

The effect of ethylene glycol methacrylate phosphate in PEG hydrogels on mineralization and viability of encapsulated hMSCs

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Received 9 May 2005; accepted 10 August 2005

Available online 1 September 2005

Abstract

Owing to their resistance to protein adsorption, poly(ethylene glycol) (PEG) hydrogels are unable to support the adhesion of human mesenchymal stem cells (hMSCs). Human MSCs attach to various substrates through cell adhesion proteins, and osteopontin is an important cell adhesion protein for both MSCs and osteoblasts found in bone. As such, we hypothesized that cell adhesion to a PEG hydrogel could be dramatically improved by actively promoting the formation of a mineral phase throughout the hydrogel. This was accomplished using the photoreactive, phosphate-containing molecule ethylene glycol methacrylate phosphate (EGMP). When EGMP was incorporated into the PEG hydrogel, it resulted in the formation of a bone-like mineral phase, as verified using compositional analysis and X-ray diffraction. Furthermore, cell viability of gel-encapsulated hMSCs was increased in the presence of EGMP from 15% in the absence of EGMP to 97% in the presence of 50 mM EGMP. This improvement in cell viability was thought to be due to the ability of mineralized hydrogels to sequester cell-secreted osteopontin. It was found that EGMP-containing PEGDA hydrogels are able to sequester the important cell adhesion protein osteopontin, and these hydrogels promoted hMSC adhesion and spreading.

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Keywords: Hydrogel; Poly(ethylene glycol); Mineralization; Tissue engineering; Ethylene glycol methacrylate phosphate; Mesenchymal stem cells

1. Introduction

The initial stages of *in vivo* biomineralization, such as the nucleation of vertebrate teeth and bone mineralization, occur through the interaction of immobilized, negatively charged functional groups with calcium and phosphate ions [1]. Although incompletely understood, all biomineralization processes are thought to be driven by deposition of an anionic, hydrophilic mineral nucleator on an organic, hydrophobic material, such as collagen in vertebrate skeletons or β -chitin in invertebrate exoskeletons [2,3]. Acidic peptide groups present in collagen and, more importantly, acidic extracellular matrix proteins that are attached to the collagen scaffold are believed to play

important roles in mineralization by serving as binding sites for calcium ions [4].

Many researchers are investigating methods in which biomaterials, from ceramics to polymers, can be better integrated with natural bone. Hydroxyapatite and phosphate-containing coatings have been studied extensively to better integrate biomaterial implants with bone for applications such as total hip replacement, dental implants, and screws for fracture fixation [5–8]. These coatings provide a bone-like mineral matrix that simulates the *in vivo* bone environment. Hydroxyapatite coatings adsorb many proteins and other macromolecules and lead to a biological layer that favors cell attachment and osteogenic differentiation [8]. Importantly, the proteins osteopontin and fibronectin are important molecules implicated in this layer [9,10].

Other approaches to integrate bone with biomaterials focus on the creation of functional polymer scaffolds that mimic the bone extracellular matrix, direct biomineralization, and stimulate cell adhesion, proliferation, migration,

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and differentiation [4,11–13]. Traditionally, a bone-like hydroxyapatite phase can be created on polymer films through a time-consuming incubation in simulated body fluid (SBF) [1], which leads to the slow growth of biominerals on the polymer surface. Unfortunately, these mineral phases present poor adhesion and lack a structural relationship with the substrate [14,15]. More recent studies have focused on chemically altering the polymer interface. This has been done by treating poly(lactide-co-glycolide) films with aqueous base in order to create pendant acid groups that can then be used as nucleators for mineralization [1]. Other strategies include thermal decomposition of urea in solution to hydrolyze hydroxyethyl methacrylate groups into acid groups for the same purpose of creating functional groups that can nucleate mineralization [4]. Unfortunately, these approaches rely on post-processing of the polymer matrix to initiate mineral nucleation (i.e., treatment with aqueous base or urea after the scaffold has been created).

Our approach to design an injectable cell delivery vehicle for tissue engineering bone utilizes photoencapsulation of human mesenchymal stem cells (hMSCs) in a poly(ethylene glycol) (PEG)-based hydrogel scaffolding system. Any post-processing of the cell/polymer construct to create acidic groups that would lead to the formation of a mineral phase would likely be detrimental to the cells. Therefore, the mineralization nucleators need to be created or incorporated into the polymer hydrogel as it is formed and need to be covalently linked to the hydrogel scaffold for maximal efficiency. There are numerous approaches to incorporate pendant functionalities in PEG gels through co-polymerization. We hypothesize that one way to incorporate mineralization nucleators into the PEG hydrogel during photoencapsulation is to combine a macromolecular monomer containing a pendant phosphate group, ethylene glycol methacrylate phosphate (EGMP), into the prepolymer solution and subsequently copolymerizing in the presence of cells. This would create a network with incorporated, covalently linked phosphate groups. These pendant phosphate groups should act as nucleators and a source of inorganic phosphate ions for mineralization. As a result, this would lead to improved rates of mineralization, even in the absence of soluble phosphates such as β -glycerophosphate, which is often used as a nucleator for *in vitro* mineral formation. Furthermore, there is significant control over the concentration of phosphate groups in the hydrogel simply by altering the amount of EGMP added to the prepolymer solution.

Another advantage of a charged group in a hydrogel is that it may lead to improved cell attachment. We hypothesize that, due to the mineralized regions created by incorporating charge in the gel, acidic adhesion proteins, such as osteopontin, are better able to interact with and adsorb to a hydrogel containing EGMP. This study aims to first study the feasibility of using EGMP to create a bone-like mineral phase in PEG-based hydrogel scaffolds and then to investigate whether or not EGMP-

incorporation leads to sequestering of charged proteins, such as the acidic sialoprotein osteopontin, and subsequent adhesion of hMSCs.

