not observed.

Human mesenchymal stem cell culture

Human mesenchymal stem cells (hMSCs) and all media and necessary cell culture reagents were obtained from Cambrex Bio Science (Walkersville, MD) and used as received. Stem cell basal media is composed of DMEM media containing 10% specially selected fetal bovine serum and antibiotics. Osteogenic differentiation media contains ascorbic acid-2-phosphate, dexamethasone, L-glutamine, β -glycerophosphate, and other ingredients.⁸ Stem cells were passaged twice prior to use by trypsinizing and subculturing at a 1:6 dilution ratio. Media was replaced twice a week and the hMSCs were cultured in 100 mm tissue culture polystyrene dishes (Corning).

MTT cytotoxicity assay

To determine the toxicity of PEG4600DA and the ultraviolet light on hMSCs, the MTT assay (methylthiazolotetrazolium, Sigma) was utilized. These experiments were similar to those conducted by Bryant et al. (2000). Human MSCs were plated in six-well plates at a concentration of approximately 5000 cells/cm². A day later, cells were exposed to combinations of ultraviolet light (5 mW/cm² at various exposure times up to 15 min) and 0.05 wt % of the photoinitiating molecule 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Darocur 2959 or D2959, Ciba-Geigy, Hawthorne, NY). These conditions are typical for many photoencapsulation experiments.¹³ In addition, the effect of adding PEG4600DA to the cell suspension was also investigated. Two days later, cell metabolic activity was characterized using the MTT assay and following standard assay procedures. Relative survival of each treatment was calculated as absorbance of each sample divided by the absorbance of the controls (no light or initiator treatment).

Photoencapsulation of hMSCs

An aqueous PEG4600DA solution was prepared in PBS (10 wt % PEG4600DA) and the photoinitiator D2959 was added at 0.05 wt %. D2959, a water-soluble photoinitiator, was used to initiate the photopolymerization of PEG4600DA in the presence of long wave ultraviolet light and has been shown to exhibit low toxicity to cells under these photoencapsulation conditions.¹³ Confluent human mesenchymal stem cells were trypsinized from cell culture and centrifuged to pellet the cells. PEG4600DA solution was added directly to the cells, and the amount of solution was adjusted so that there were 25 million cells per mL of the PEG4600DA solution. Approximately 40 μ L of cell/polymer solution was then placed into a sterile 1-mL syringe that had its tip cut off, and syringes were placed under ultraviolet light for 10 min at room temperature to polymerize the samples. Upon polymerization, the disk-shaped constructs were removed from the syringes and placed into either control media (stem cell basal media, CON) or osteogenic differentiation media (OST). Cell/polymer constructs were cultured for up to 6 weeks at 37°C and 5% carbon dioxide and media was replaced twice a week. A control gel (0 week Gel) was constructed, and immediately after polymerization, the gel was preserved for further analysis. For each experiment, three identical cell/polymer constructs were made. Figure 3 depicts the photoencapsulation scheme.

Two-dimensional monolayer controls

Tissue culture controls were performed in parallel with the photoencapsulation experiments to examine the behavior of cells in monolayer culture. At the beginning of each experiment, hMSCs were plated at a 1:6 dilution ratio (~5000 cells/cm²) in six-well tissue culture plates and allowed to grow to confluency. Once confluent, cells were not

passed and media was replaced twice a week for the duration of the experiments. Analysis was performed on cells 1 week prior to photoencapsulation (-1 wk), at encapsulation (0 wk), and various time points after photoencapsulation (up to 6 weeks). Cells were grown in either hMSC basal media as the control (CON) or osteogenic differentiation media (OST). Human mesenchymal stem cells were also grown in the presence of chondrogenic differentiation media to compare gene expression of collagen type II (characteristic of chondrocytes) to that of hMSCs cultured in osteogenic and control media.

Live/dead cell assay

To verify viability of hMSCs upon photoencapsulation, a Live/Dead cell assay (Molecular Probes, Eugene, OR) was employed. This assay is based upon the fluorescence of two dyes, ethidium homodimer (EthD-1) and calcein AM. The nonfluorescent, cell-permeant calcein AM is converted rapidly into the intensely fluorescent calcein, which is retained within living cells, producing intense green fluorescence in live cells. EthD-1 is able to enter cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids. Thus, dead cells fluoresce bright red. At various time points, cell/polymer constructs were removed from cell culture, rinsed briefly with PBS, and 200- μ m sections were prepared using a vibratome (Energy Beam Sciences). These thin sections were then incubated for 30 min in a solution containing 1 mL PBS, 0.5 μ L calcein AM, and 2 μ L ethidium homodimer (EthD-1). The sections were transferred to fresh media for 30 min and were then mounted in PBS on glass slides. A laser scanning confocal microscope (Zeiss Axioplan 2 with LSM 5 Pascal laser) and associated software were used to visualize the relative viability of cells and their distribution in the gel.

