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Synthesis and characterization of a fluvastatin-releasing hydrogel delivery system to modulate hMSC differentiation and function for bone regeneration

Danielle S.W. Benoit[a], Charles R. Nuttelman[a], Stuart D. Collins[a], Kristi S. Anseth[a,b,*]

[a]*Department of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80309-0424, USA*
[b]*Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309-0424, USA*

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Abstract

Increases in bone formation have been demonstrated in mice and rats treated with statins, a group of molecules that increase the production of bone morphogenetic proteins-2 (BMP2) by stimulating its promoter. However, clinical use of statins (e.g., fluvastatin) is limited by the lack of a suitable delivery system to localize and sustain release. To harness the therapeutic effect of statins in orthopedic applications, a fluvastatin-releasing macromer was synthesized. When copolymerized with a dimethacrylated poly(ethylene glycol) solution, this fluvastatin-containing molecule was covalently incorporated into hydrogel networks, and hydrolysis of lactic acid ester bonds resulted in the release of the pendantly tethered fluvastatin from the hydrogel into the surrounding solution. The rate of fluvastatin release was controlled by the length of lactic acid spacer (2–6 repeats), and the dose was controlled by the initial comonomer composition (5–500 μg fluvastatin/gel). Released fluvastatin increased human mesenchymal stem cell (hMSC) gene expression of CBFA1, ALP, and COL I by 34-fold, 2.6-fold, and 1.8-fold, respectively, after 14 days of in vitro culture. In addition, treating hMSCs with the released fluvastatin resulted in an average of 2.0- and 1.5-fold greater BMP2 production whereas mineralization increased an average of 3.0-fold and 2.5-fold for 0.01 and 0.1 μM fluvastatin, respectively, over the 2 week culture period. Therefore, fluvastatin-releasing hydrogels may be useful in bone tissue engineering applications, not only for triggering osteogenic differentiation of hMSCs, but also by modulating their function.

Keywords: Controlled drug release; Statin; Mesenchymal stem cells; Bone tissue engineering

1. Introduction

In vivo, bone morphogenetic proteins (BMPs) are potent effectors of osteoblast function and human mesenchymal stem cells (hMSC) osteogenic differentiation and have been used extensively to stimulate bone regeneration [1–3]. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly referred to as statins, were originally developed to treat hypercholesterolemia and have been safely administered for nearly two decades. In addition, many studies have found that statins increase BMP2 expression and stimulate bone formation in vivo and in vitro [4–9]. In two different studies, Meier et al. (2000) [9] and Wang et al. (2000) found that the risk of hip fractures decreased with increases in statin use [10]. Like recombinant BMPs, statins are approved by the Food and Drug Administration [11]; however, the recombinant BMP levels required for bone regeneration are over six magnitudes higher than when BMPs are produced and released naturally by cells in the body [12]. Statins are small, stable molecules that are not susceptible to proteolytic degradation and are easily synthesized, making them 16,000-fold cheaper than recombinant BMPs to produce [6].

The major limitation in the clinical application of statins for bone regeneration is an appropriate delivery system. Due to the ease of absorption of statins into the

*Corresponding author. Tel.: +303 492 7471; fax: +303 492 4341.
E-mail address: kristi.anseth@colorado.edu (K.S. Anseth).

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bloodstream by the digestive system, oral administration is effective in controlling blood cholesterol levels. However, since the systemic availability is only ~2.4% and likely far less in bone tissue, this effectiveness is not expected for healing bone defects [13]. Another delivery option is injection at the site of the bone fracture or defect; however, when statins are injected locally at the doses required for bone regeneration, cytotoxicity, due to the dramatically reduced production of cholesterol, a molecule required for membrane integrity, has resulted [11]. In addition, injections are inefficient due to the rapid diffusion of the low molecular weight statin, requiring repeated injections to obtain the desired effect.

The covalent incorporation of dexamethasone in poly(ethylene glycol) (PEG) hydrogels has recently been reported. Degradation of lactic acid bonds leads to dexamethasone release and osteogenic differentiation of hMSCs [14]. Similar studies in affecting osteogenic differentiation of mesenchymal stem cells (MSCs) were conducted by Whang et al. (2005), who delivered simvastatin by grafting it to the hydrolytically degradable poly(lactide-*co*-glycolide) (PLG) and subsequently formed films that released statin for up to 6 weeks [11]. Kim et al. (2003) developed a method to deliver ascorbate-2-phosphate and dexamethasone from PLG scaffolds and found that these components were released for at least 4 weeks and had osteogenic effects on rabbit MSCs [15]. However, the loading and release of molecules from PLG microparticles and films are often difficult to control. In addition, microparticle delivery is often most advantageous for molecules of high molecular weight, such as proteins, where the required dose is large and the molecule must be protected due to stability issues. Because statins are stable, potent compounds, the opportunity to explore hydrogel delivery systems is possible and provides further advantages with respect to simultaneous cell delivery. However, the sustained release of low molecular weight compounds is difficult to achieve in highly swollen gels, so new approaches to sequester these molecules in a manner that allows control of their loading and release are needed.