2. Materials and methods

2.1. Synthesis of phosphate-containing gels

EGMP (Aldrich, see Fig. 1) contains a photoreactive carbon-carbon double bond and a pendant phosphate group. To create phosphate-containing PEG-based hydrogels, EGMP was combined with a 10 wt% solution of di-acrylated PEG of molecular weight 3400 Da (PEG3400DA, Nektar Therapeutics) dissolved in sterile phosphate buffered solution (PBS, Gibco). The solution also contained 0.05 wt% photoinitiating molecule I2959 (Ciba-Geigy). EGMP was first dissolved in PBS at a concentration of 100 mM and the pH was adjusted to 7.4. The concentration of EGMP in the gels was varied from 0 to 10 mM. Upon ultraviolet light exposure ($\sim 5 \text{ mW/cm}^2$), the solution polymerizes, resulting in a covalently crosslinked hydrogel.

2.2. Human mesenchymal stem cell culture

hMSCs were purchased from Cambrex Bio Science (Walkersville, MD) and cultured in low-glucose Dulbecco's modified eagle medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.25% gentamicin (Gibco), and 0.25% fungizone (Gibco). hMSCs after passage 3 were used in this study.

2.3. Cytotoxicity of ethylene glycol methacrylate phosphate

During photoencapsulation, carbon radicals propagate through the carbon-carbon double bonds of EGMP, resulting in the covalent attachment of the negatively charged phosphate group into the hydrogel. During the encapsulation process, cells are exposed to the EGMP monomers for a period of several minutes, so the cytotoxicity of the EGMP monomer must be assessed. To determine the toxicity of EGMP, the MTT assay (methylthiazolotetrazolium, Sigma) was utilized. An aqueous solution containing 100 mM EGMP was created by dissolving 16 μl EGMP (Sigma) in 984 μl hMSC media and adjusting the pH to 7.4 using a 6 N sodium hydroxide solution. hMSCs were plated in 6-well plates at a concentration of approximately 5000 cells/cm². A day later, cells were exposed to media containing the following concentrations of EGMP: 0, 10, 100 μM , 1, 5, or 10 mM. Two days later, cell metabolic activity was analyzed using the MTT assay following standard assay procedures. Relative survival of each treatment was calculated as absorbance of each sample divided by the absorbance of the controls (no initiator treatment) at a wavelength of 560 nm.

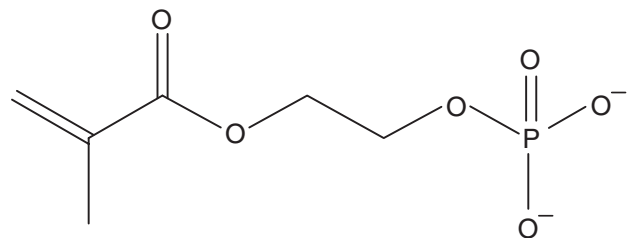


Fig. 1. The chemical structure of ethylene glycol methacrylate phosphate (EGMP). Under free radical propagation reactions, this molecule is incorporated into the PEG hydrogel, resulting in pendant, negatively charged phosphate groups.

2.4. LIVE/DEAD assay for viability of hMSCs encapsulated within EGMP-containing hydrogels

hMSCs were photoencapsulated in a 10 wt% monomer solution in PBS; the solution contained PEG3400DA and 0.05 wt% photoinitiator I2959. In addition, EGMP was added at varying concentrations (0, 10, and 50 mM) to introduce phosphate groups at these same concentrations within the hydrogel. The cell/monomer solution (40 μ l) containing 25×10^6 cells/ml was placed into 1-ml syringes that had their tips cut off. Upon ultraviolet light exposure (~ 5 mW/cm², 10 min), the solution polymerizes, physically entrapping the cells within the hydrogel. After polymerization, disks (diameter = 5 mm, thickness = 2 mm) were pushed out of the syringe using the plunger and placed in hMSC media and cultured at 37 °C and 5% CO₂, replacing the media every 3–4 days. After 1 week, cell/polymer constructs were removed from culture, sectioned with a vibratome into 200- μ m sections, and sections midway between the top and bottom gel surfaces were stained using the LIVE/DEAD assay (Molecular Probes) according to the manufacturer's instructions. Cell viability and distribution were visualized using a laser scanning confocal microscope. Living and dead cells were counted in photomicrographs for several samples and the total percentage of viable cells was determined.

2.5. DNA content in hydrogels formed with or without EGMP

The PicoGreen assay (Molecular Probes) was used to measure the DNA content of the same PEG3400DA hydrogels as above (Section 2.2) containing hMSCs (25×10^6 cells/ml) and 0 mM EGMP or 50 mM EGMP were cultured in hMSC media for 2–3 weeks. At various time points, samples were removed from culture and the cell/polymer constructs were homogenized in PBS with a tissue homogenizer. These homogenates were then sonicated for 1 min to disperse the DNA throughout the solution. After centrifuging the supernatants, the DNA content of each cell/polymer construct was measured using the PicoGreen assay according to the manufacturer's instructions. Results are presented as ng DNA/gel, and samples were performed in triplicate.

2.6. Mineralization of PEG hydrogels containing EGMP

Solutions containing 10 wt% PEGDA ($M_n \sim 3400, 4600, \text{ and } 8000$), 0.05 wt% I2959, and either 0 or 50 mM EGMP but in the absence of cells were prepared and disks (diameter = 5 mm, thickness = 2 mm) were polymerized under ultraviolet light for 10 min. Disks were then placed in hMSC media containing 0 or 10 mM β -glycerophosphate (β -GP). At various time points (2, 4, 7, 9, 11, and 14 days), gels were removed from the media, rinsed $3 \times$ with PBS, and placed in 1 ml 0.9 N H₂SO₄ overnight at 4 °C. The next day, 2 μ l of the supernatant was combined with 8 μ l dH₂O, and this solution was added to 100 μ l of a solution containing 1 part calcium binding reagent (0.024% *o*-cresolphthalein complexone and 0.25% 8-hydroxyquinone in dH₂O) and 1 part calcium buffer (500 mmol/l 2-amino-2-methyl-1,3 propanediol in dH₂O). The absorbance of each solution was then measured at 560 nm using a plate reader, and based on a standard curve of known concentrations of calcium chloride, the total amount of calcium deposited in each hydrogel was determined. The molecular weight of starting macromer was varied in order to determine if differences in diffusion characteristics affected mineralization nucleation and/or rate. Samples were performed in triplicate, and error was represented as standard deviation.