Isolation of total RNA

At various time points, constructs were removed from culture and the gene expression of encapsulated cells was analyzed. Constructs were placed in 1.0 mL TRI REAGENT (Sigma) in RNase-free, 1.5-mL test tubes and were crushed and homogenized with a tissue homogenizer. Total RNA was obtained by following instructions that were supplied with the TRI REAGENT. To quantify the total RNA, the pellets were dissolved in 100 μ L of DEPC (diethyl pyrocarbonate)-treated water (0.1%) at 60°C for 10 min. A 10- μ L sample from each RNA tube was combined with 990 μ L DEPC-treated water in 1-mL spectrophotometer cuvettes (Fisher). The cuvettes were then inverted to mix and the absorbance at 260 nm was measured. The concentration of RNA in each tube was calculated (an absorbance of 1.0

TABLE I
Sequences of Primers Used in the Polymerase Chain Reaction and Product Size

Primer	Nucleotide Sequence (5'-3')	Product Size (bp)	Reference
Osteonectin (ON)	S: ACATGGGTGGACACGG A: CCAACAGCCTAATGTGAA	405	18
Osteopontin (OPN)	S: AGCCGTGGGAAGGACAGTTATG A: GGAGTTTCCATGAAGCCACAAAAC	473	19
Alkaline phosphatase (ALP)	S: ACGTGGCTAAGAATGTCATC A: CTGGTAGGCGATGTCCTTA	475	20
Collagen type II (COL2)	S: TTCCCAGGTCAAGATGGTC A: CTTCAGCACCTGTCTCACCA	377	21
GAP dehydrogenase (GAPDH)	S: CCATGGAGAAGGCTGGGG A: CAAAGTTGTCATGGATGACC	194	20

Sense primers are designated with S and antisense with A.

corresponds to a RNA concentration of 40 $\mu\text{g}/\text{mL}$), and DEPC-treated water was added to adjust the final concentration of each RNA tube to 10 ng/ μL . This RNA was then used in reverse transcription polymerase chain reaction (RT-PCR).

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad CA). Instructions supplied with the kit were followed and 5 ng total RNA was used for each sample. A control containing no reverse transcriptase was also performed to verify absence of contaminating genomic DNA. Reverse transcription yielded cDNA, which was then utilized in the polymerase chain reaction (PCR).

PCR was used to determine the gene expression of osteonectin (ON), osteopontin (OPN), and alkaline phosphatase (ALP), all of which are characteristic of osteoblasts. Gene expression of collagen type II (COL2), which is expressed by chondrocytes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also evaluated. GAPDH is an enzyme utilized in cellular metabolism and is assumed to be expressed at the same level in most cells; therefore, gene expression of GAPDH was used as an internal control to normalize out any differences in amount of total RNA isolated. Primer sequences as well as product size and references are given in Table I.

Polymerase chain reactions were conducted using reagents and primers obtained from Invitrogen according to instructions provided with the SuperScript First-Strand Synthesis System for RT-PCR. Thermal cycling conditions (using an Eppendorf Mastercycler Personal thermal cycler) for ON, OPN, ALP, COL2, and GAPDH were obtained from the literature (see Table I). Samples were run on 1.5% agarose gels to analyze gene products and a photograph was taken with a digital camera. To quantify the gene expression results, NIH Image software was utilized to measure DNA gel band intensities. Relative gene expression of ON, OPN, and ALP were calculated by dividing band intensities by GAPDH band intensity to obtain a numerical result that is proportional to gene expression in each particular sample.

Histology

At various time points, samples were removed from *in vitro* culture and fixed overnight in 10% formalin. The samples were then dehydrated through a sequence of 1-h steps: 80% ethanol, 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 50% ethanol/50% Citrisolve (Fisher), 100% Citrisolve, 100% Citrisolve, molten paraffin wax at 60°C, and molten paraffin wax overnight at 60°C.