The overall objective of this study was to synthesize a fluvastatin-releasing monomer that could be copolymerized into a gel delivery system and test the osteogenic response of this released fluvastatin on hMSCs. First, hydrolytically degradable lactic acid linkages were grafted from PEG macromers and subsequent covalent conjugation of fluvastatin was performed through standard carbodiimide chemistry. The resulting macromer allowed the delivery of fluvastatin where the release is controlled by length of lactic acid linkages and dose is controlled by the original comonomer loading concentration. The resulting fluvastatin-releasing hydrogels were analyzed for their ability to affect the osteogenic differentiation of hMSCs, as measured by gene expression, as well as hMSC BMP2 production and mineralization for evaluation as a bone tissue engineering material.

2. Materials and methods

All materials were obtained from Sigma-Aldrich unless otherwise specified.

2.1. Synthesis of PEG526MMA-LA

Poly(ethylene glycol) monomethacrylate (MW~526 g/mol, PEG526MMA) and D,L-lactides (LA, Polysciences, Inc.) were added in stoichiometric ratios (PEG526MMA:LA; 1:1, 1:2, or 1:3) to a 50 ml round bottom flask. After addition of trace hydroquinone, a free-radical inhibitor, the reaction was heated in an oil bath to melt the lactides. Once melted, the catalyst stannous octoate (at 0.005 molar ratio to the PEG526MMA) was added. The flask was sealed, purged with argon for approximately 5 min, placed in a 110 °C oil bath and stirred.

After 2 h, dichloromethane (Fisher) was added to the reaction mixture and the resulting polymer mixture was extracted twice with 2.0 M hydrochloric acid and once with 10 wt% aqueous sodium chloride. Sodium sulfate was added to the product to remove any residual water and the polymer solution was separated from the sodium sulfate by filtration (Whatman 40 filter paper). The polymer solution was rotoevaporated to remove the dichloromethane and stored at 4 °C under argon. Depending on the ratio of lactides to PEG526MMA (1:1, 1:2, or 1:3), different products containing 2 (PEG526MMA-2LA), 4 (PEG526MMA-4LA), or 6 (PEG526MMA-6LA) lactic acid repeats were synthesized.

2.2. Addition of fluvastatin to PEG526MMA-LA

PEG526MMA-LA was placed in a 25 ml round bottom flask and dissolved in 20 ml of methanol (Fisher). Fluvastatin (Calbiochem), in a 1:1.1 PEG526MMA-LA:fluvastatin stoichiometric ratio and 1,3-diisopropyl carbodiimide (catalyst) in a 1:1 stoichiometric ratio were added. The flask was sealed, purged with argon, and reacted overnight. The progress of the reaction was monitored using thin layer chromatography with ethyl acetate as the solvent.

2.3. Purification of PEG526MMA-LA–fluvastatin

The PEG526MMA-LA–fluvastatin solution was rotoevaporated to remove the methanol and resuspended in dichloromethane. The mixture was extracted twice with 2.0 M hydrochloric acid and once with 10 wt% aqueous sodium chloride. Sodium sulfate was added to the product to remove any residual water and the polymer solution was separated from the sodium sulfate by filtration (Whatman 40 filter paper). To remove unreacted fluvastatin, the polymer solution was rotoevaporated in the presence of 5 g silica gel, loading the gel with free and conjugated fluvastatin. The loaded silica gel was then added to a chromatography column packed with 30 g of silica gel. Ethyl acetate (4 × 50 ml) was used to elute the unreacted fluvastatin. PEG526MMA-LA–fluvastatin was eluted with methanol (3 × 75 ml), and the methanol fractions were combined and rotoevaporated to isolate the product. The addition of fluvastatin to PEG526MMA-LA (i.e., milligrams fluvastatin per milligrams total product) was evaluated via proton NMR.

2.4. Synthesis of poly(ethylene glycol) dimethacrylate (PEGDM)

PEGDM was synthesized as described elsewhere [16]. Briefly, PEG (MW~4600 g/mol) was dissolved in dichloromethane. Triethylamine at 20% molar excess was added dropwise, and the solution was mixed under argon for 5 min. Methacryloyl chloride at 20% molar excess was mixed with dichloromethane and added dropwise to the PEG/TEA solution, and the final mixture was stirred overnight. The product was precipitated in ice-cold diethyl ether, filtered, and dried in a desiccator. After drying, the PEGDM was redissolved in distilled water and dialyzed (Spectrum, 1000 MW cutoff) over 24 h with two distilled water exchanges. The dialyzed PEGDM was lyophilized and stored at 4 °C until use.