The supernatants used in the above calcium assay were also used to assay for phosphate content of the mineralized gels using a procedure developed by Van Veldhoven et al. [16]. Reagent A consisted of 1.75% ammonium heptamolybdateheptahydrate in 6.3 N H₂SO₄, and Reagent B consisted of 0.035 wt% malachite green (Aldrich) and 0.35% polyvinyl alcohol in dH₂O. Sample solutions (10 μ l) were combined with 90 μ l 0.9 N H₂SO₄ in triplicate in 96-well plates. Reagent A (20 μ l) and Reagent B (20 μ l) were added to each well. After 20 min of incubation at room temperature, the absorbance of each well was measured using a plate

reader at 590 nm. Using a range of K₂HPO₄ standards, the concentration of phosphate in each gel could be calculated. Combining the calcium assay results (Section 2.3) with these experiments, the ratios of calcium to phosphate (Ca/P) were then determined and compared to typical hydroxyapatite composition.

2.7. X-ray diffraction of mineralized regions

PEG3400DA hydrogels containing 50 mM EGMP were mineralized by placing in hMSC media with or without β -glycerophosphate for 2 weeks. X-ray diffraction (XRD, using an Inel 1150 X-ray diffraction machine) was then used to determine whether the structure of the calcium/phosphate mineral phase was similar to that of organic apatites. XRD spectra were obtained for 2θ angles from 20° to 40°.

2.8. Osteopontin sequestering by EGMP-containing hydrogels

Recombinant human osteopontin (R&D Systems, Minneapolis) was dissolved at a concentration of 1.25 μ g/ml (equivalent to 50 ng/gel) in 10 wt% PEG3400DA solutions containing 0.05 wt% I2959 and 0 mM or 50 mM EGMP. These solutions were then polymerized under ultraviolet light for 10 min. Each gel was transferred to cryovials containing 1 ml PBS supplemented with 0.1% bovine serum albumin (to prevent non-specific adsorption to the tube walls) and 0 or 200 mg/l calcium chloride (Fisher) to promote mineralization. At various time points (2, 4, 8, 14, 24, 36, and 48 h), supernatants surrounding the gels were removed (in triplicate) and stored at –20 °C for later analysis.

An enzyme-linked immunosorbent assay (ELISA) was used to quantify the amount of osteopontin that diffused out of each hydrogel with time. After the final time point (48 h), all supernatants were thawed at room temperature, and the concentrations of osteopontin were measured using a human osteopontin ELISA kit (Quantikine osteopontin-specific ELISA, R&D Systems) based on the manufacturer's instructions.

2.9. Adhesion of hMSCs on osteopontin-sequestered EGMP-containing hydrogels

Hydrogel disks were fabricated from solutions containing 10 wt% of PEG3400DA, 0.05 wt% I2959, and either 0, 10, or 50 mM EGMP. The resulting solutions were polymerized in sterile molds with ~ 5 mW/cm² ultraviolet light. A solution of osteopontin (4 μ g/gel) in hMSC media (without FBS) was added to half of the gels, half remaining as negative controls, and allowed to adsorb for 48 h. hMSCs were trypsinized from culture plates, counted, centrifuged, resuspended, and seeded onto the sterile disks at a density of 2×10^4 cells/cm² in low serum (1% FBS) containing media to reduce any protein adsorption beyond osteopontin. After 6 h, the disks were rinsed with PBS and cells were fixed in 4% paraformaldehyde in PBS for 10 min. Phase contrast images (Nikon Eclipse TE300) were taken of the fixed hMSCs. Attached cells were counted on a minimum of three random fields (0.0026 cm²) per disk ($n = 4$ per composition).

3. Results and discussion

3.1. Cytotoxicity of ethylene glycol methacrylate phosphate

Fig. 2 shows the relative metabolic activities, as measured using the MTT assay, of hMSCs cultured in media containing various concentrations of EGMP for 2 days. All results are normalized to the control (0 μ M EGMP). As can be seen, with increasing concentration of EGMP, metabolic activity, which is proportional to relative survival, decreases. At a concentration of 1 mM, the metabolic activity drops $\sim 10\%$, and this difference is

statistically significant ($p < 0.05$). Despite the apparent decrease in hMSC viability with increasing EGMP concentration, these results were very promising. In general, methacrylate-containing small molecules, such as EGMP, are relatively toxic to cells. These studies represent a worst-case scenario, as the cells were in contact with soluble EGMP for 48 h. However, during photoencapsulation of hMSCs in the presence of EGMP, the cells are only in contact with the EGMP for at most 15 min. Subsequently, the gels are placed in a large excess of media, and this helps to quickly eliminate any un-reacted EGMP. Further, while we preferred to use the commercially available EGMP, the synthesis of a higher molecular weight poly(EGMP) is possible, and the cytocompatibility of this molecule would be expected to increase dramatically as the molecular weight increases to at least 2000–3000 Da. Based on these studies, we surmised that under photoencapsulation conditions (i.e., limited exposure of the cells to soluble EGMP), the monomer would be relatively non-toxic to the cells. Moreover, it was likely that a higher concentration of EGMP, if needed, could successfully be utilized during the photoencapsulation of hMSCs.

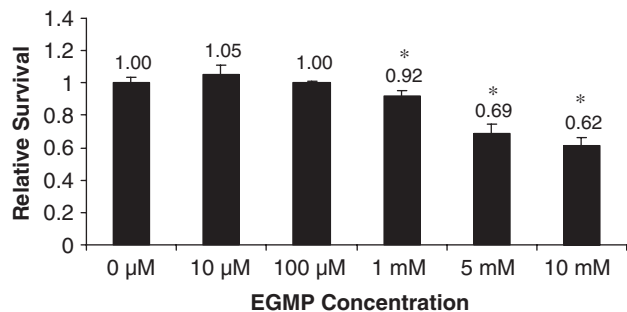


Fig. 2. Cytotoxicity of EGMP on hMSCs in vitro. Relative metabolic activity is plotted as a function of EGMP concentration. Cells were cultured with various concentrations of EGMP for two days prior to evaluating their metabolic activity using the MTT assay. Asterisk denotes results that are significantly different than the 0 μ M results ($p < 0.05$).