Samples were embedded in paraffin wax using a tissue embedding machine (Leica, model EG 1160) and sectioned at 10 μm using a microtome (Leica, model RM 2125). Sections were placed on glass slides, warmed at 60°C for 2 min, and dried at room temperature overnight. The slides were then dried an additional 30 min on a slide warmer at 60°C.

Slides containing sections were stained using standard histological techniques. Safranin-O and Fast Green stains were used to visualize cells, and the von Kossa method was utilized to evaluate calcification. A light microscope was used to visualize the cells and photomicrographs were obtained using a digital camera.

RESULTS AND DISCUSSION

MTT cytotoxicity assay

Figure 2 shows the effects of ultraviolet light, photoinitiator, PEG4600DA, and combinations of these factors on the relative survival of hMSCs, as measured and related to their metabolic activity. In the panel on the left, cells were exposed to the photoencapsulating light for 10 min (except for the D2959 sample, which was conducted in the absence of light). As can be seen, both UV light and the photoinitiator molecule alone are relatively benign to the cells under these conditions; however, upon generation of the initiator radicals (i.e., exposure to UV light and D2959), the cell viability, as correlated to metabolic activity, reduced to 88% of the control. As observed with other cell types, cell viability increases in the presence of reac-

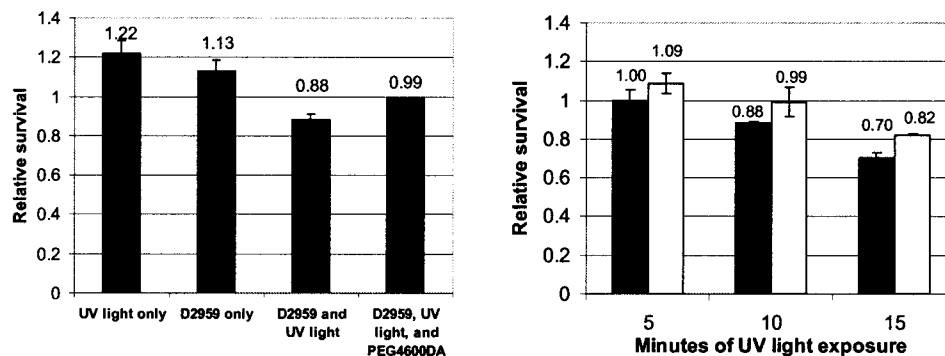


Figure 2. In the left panel, various combinations of ultraviolet light (5 mW/cm² for 10 min), photoinitiating molecule (D2959), and poly(ethylene glycol) diacrylate of molecular weight 4600 were exposed to human mesenchymal stem cells and the relative survival compared to the control (no treatment) was measured for each sample using the MTT assay. The right panel shows the relative survival of cells treated with D2959 as a function of time of UV light exposure; ■: UV light and D2959, □: UV light, D2959, and PEG4600DA. The macromer is thought to “protect” the cells from free radical damage. Error bars represent standard deviation of samples performed in triplicate.

tion macromer (PEG4600DA), presumably due to some type of protection mechanism. As can be seen in the left panel of Figure 2, cell survival is returned to near 100% in the presence of PEG4600DA. The right panel of Figure 2 shows the effect of UV light exposure time on cell survival of hMSCs in the presence of D2959 (left bars) and D2959 with PEG4600DA (right bars). These experiments verify the cytocompatibility of the conditions used to photoencapsulate hMSCs. Cell survival and metabolic activity remain high and unaffected by the presence of D2959 and PEG4600DA after 10 min of UV light exposure.

Photoencapsulation of hMSCs

In the presence of a suitable initiator, the carbon-carbon double bonds of the acrylate group undergo chain polymerization to form hydrogel networks. When ultraviolet light is absorbed by D2959, two primary radicals are generated. These radicals propagate through the electron-rich carbon-carbon double bonds of the acrylate groups, which leads to the formation of kinetic chains that are crosslinked via PEG. Because of the high molecular weight of the PEG and its low concentration (10 wt %) during gel formation, a loosely crosslinked and highly swollen gel results. This gel contains approximately 90–95% water by weight. The gel properties provide suitable mechanical strength of the cell/polymer construct while conferring facile cellular exchange of nutrients and wastes with the surrounding environment. Figure 3 depicts the photoencapsulation scheme of hMSCs using a macromer solution containing PEG4600DA. Note: the figure is not to scale; the size scale of the polymer chains (hundreds of angstroms) is much smaller than the size scale of cells (micrometers).