2.5. Copolymerization of PEG526MMA-LA–fluvastatin with PEGDM and degradation-controlled release of fluvastatin

PEG hydrogels with tethered fluvastatin were prepared by mixing 10 wt% PEG4600DMA, 0.05 wt% I2959 (Ciba-Geigy) initiator, and PEG526MMA-LA–fluvastatin in dimethyl sulfoxide. About 12.5 mg/ml of PEG526MMA-LA–fluvastatin was added to synthesize a series of gels used to examine the effect of lactic acid length, and about 12.5, 1.25, and 0.125 mg/ml of PEG526MMA-2LA–fluvastatin was added for a series of gels examining the effect of initial loading concentration on fluvastatin release behavior and profile. The solution was polymerized under ultraviolet light (365 nm, ~5 mW/cm^2) for 10 min in molds that yielded cylindrical hydrogels 5 mm wide and 2 mm thick.

Each disk (samples in quadruplet) was added to 1 ml of phosphate-buffered saline (PBS) and fluvastatin was allowed to release over time at 37 °C. At each time point, the entire supernatant was collected and replaced with fresh PBS to ensure sink conditions. Fluvastatin release was quantified from the collected samples using high-performance liquid chromatography (HPLC, Waters Delta Prep 4000). Briefly, 300 μl of each sample was injected into the HPLC inlet, and peaks were analyzed with an ultraviolet detector set at $\lambda = 310$ nm (Waters 2487). The mobile phase consisted of 67% acetonitrile and 33% of 0.1 mM ammonium acetate and the column was a Waters Nova-Pak C18 (3.9 mm × 150 mm). Fluvastatin concentration was calculated from peak area by injecting samples of known concentration and preparation of a standard curve correlating concentration with peak area.

2.6. Effect of fluvastatin delivery on hMSC osteogenic differentiation

hMSCs were purchased from Cambrex 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). Media was not supplemented with osteogenic factors, such as ascorbic acid, dexamethasone, and β-glycerophosphate, to provide a more clinically relevant condition; in the case of an in vivo system to deliver cells to a bone defect, these molecules will not be present in any significant concentration. hMSCs at passage 3 were used in this study.

Samples of released fluvastatin were obtained from supernatants surrounding the disks (Section 2.5); the concentration was adjusted to 0.01 or 0.1 μM by diluting in media. In general, since the concentrations of fluvastatin in the release samples were several orders of magnitude greater than the concentration needed for in vitro studies, the media composition was not altered significantly. hMSCs were seeded in wells of 12-well tissue culture plates and cultured in media containing 0, 0.01, or 0.1 μM released fluvastatin. hMSC osteogenic differentiation was measured using reverse transcription polymerase chain reaction after 1 day, 7 days, and 14 days of culture, monitoring core binding factor α1 (CBFA1), collagen type I (COL I), and alkaline phosphatase (ALP) genes. CBFA1, also known as RUNX2, is a transcription factor that is required for osteogenic differentiation of mesenchymal progenitors [17]. During osteogenic differentiation, other markers of osteoblast phenotype appear, such as accumulation of bone extracellular matrix proteins, of which COL I is the most prevalent, and expression of ALP [18]. At days 1, 7, and 14, cells were rinsed three times with PBS. Total RNA was isolated using a guanidinium thiocyanate/phenol reagent (TRI reagent) and standard manufacturer's protocols. After allowing the RNA pellet to dry, it was resuspended in nuclease-free water, and any residual genomic DNA in the samples was digested (DNase I, Invitrogen). RNA was then quantified using the RiboGreen assay (Molecular Probes) based on the manufacturer's instructions.

Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad). A 15 ng total RNA sample was used for the single strand cDNA synthesis. The reverse transcription reaction was incubated at 25 °C for 5 min, 42 °C for 30 min, and terminated at 85 °C for 5 min. Polymerase chain reaction (PCR) was conducted using the iCycler real-time PCR machine (Bio-Rad), and primers and probes were designed using the Beacon Designer primer design program (Table 1). Primers (Invitrogen) and probes (Integrated DNA Technologies) for CBFA1, ALP, COL I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used. The following PCR parameters were utilized: 95 °C for 90 sc followed by 45 cycles of 95 °C for 30 s and 55 °C for 60 s. Threshold cycle (C_T) analysis was used to quantify the PCR products, which were subsequently normalized to GAPDH.

2.7. Effect of fluvastatin delivery on hMSC function

hMSCs were seeded in wells of 12-well tissue culture plates and cultured in media containing 0, 0.01, or 0.1 μM released fluvastatin. At various time points (1, 7, and 14 days), media was collected and stored at −80 °C until assayed. The cells were rinsed, trypsinized, and quantified with a Multisizer 3 Coulter Counter (Beckman Coulter, Inc., Fullerton, CA). The stored media was thawed and assayed for BMP2 production with an ELISA (Quantikine, R & D Systems) using standard manufacturer's instructions.