3.2. LIVE/DEAD cell assay for viability of hMSCs encapsulated within EGMP-containing hydrogels

Fig. 3 shows laser scanning confocal microscopy images of hMSCs that had been photoencapsulated in PEG3400-DA hydrogels containing 0, 10, and 50 mM EGMP and cultured for 1 week. The cells were stained using the LIVE/DEAD cell assay. Based on results from Fig. 2, it was believed that cell viability would be highest in the gels containing 0 mM EGMP, moderate in the gels containing 10 mM EGMP, and lowest in the gels containing 50 mM EGMP. To our surprise, viability was always improved when EGMP was incorporated into the gel, at least up to 50 mM. On the other hand, viability in the gels containing no EGMP was very low. Maintenance of viability of hMSCs in PEG-based hydrogels has been found to be significant problem [17]. 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 PEG3400DA hydrogels and high viability was seen after 2 weeks [18,19] and in work by Wang et al. [20] using goat mesenchymal stem cells encapsulated in PEG-based hydrogels, viability was maintained at $\sim 100\%$ up to 3 weeks. Also, $\sim 60\%$ viability after 1 week is achieved with hMSCs isolated from a different donor [17] demonstrating viability is both highly dependent on cell type, species, and donor. Monoacrylated PEG molecules with pendant RGD groups were incorporated into similar networks, and viability is rescued to $\sim 75\%$ after 1 week [21], indicating anoikis, apoptosis due to lack of cell-material interactions, is occurring. Based on these results presented here and in [21], the charged phosphate groups on EGMP is likely altering the cell environment in a manner that leads to an improvement in cell viability in PEG3400DA hydrogels. Despite our findings that exposure to higher concentrations of the un-reacted EGMP monomer leads to reduced viability in monolayer culture after 48 h, these studies

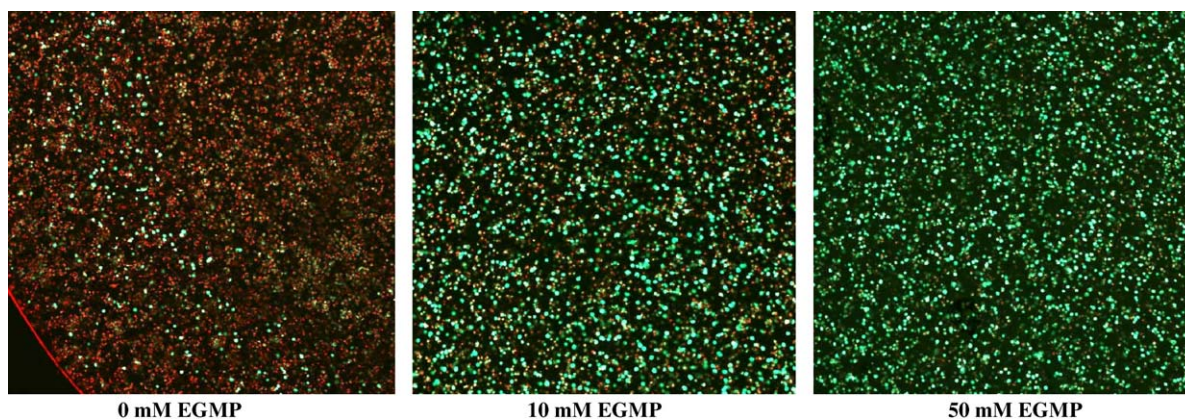


Fig. 3. Human MSCs were photoencapsulated in 10 wt% PEG3400DA hydrogels containing 0 mM (left), 10 mM (center), or 50 mM (right) EGMP and cultured in CON media. Viability was assessed using the LIVE/DEAD assay. Living cells fluoresce green whereas dead cells fluoresce red. Cells are viable upon photoencapsulation in the presence of 10 mM ($74 \pm 5\%$ of cells viable) and 50 mM EGMP ($97 \pm 7\%$ of cells viable) as compared to 0 mM EGMP ($15 \pm 7\%$ of cells viable). Surprisingly, the viability of cells encapsulated in the presence of EGMP is greater than in the absence of EGMP.

show that the low exposure time (<15 min) to soluble EGMP during photoencapsulation has negligible cytotoxicity implications; in contrast, it actually improves viability of encapsulated hMSCs when reacted into PEG hydrogels.

3.3. DNA content in hydrogels formed with or without EGMP

Owing to the fact that cell viability drops in PEG gels over time and this viability can be rescued using EGMP, the amount of DNA should also remain high over time in gels containing 50 mM EGMP. DNA content of hydrogels containing hMSCs was assessed using the PicoGreen assay and the results are shown in Fig. 4. DNA content decreases with time in the gels containing 0 mM EGMP, whereas there appears to be a sustained amount of DNA in the gels containing 50 mM EGMP. The results from Fig. 3 indicate that cell viability drops tremendously after 7 days in the absence of EGMP, while the results from Fig. 4 show that after 7 days there is still a large quantity of DNA within the gels. The time lag associated with the DNA decrease in the PEG gels is believed to result from the lag between cell death and release of DNA from the gel. The assay used to quantify DNA does not discriminate between DNA from living and dead cells, so DNA that is measured does not necessarily have to come from living cells. Despite this fact, DNA content in gels without EGMP continues to drop over time. In contrast, DNA content of gels containing cells encapsulated in the presence of EGMP remains relatively constant over time, indicating that this environment is suitable for the sustained viability of hMSCs up to at least 3 weeks.

3.4. Mineralization of PEG hydrogels containing EGMP

Complementary to the observation that phosphate containing gels enhance hMSC viability, the potential of the phosphate groups to promote mineralization of the hydrogels was expected. Fig. 5 shows the mineralization of

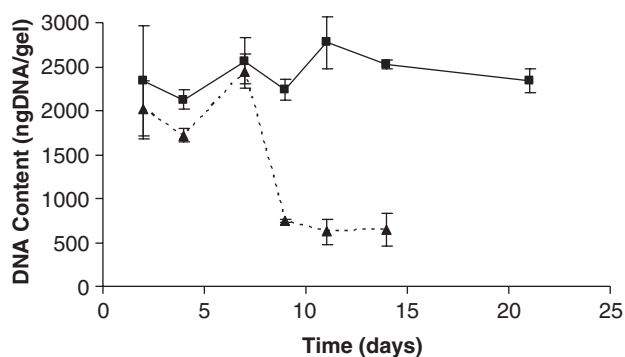


Fig. 4. DNA content of cell/polymer constructs versus culture time. Human MSCs were photoencapsulated in PEG3400DA hydrogels containing 0 mM EGMP (▲ and dotted lines) or 50 mM EGMP (■ and solid line). At various time points, total DNA was quantified for each construct. Samples were performed in triplicate.