Live/dead cell assay

Figure 4 shows confocal images of hMSCs photoencapsulated in PEG4600DA gels and cultured *in vitro* for up to 4 weeks and stained using the Live/Dead assay. All constructs were cultured in hMSC basal media. Within an hour of photoencapsulation (Fig. 4, panel A), nearly 100% of the cells are green, indicating that they are viable and have survived the photoencapsulation process (i.e., the photoencapsulation conditions are cytocompatible). However, cell viability decreases with time in a spatially dependent manner. Cell viability is >95% after 1 day (panel B), but drops to ~60% after 1 week in culture (panel C). In addition, the distribution of viable cells is inhomogeneous and varies spatially throughout the gel. Cell viability decreases radially from the exterior to interior of the gel; 90% of the cells are viable at the edge of the disk, but less than 10% of cells are viable in the center of the disk. At later time points (3 and 4 weeks, panels D and E), cell viability is reduced further, and living cells are confined to the outer perimeter of the disk (within roughly 500 μm of the polymer surface).

It is important to note that the cross-sectional distribution of viable cells varies along the height of the construct (i.e., the axis of the disk); at the very top and very bottom of the disk cell viability is homogeneous and near 100% even at later time points. Although it is unclear at this point what is causing the large difference in cell viability between the interior and surface of the constructs, it is hypothesized that diffusion of nutrients and wastes between the interior cells and the surrounding environment may be affecting their survival. Both osteoblasts and chondrocytes have been photoencapsulated in PEG4600DA hydrogels and high viability throughout the scaffold was seen after 2 weeks,^{14,15} much greater than seen with hMSCs.

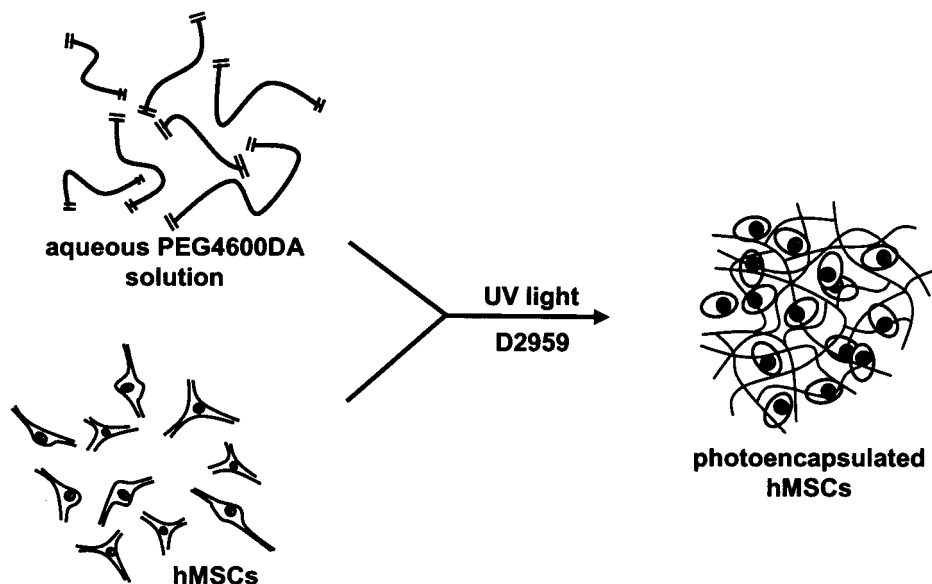


Figure 3. Photoencapsulation scheme. A sterile 10 wt % aqueous PEG4600DA solution is prepared and aseptically combined with isolated human mesenchymal stem cells (hMSCs). This cell/polymer solution is then photopolymerized using ultraviolet light and the photoinitiator D2959. As a result, the stem cells are encapsulated or immobilized in the PEG hydrogel.

Therefore, it is unlikely that viability of encapsulated hMSCs is purely limited by nutrient diffusion. It is possible that hMSCs are more metabolically active than either osteoblasts or chondrocytes. In this case, hMSCs on the periphery of the cell/polymer constructs would consume nutrients at a higher rate, preventing nutrients from reaching the interior of the hydrogel. In addition, these viability experiments were conducted using hMSCs encapsulated and cultured in control media. It is likely that if the cells were encapsulated and cultured in osteogenic differentiation media, their metabolic rate would decrease as they differentiate and, as a result, viability in the interior of the gel would increase. Studies are currently under way to investigate these relationships between viability and cellular metabolism of hMSCs and osteoblasts.

Cell culture of cell/polymer constructs

Photopolymerized PEG4600DA constructs containing encapsulated hMSCs were cultured *in vitro* for up to 6 weeks. Figure 5 shows macroscopic images of the constructs after 1 and 6 weeks. As can be seen, after 1 week in culture the general appearance of the OST gel (cultured in osteogenic differentiation media) is nearly transparent. Similarly, the 6-week control gel (no differentiation media) is transparent. In contrast, the 6-week OST gel is opaque. This opacity is attributed to mineralization and tissue formation by encapsulated hMSCs that have been transformed to osteoblasts in-

side the hydrogel, as confirmed by the following results.

Reverse transcription polymerase chain reaction (RT-PCR)

To determine gene expression of encapsulated hMSCs, RT-PCR was utilized. Primers were specific for osteonectin (ON), osteopontin (OPN), and alkaline phosphatase (ALP), as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Figure 6 presents results of RT-PCR experiments. Presence of a band indicates expression of that particular gene by the encapsulated hMSCs, and band intensity is proportional to amount of gene expressed. Table II shows semiquantitative RT-PCR results calculated by normalizing the OPN, ON, and ALP band intensities by the GAPDH band intensity as measured using NIH Image software. Shown are results for hMSCs in monolayer conditions 1 week prior to encapsulation (Monolayer, -1 wk) and at the time of encapsulation (Monolayer, 0 wk), as well as gene expression of monolayer hMSCs grown in control media (Monolayer, 1 wk CON) and osteogenic differentiation media (Monolayer, 1 wk OST) for an additional week. Results also show gene expression of hMSCs that were photoencapsulated in PEG4600DA gels and either immediately removed for RNA isolation (PEG4600DA Gel, 0 wk) or subsequently incubated for 1 week in control media (PEG4600DA Gel, 1 wk CON) and osteogenic differentiation media (PEG4600DA Gel, 1 wk

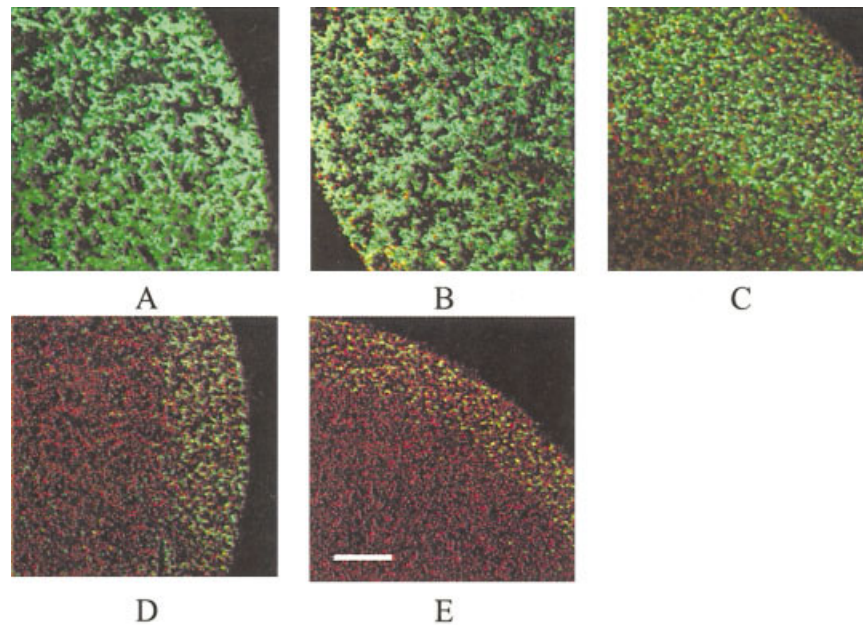


Figure 4. Human mesenchymal stem cells were photoencapsulated in PEG4600DA hydrogels and cultured in hMSC basal media for up to 4 weeks. At various time points, the cell/polymer constructs were removed from culture, sectioned with a vibratome, and stained using the Live/Dead cell assay, and imaged with a laser scanning confocal microscope. Living cells stain green, whereas dead cells stain red (100× original magnification). (A) Less than 1 h, (B) 1 day, (C) 1 week, (D) 3 weeks, (E) 4 weeks after photoencapsulation. Cell viability decreases with time from nearly 100% initially to less than 10% at 4 weeks. Bar is 500 μm .