PEG hydrogels formed from PEG3400DA, PEG4600DA, and PEG8000DA in the presence or absence of 50 mM EGMP. The neutral or phosphate-containing gels were then cultured in media containing 0 or 10 mM β -GP (the mineral nucleator found in osteogenic media). In general, tethered EGMP helps to increase both the rate of mineralization and the extent of mineralization in PEG-based hydrogels when β -GP is absent. When β -GP is present, it leads to significant mineralization, and this mineralization is enhanced when EGMP is incorporated within the hydrogel. In PEG3400DA, it appears that the presence of EGMP is more important than the presence of β -GP; even in the absence of soluble β -GP the rate of mineralization is similar to the rate with β -GP as long as EGMP was copolymerized within the gel.

In the absence of EGMP (left column of Fig. 5), the rate of mineralization in the presence of β -GP is greater in PEG4600DA and PEG8000DA hydrogels than PEG3400-DA hydrogels. However, the eventual extent of mineralization in these three gel compositions remains the same ($\sim 200 \mu\text{g Ca}^{2+}/\text{gel}$), indicating that diffusional limitations might be hindering the initial nucleation but not the extent of mineralization. When EGMP is incorporated within the gel (right column), mineralization is improved in the absence of β -GP over gels containing no EGMP. When β -GP is present in combination with EGMP, the biggest effect is seen in PEG4600DA hydrogels (increased from $140 \mu\text{g Ca}^{2+}/\text{gel}$ in the absence of β -GP to $200 \mu\text{g Ca}^{2+}/\text{gel}$ in the presence of β -GP). From a mineralization standpoint, it appears that tethered EGMP can improve mineralization tremendously, especially in the absence of β -GP. In the case of an in vivo injectable system to deliver hMSCs to a bone defect, β -glycerophosphate will not be present in the required concentrations to induce mineralization and any physically entrapped β -glycerophosphate would quickly diffuse away from the site of delivery. Instead of relying on β -glycerophosphate in the surrounding media to trigger mineralization, this approach utilizes a tethered β -glycerophosphate analog, EGMP, to augment mineralization, a more clinically relevant approach.

In vitro, mineralization is induced in osteogenic cultures using the water-soluble phosphate β -glycerophosphate. Although studied extensively to date, the exact mechanism by which β -glycerophosphate induces mineralization is still unclear, but it is believed to be closely related to the ability of alkaline phosphatase to hydrolyze organic phosphate and release inorganic phosphate [22]. This free inorganic phosphate can then provide the chemical potential for promoting mineral deposition.

In a similar manner, the alkaline phosphatase present on the cell surface may be capable of hydrolyzing the pendant phosphate groups provided in the gel by EGMP to inorganic phosphates. The phosphates may then be involved in promoting mineral formation inside the gel. However, our results above (Fig. 5) show that significant mineralization occurs in the *absence* of cells. Therefore, one would expect that alkaline phosphatase is not required for

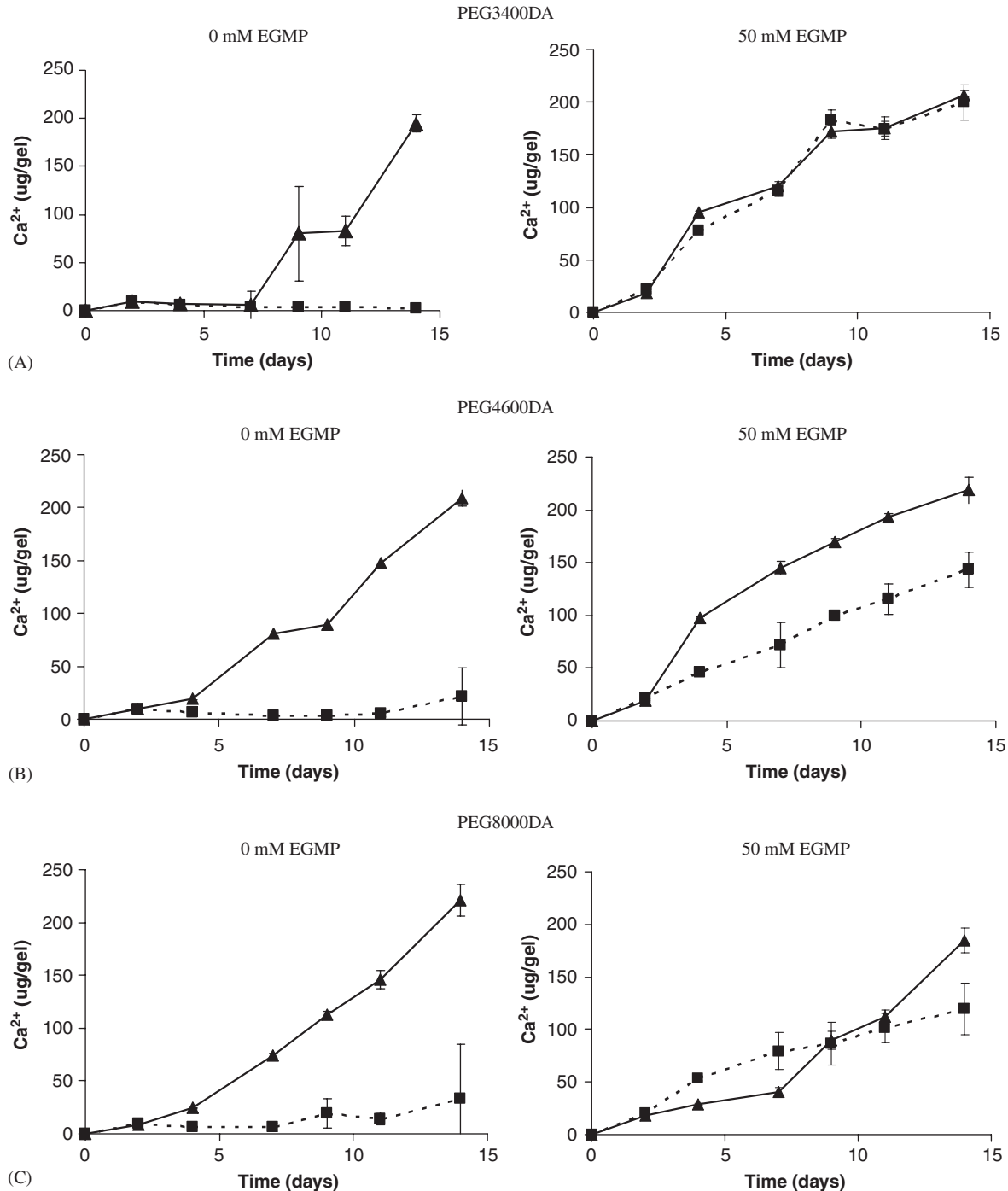


Fig. 5. Mineralization of PEG hydrogels polymerized from 10 wt% solutions of (A) PEG3400DA, (B) PEG4600DA, or (C) PEG8000DA. Gels contain 0 or 50 mM EGMP and were cultured in media containing 0 mM (■ and dotted lines) or 10 mM β -glycerophosphate (β -GP, ▲ and solid lines).

EGMP to promote mineralization in these hydrogels. We have noticed that mineralization does not occur without serum in the surrounding media during mineralization of EGMP-containing PEG hydrogels. Serum contains many diverse proteins; perhaps some of these proteins have phosphatase activity and are capable of hydrolyzing the organic phosphates to inorganic phosphates capable of initiating mineral formation. At this point, however, the

exact mechanism as to how EGMP promotes mineralization in these hydrogels remains unclear.

3.5. Mineralization of PEG hydrogels containing EGMP

While mineralization can be enhanced in phosphate-containing gels, the composition of the mineral phase is important to characterize. Using an assay specific for

Table 1
Calcium/phosphate (Ca/P) ratios of various pre-mineralized PEG3400DA hydrogels. Gels were incubated in various media for 2 weeks

Gel composition	Media	Ca ²⁺ (μmol/gel)	PO ₄ ²⁻ (μmol/gel)	Ca/P
0 mM EGMP	0 mM β-GP	(Not measurable)	(Not measurable)	—
0 mM EGMP	10 mM β-GP	4.83 ± 0.06	2.94 ± 0.21	1.64 ± 0.02
50 mM EGMP	0 mM β-GP	4.71 ± 0.18	2.96 ± 0.23	1.59 ± 0.06
50 mM EGMP	10 mM β-GP	4.59 ± 0.06	2.87 ± 0.30	1.60 ± 0.02

calcium and another assay specific to phosphate [16], the absolute amounts of these minerals and the Ca/P ratios were determined for gels containing 50 mM EGMP that were incubated in the presence of 10 mM β-glycerophosphate. The results are summarized in Table 1. Hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂] is the major component of bone and other biological apatites; therefore, the theoretical stoichiometric ratio of calcium to phosphate (Ca/P) is 1.67 [23]. Due to imperfections and other factors, the actual Ca/P ratio found in bone is around 1.60 [1,4]. The Ca/P ratios in amorphous calcium phosphate [Ca₃(PO₄)₂], brushite [(CaHPO₄)₃·H₂O], and octacalcium phosphate [Ca₈(PO₄)₆H₂] are 1.5, 1.0, and 1.33, respectively [23]. These results indicate that the mineral regions of mineralized PEG hydrogels have similar composition to biological apatite.

3.6. X-ray diffraction of mineralized regions

Given the enhanced mineralization and the fact that the composition of this mineralization is similar to biological apatites, we wanted to compare and confirm that the structure is similar to biological apatites. Fig. 6 shows a typical X-ray diffraction (XRD) spectrum of pre-mineralized PEG3400DA hydrogels containing tethered EGMP. The XRD spectra of mineralized PEG3400DA hydrogels were similar either with or without EGMP, and were similar to the XRD spectra of biological apatites presented in the literature, especially with respect to the peak at 32° [24].

These results, and the results presented in Section 3.5, indicate that the calcium/phosphate mineral phases created in PEGDA hydrogels are similar in composition and structure to biological apatites. Due to the fact that PEG hydrogels mineralize in a manner similar to biological mineralization, these gels have great promise as osteogenic scaffolds for hMSC delivery and bone regeneration.

3.7. Osteopontin-sequestering by EGMP-containing hydrogels

To better understand the enhanced cell viability in the charged, EGMP-containing gels, the ability of proteins to interact with and adsorb to the modified PEG gels was explored. PEG is traditionally very resistant to protein adsorption; however, charged and mineralized PEG gels present an entirely different environment. Initially, experi-

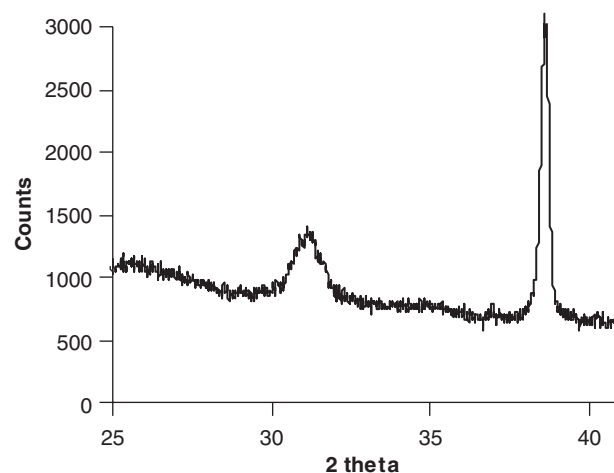


Fig. 6. X-ray diffraction spectrum of PEG3400DA gels containing 50 mM EGMP and cultured in hMSC media for 2 weeks.

ments were conducted to examine osteopontin adsorption in these gel formulations, given osteopontin's prominent role in osteoblast adhesion [9] and its role in osteogenic differentiation [8].