OST). Using the monolayer control RNA at 1 week, reverse transcriptase enzyme was left out of the reaction mixture to guarantee the absence of contaminating DNA (No RT).

Osteonectin, a glycoprotein that binds to calcium, hydroxyapatite, and collagen and is believed to be a nucleator for matrix mineralization,¹⁶ is expressed by osteoblasts. However, ON is also found in hMSCs. ON was expressed to approximately the same extent in all samples; therefore, ON does not necessarily indicate osteogenic differentiation.

In contrast, OPN was found to be indicative of early osteoblastic differentiation. The tissue culture controls (Monolayer) indicate gradual loss of OPN expression with time as indicated by OPN band weakening. Osteopontin is involved with general cell attachment to the bone matrix,¹⁶ and its expression varies temporally during bone development. OPN expression is upregulated in the presence of osteogenic differentiation media after 1 week in monolayer, as well as after 1 week in gel-encapsulated hMSCs both in the presence and absence of osteogenic differentiation media. The strong expression of osteopontin in the control gels was unexpected, but may indicate that forcing the hMSCs to assume a rounded, three-dimensional shape (through photoencapsulation) can induce stem-cell differentiation.

Alkaline phosphatase (ALP) is secreted by osteoblasts and is responsible for crystal formation in bone.¹⁷ ALP is expressed in all tissue culture experi-

ments, including hMSCs cultured in control media, as well as osteogenic gels at 1 week, but it is not expressed by hMSCs in control gels. Although cells in

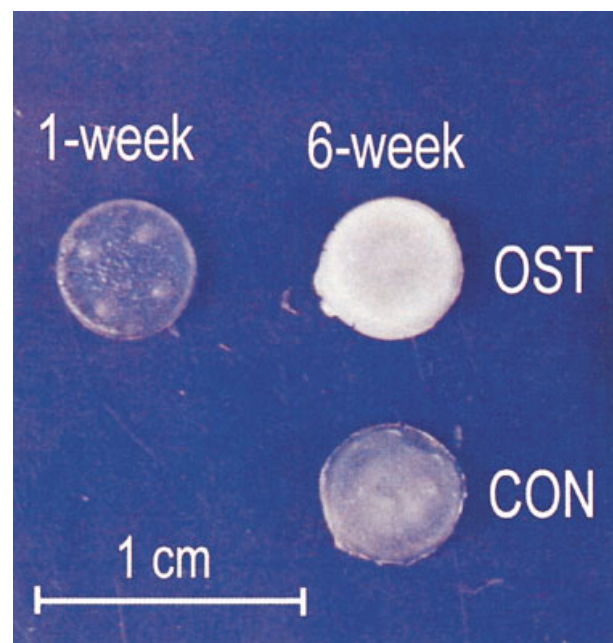


Figure 5. Human mesenchymal stem cells were photoencapsulated in PEG4600DA hydrogels and cultured *in vitro* for up to 6 weeks in hMSC basal media (CON) and hMSC osteogenic differentiation media (OST). The opacity of the 6-week OST gel is due to mineralization inside the gel.

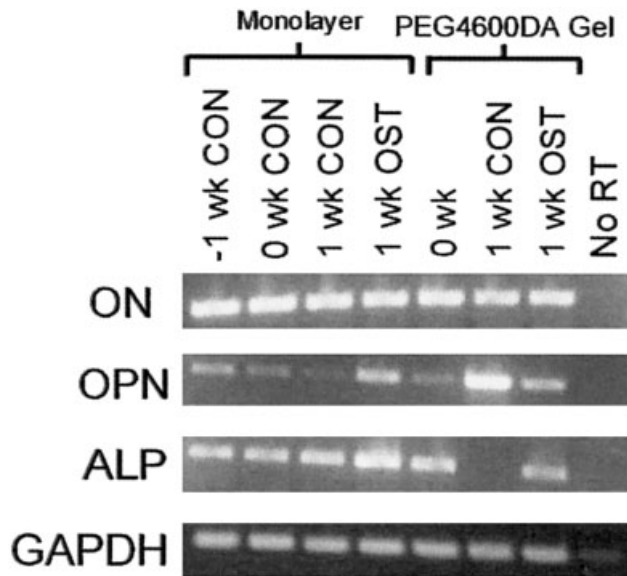


Figure 6. Total RNA was isolated from monolayer hMSC tissue culture samples and 1-week cell/polymer constructs and isolated RNA was reverse transcribed into cDNA using reverse transcriptase. Using primers specific for osteonectin (ON), osteopontin (OPN), alkaline phosphatase (ALP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the polymerase chain reaction was performed and products were run on an agarose gel. Shown are results for monolayer controls 1 week prior to photoencapsulation (–1 wk CON), at the time of encapsulation (0 wk CON), and 1 week after encapsulation cultured in hMSC basal media (1 wk CON) and osteogenic differentiation media (1 wk OST); cell/polymer gels at time of encapsulation (0 wk) and cultured 1 week in hMSC basal media (1 wk CON) or osteogenic differentiation media (1 wk OST). A negative control in which reverse transcriptase was left out (No RT) was also performed. Total RNA from three samples were pooled together for each experiment.

the control gels express osteopontin, which is characteristic of osteoblasts, they do not express alkaline phosphatase, which would be expected of differentiated osteoblasts. It is believed, however, that the difference in opacity between 1- and 6-week OST Gels (see Fig. 5) is due to mineralization, induced by alka-

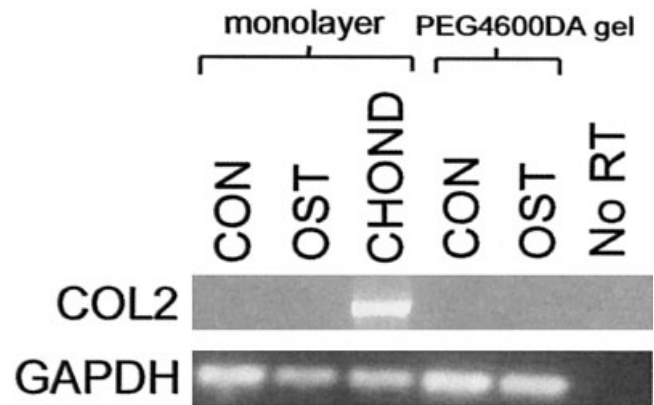


Figure 7. Reverse transcription polymerase chain reaction results for collagen type II (COL2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after either 1 week in monolayer or 1-week after photoencapsulation in PEG4600DA hydrogels. Gene expression of COL2 was analyzed to confirm that chondrogenic differentiation of hMSCs was not occurring inside PEG gels (Gel). Samples are hMSCs cultured in basal media (CON monolayer), hMSCs cultured in osteogenic differentiation media (OST monolayer), hMSCs cultured in chondrogenic differentiation media (CHOND monolayer), hMSCs photoencapsulated in PEG gels and cultured in hMSC basal media (CON gel), hMSCs photoencapsulated in PEG gels and cultured in osteogenic differentiation media (OST gel), and hMSCs cultured in chondrogenic differentiation media but reverse transcriptase was omitted during reverse transcription (No RT) to confirm that there was no contaminating genomic DNA.

line phosphatase activity. These conflicting results are the focus of further experiments.

Because the encapsulated cells are forced into a spherical geometry, we were interested in characterizing the effect that cell morphology might have on hMSC differentiation, in particular, chondrogenic differentiation. To verify that chondrogenic differentiation was not occurring in the gels, PCR for collagen types II were conducted. As can be seen from Figure 7, hMSCs that were cultured in chondrogenic differentiation media express type II collagen, which is characteristic of chondrocytes. Gene expression of collagen

TABLE II
Semiquantitative RT-PCR Gene Expression Results of Osteonectin (ON), Osteopontin (OPN), and Alkaline Phosphatase (ALP).

	Monolayer				PEG4600DA Gel			
	–1 Week CON	0 Week CON	1 Week CON	1 Week OST	0 Week	1 Week CON	1 Week OST	No RT
ON	1.77 ± 0.03	1.69 ± 0.07	1.62 ± 0.07	1.34 ± 0.07	1.51 ± 0.07	1.37 ± 0.01	1.54 ± 0.03	0
OPN	0.42 ± 0.02	0.17 ± 0.02	0.08 ± 0.02	0.73 ± 0.02	0.26 ± 0.01	1.84 ± 0.26	0.82 ± 0.06	0
ALP	0.79 ± 0.03	0.85 ± 0.04	1.16 ± 0.04	1.50 ± 0.04	1.00 ± 0.01	0	0.80 ± 0.02	0

Band intensities of the gel shown in Figure 5 were analyzed using NIH Image software to obtain quantitative measures of gene expression relative to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Numbers shown are ratios of band intensity of particular gene to band intensity of GAPDH. Error represents standard deviation of samples in triplicate.