One hypothesis is that by creating mineral regions throughout the hydrogel, EGMP leads to sequestering of osteopontin in the hydrogel, which influences hMSC survivability. To test this hypothesis, osteopontin was encapsulated within PEG3400DA hydrogels in the presence of 0 or 50 mM EGMP. Gels were then incubated for up to 46 h in PBS containing 0 or 200 mg/l CaCl₂ to promote accelerated mineralization. The diffusion of encapsulated osteopontin into solution versus sequestering or adsorption into the gel was measured using an osteopontin-specific ELISA, and the results are shown in Fig. 7.

The dotted line shows the release of osteopontin in unmodified PEG3400DA hydrogels, and the release profile follows a simple Fickian diffusion mechanism. Based on this data and one-dimensional Fickian diffusion through the gel, the diffusivity of osteopontin through the hydrogel was calculated to be $\sim 7.5 \times 10^{-9} \text{ cm}^2/\text{s}$ (r^2 of 1.00). Using the same Fickian diffusion model, the diffusion of osteopontin in PEG3400DA hydrogels containing 50 mM EGMP was estimated to be $\sim 3.3 \times 10^{-8} \text{ cm}^2/\text{s}$ ($r^2 = 0.98$), which is 4.4 times faster than that in the absence of EGMP. We hypothesize that the diffusion coefficient of osteopontin in the negatively charged, EGMP-containing gels is

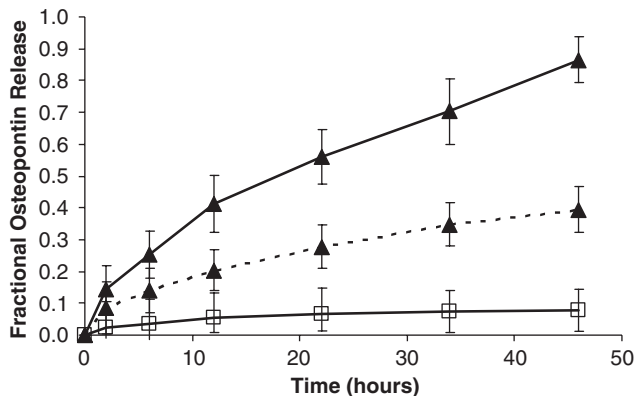


Fig. 7. Cumulative fractional osteopontin release as a function of time. Osteopontin was loaded at a concentration of 50 ng/gel into PEG3400DA hydrogels containing 0 or 50 mM EGMP and cultured in PBS containing 0 or 200 mg/l CaCl₂; 0 mM EGMP and 0 mg/l CaCl₂ (▲, dotted line), 50 mM EGMP with 0 mg/l CaCl₂ (▲, solid line), and 50 mM EGMP with 200 mg/l CaCl₂ (□, solid line). Osteopontin released into solution was measured using an osteopontin-specific ELISA.

greater due to electrostatic interactions between the negatively charged osteopontin molecules and the negative charge on the phosphate groups within the gel. Alternatively, repulsive electrostatic interactions between pendant phosphate groups within the gel could be leading to increased swelling in EGMP-containing hydrogels, which would result in greater mesh size and more facile diffusion of osteopontin. However, the mass swelling ratio of gels containing 50 mM EGMP was statistically the same as gels containing no EGMP (unpublished findings), refuting this hypothesis.

Using the Stokes–Einstein equation and an estimated radius of 22 Å (radius estimated from a peptide properties calculator at www.basic.nwu.edu/biotools/protein-calc.html), the diffusivity of osteopontin in water is estimated to be $\sim 1.3 \times 10^{-6}$ cm²/s at 37 °C, which is on the same order of magnitude as the diffusion of other proteins through hydrogels [25,26]. Osteopontin diffusion in PEG hydrogels is about two orders of magnitude lower than in aqueous solution. The diffusion coefficient of a solute in a gel is dependent upon its size (i.e., diameter) and the average mesh size of the hydrogel. As the solute size approaches the average mesh size of the gel, the diffusion coefficient decreases and ultimately the macromolecule is unable to diffuse through the gel. These data indicate that the size of osteopontin is approaching the average mesh size of the hydrogel.

After 48 h of release, the fraction of osteopontin released increased from ~ 0.40 in the absence of 50 mM EGMP to 0.86 in the presence of EGMP. When CaCl₂ was added in the surrounding solution, this dropped to 0.08. Thus, the presence or absence of CaCl₂ in the surrounding fluid appears to play an important role in altering the release rate of osteopontin from the charged, EGMP-containing hydrogels. In the absence of CaCl₂, osteopontin diffusion into the surrounding fluid is more rapid from gels containing tethered EGMP than gels containing no

EGMP. While initially this might seem unexpected, repulsive electrostatic interactions between the negatively charged phosphate groups and the acidic, negatively charged osteopontin molecules may cause the osteopontin to diffuse out of the network quickly. On the other hand, if CaCl₂ is present in the surrounding fluid (bottom line in Fig. 7), osteopontin diffusion from EGMP-containing hydrogels is very slow. In this case, it is believed that the positively charged, divalent Ca²⁺ ions in solution act as “bridges” between the negatively charged tethered EGMP molecules and the negatively charged osteopontin molecules. In addition, the EGMP groups aid in mineralization, as seen in Section 3.4 above. Both of these mechanisms explain the ability of EGMP-containing PEGDA hydrogels to sequester osteopontin.

3.8. Adhesion of hMSCs on osteopontin sequestered EGMP-containing hydrogels

In order to further understand hMSC interaction with EGMP and test the hypothesis that, because osteopontin can be sequestered by EGMP, hMSCs can interact with and adhere to EGMP-containing hydrogels, osteopontin was sequestered in EGMP-containing hydrogels and hMSC adhesion was analyzed. Fig. 8a shows improvement of attachment of hMSCs to hydrogels incorporating EGMP-sequestered osteopontin over hydrogels with no modification. In addition, as shown in Fig. 8b, attachment increases with EGMP concentration, finding that very few cells attached to 0 mM EGMP (865 cells/cm²) after 6 h of incubation whereas 3285 cells/cm² and 6321 cells/cm² attached to 10 and 50 mM EGMP-containing hydrogels. No statistical differences in cell density were found when comparing different EGMP concentrations and no osteopontin adsorption (data not shown). In light of these results, it is likely that, in addition to promoting a bone-like mineral phase throughout the PEGDA hydrogel, EGMP promotes cell adhesion to the hydrogel through the indirect immobilization of important cell adhesion proteins, such as osteopontin.