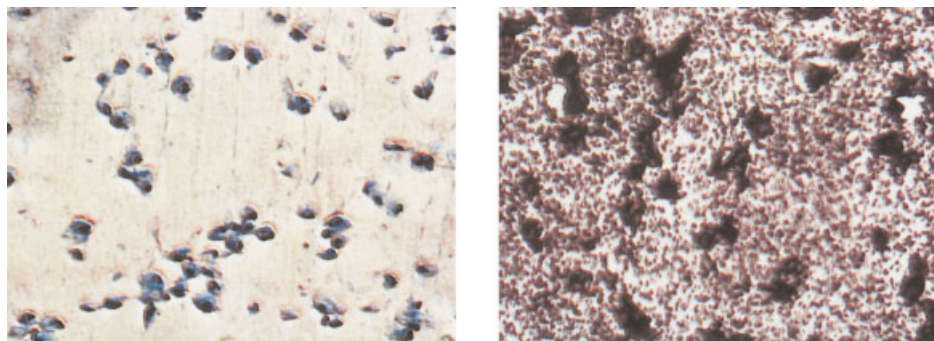


Figure 8. Human mesenchymal stem cells were photoencapsulated in PEG4600DA hydrogels and cultured in hMSC basal media (left) and hMSC osteogenic differentiation media (right) for 4 weeks. The cell/polymer constructs were then fixed in 10% formalin and sectioned using a microtome. Sections of thickness 10 μm were stained with Safranin-O and Fast Green. Cell cytoplasm stains bluish green and cell nuclei stain black. In addition, both samples were stained using the Von Kossa method in which mineralization stains black (200 \times magnification).

type II was not detected in any of the tissue culture controls or cell/polymer constructs, verifying that chondrogenic differentiation had not occurred.

It is also important to note that these RT-PCR experiments measure the cell/polymer construct population as a whole. It is likely that a mixture of differentiated and undifferentiated cells exist. Total RNA is isolated from all the cells that reside in the particular construct and using this technique it is impossible to determine if there is a single population of cells or a mixture of cell types. For this reason, *in situ* hybridization experiments may prove to be particularly valuable for analysis of three-dimensional cell constructs.

Histology

Figure 8 shows Safranin O and Fast Green histological staining of 10- μm sections of 4-week CON (left) and OST (right) gels. Cell cytoplasm stains bluish green and cell nuclei stain black. In addition, both samples were stained using the von Kossa method in which mineralization stains black. As can be seen, after 4 weeks of *in vitro* culture, there is exceptional mineralization in the OST gel compared to the control, demonstrating that hMSCs encapsulated in PEG4600DA gels transform to an osteoblastic phenotype and are capable of mineral formation.

CONCLUSIONS

In this article, the first steps towards designing a PEG-based, photopolymerizable scaffolding system for the regeneration of bone tissue using human mesenchymal stem cells were investigated. First, the cyto-compatibility of the photoencapsulation system was demonstrated using the MTT assay. Second, hMSCs

were successfully photoencapsulated in PEG4600DA hydrogels and their viability upon encapsulation and during culture up to 4 weeks was assessed. Third, the differentiation of encapsulated hMSCs into osteoblasts was examined by analyzing gene expression via the reverse transcription polymerase chain reaction (RT-PCR). In the presence of osteogenic differentiation media, encapsulated hMSCs exhibited gene expression of osteonectin, osteopontin, and alkaline phosphatase, all of which are characteristic of osteoblasts. Furthermore, osteogenic-specific differentiation (as opposed to chondrogenic differentiation) was verified by analyzing chondrocyte-specific gene expression; gene expression of collagen type II was not detected. Finally, histological analysis revealed extensive mineralization in osteogenic hydrogels as detected by the von Kossa mineralization method. In light of these results, it can be concluded that hMSCs photoencapsulated in PEG4600DA hydrogels do, in fact, differentiate to osteoblasts inside the gel and are able to secrete extracellular matrix molecules found in bone tissue. Although PEG4600DA is essentially nondegrading, the starting PEG macromer can easily be modified to incorporate degradable linkages, enabling tailored degradation of the polymer scaffold to match tissue evolution. These experiments represent the first steps in developing an *in situ* method based on photocrosslinkable PEG hydrogels and human mesenchymal stem cells for bone tissue regeneration and repair.

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