4. Conclusions

In the research presented herein, we first demonstrated that EGMP is cytotoxic at concentrations greater than 10 mM to hMSCs cultured in monolayer in vitro conditions. Despite the apparent negative effect of EGMP on hMSC viability, the viability of encapsulated hMSCs in EGMP-containing gels (10 and 50 mM) was shown to be much higher than expected, based on monolayer studies, even higher than cell viability in gels without EGMP. The concentration of EGMP was increased up to 50 mM, and there was little evidence of toxicity on encapsulated hMSCs. In fact, the findings were quite opposite, as cell viability increased from 15% in gels containing 0 mM EGMP to 74% with 10 mM EGMP and 97% with 50 mM EGMP. These studies indicate that EGMP may, in fact,

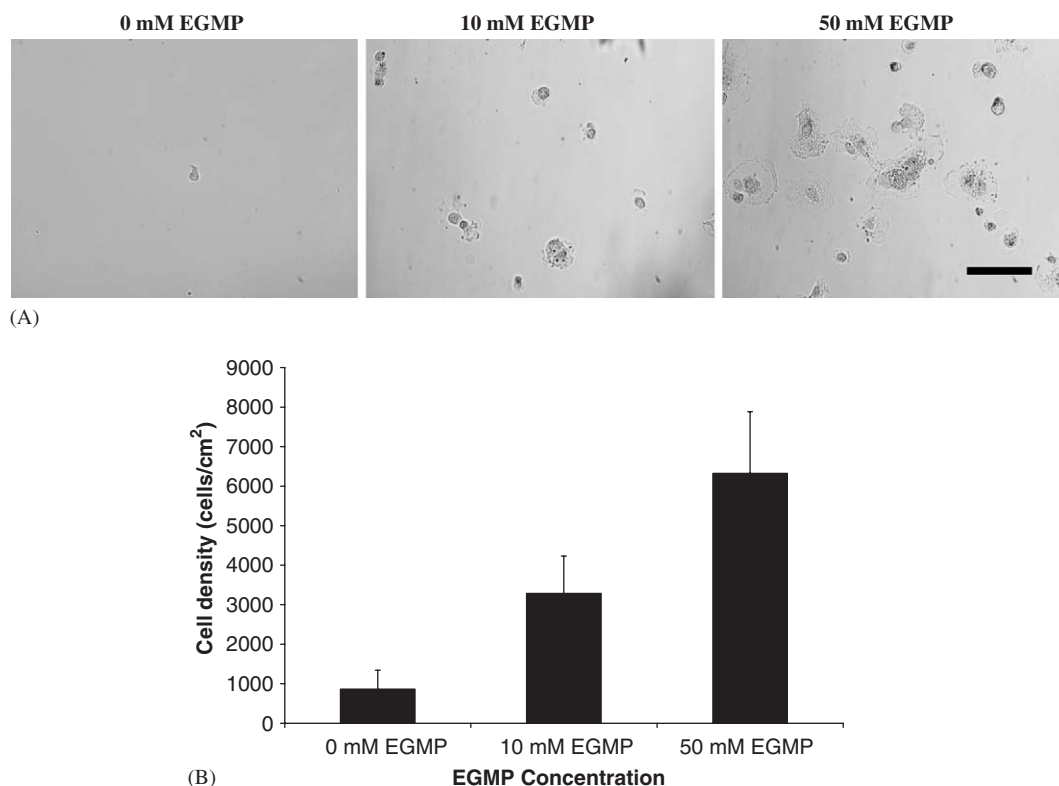


Fig. 8. (A) hMSCs were seeded onto various two-dimensional PEG surfaces at a concentration of 20,000 cells/cm². After 6 h, cells were fixed with 4% paraformaldehyde and visualized for attachment and spreading on the various substrates. Shown are cells that have been seeded on PEG3400DA only, PEG3400DA with 10 mm ethylene glycol methacrylate phosphate (EGMP), and PEG3400DA with 50 mM EGMP (bar = 100 μm). Surfaces were incubated in media without FBS supplemented with 4 μg/gel osteopontin for 48 h prior to cell seeding. Cells seeded on the above surfaces without osteopontin showed no significant attachment or spreading as compared with PEG3400DA surfaces. (B) Cell density as a function of EGMP concentration. Osteopontin was incubated with the gels at a concentration of 4 μg/gel. Values reported as the average of five random fields for four hydrogels per composition (error bars designate standard deviation).

improve hMSC survivability over un-modified PEGDA hydrogels.

Secondly, it was shown that the rate of mineralization and the eventual extent of mineralization of various PEGDA hydrogels could be increased through the incorporation of EGMP. Furthermore, the calcium/phosphate mineral phase created in these hydrogels was shown to be similar to biological apatites, both in the structure and atomic composition.

Finally, it was found that EGMP-containing PEGDA hydrogels are able to sequester negatively charged proteins, such as the important cell adhesion protein osteopontin, in the presence of CaCl₂, and osteopontin-sequestering EGMP-containing hydrogels promoted hMSC adhesion and spreading. For tissue engineering applications, it is likely that the ability of these hydrogels to sequester osteopontin and other negatively charged proteins promotes cell–gel interactions and, as a result, improves viability of encapsulated hMSCs.

Acknowledgements

The authors would like to thank Drew Watkins for his help with the diffusion coefficient calculations and Xiaoyun

Lu for performing the X-ray diffraction spectra. This work has been supported by the Howard Hughes Medical Institute and a grant from the National Institute of Health (DE016523). Fellowship assistance to CRN and DSWB is awarded graciously and the National Science Foundation and the Department of Education's Graduate Assistantships in Areas of National Need (GAANN).

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