Mineralization by hMSCs was analyzed using a calcium assay. At days 1, 7, and 14, cells were removed from culture and rinsed 3 times with HEPES buffer solution (Invitrogen). Then, 1 ml 0.9 N H_2SO_4 was added to each well, and plates were incubated overnight at 37 °C to dissolve all deposited calcium. The supernatant, in triplicate, was added to 100 μl of a solution containing 1 part calcium-binding reagent (0.024 wt% *o*-cresolphthalein and 0.25 wt% 8-hydroxyquinone in diH_2O) and 1 part calcium buffer (500 mM 2-amino-2-methyl-1,3 propanediol in diH_2O). 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 was determined. BMP2 production and mineralization was normalized to number of cells to eliminate variations due to cell density differences, then reported relative to levels of cells cultured in the absence of fluvastatin.

2.8. Statistical analysis

Statistical analysis was performed using a one-way ANOVA with $\alpha = 0.05$. Data are presented as mean ± standard deviation.

3. Results

3.1. Synthesis of PEG526-MMA-LA–fluvastatin macromer

Fig. 1A shows the macromolecular monomer, PEG526-MMA-LA–fluvastatin. The number of lactic acid units, *m*, was varied by controlling the molar ratio of PEG526MMA to lactide in the first step of the synthesis. For the work detailed here, three different monomers were synthesized: PEG526MMA-2LA–fluvastatin, PEG526MMA-4LA–fluvastatin, PEG526MMA-6LA–fluvastatin, corresponding to theoretical numbers of lactic acid repeat units of 2, 4, and 6, respectively. The actual number of lactic acid repeat units added was analyzed with proton nuclear magnetic resonance spectroscopy (NMR, Bruker 500 MHz) and the results are listed in Table 2. In addition, Table 2 lists the % yield (e.g., the % of molecules of PEG526MMA-mLA with fluvastatin conjugated) and milligrams of fluvastatin per mg of product of the fluvastatin addition reaction, as the product is a mixture of the fluvastatin-conjugated product as well as unreacted PEG526MMA-mLA, as analyzed with proton NMR. As lactic acid repeats increased, the % yield

Table 1
Primer and probe sequences designed by Beacon Designer software and utilized for hMSC real-time PCR

Gene	Sense primer	Anti-sense primer	Probe
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5′-GCAAGAGCACAAGAGGAAGAG-3′	5′-AAGGGGTCTACATGGCAACT-3′	5′-ACCCTCACTGCTGGGGAGTCC-3′
Core binding factor α1 (CBFA1)	5′-GGTATGTCCGCCACCACTC-3′	5′-TGACGAAGTGCCATAGTAGAGATA-3′	5′-CTACCACACCTACCTGCCACCACC-3′
Collagen type I (Col I)	5′-GGGCAAGACAGTGATTGAATACA-3′	5′-GGATGGAGGGAGTTTACAGGAA-3′	5′-CCAAGTCCTCCCGCCTGCCCATC-3′
Alkaline phosphatase (ALP)	5′-GTGGAGTATGAGAGTGACGAGAA-3′	5′-AGATGAAGTGGGAGTGCTTGTAT-3′	5′-CCTGGACCTCGTTGACACCTGGAAG-3′

dropped from 100 to 91 to 23%. Since the carboxylic acid functionality of fluvastatin's tether is hydrophilic and lactic acid repeats are hydrophobic, there is significant repulsion that increases with increasing lengths of lactic acid, resulting in lower yields. The monomer was copolymerized with PEG4600DMA to form hydrogels with pendant fluvastatin grafts as pictured in Fig. 1B. As the lactic acid bonds are hydrolyzed, fluvastatin is released and available for uptake by cells.

3.2. Fluvastatin release as a function of lactic acid repeat unit

By altering the number of lactic acid repeat units within the initial monomer, the rate of release of fluvastatin from hydrogel networks can be controlled. The results of fluvastatin release into PBS when different fluvastatin-containing monomers were copolymerized with PEG4600DMA are shown in Fig. 2. The rate of release of fluvastatin from the PEG526MMA-2LA–fluvastatin and the PEG526MMA-4LA–fluvastatin hydrogels was slower than the PEG526MMA-6LA–fluvastatin. This trend agrees with the hypothesis that increasing the number of lactic acid units in the poly(lactic acid) (PLA) block increases the probability that the fluvastatin is unbound and releasable at any given time. However, no statistical difference was observed in the rate of release between the PEG526MMA-2LA–fluvastatin and PEG526MMA-4LA–fluvastatin. This could be due to the lactic acid lengths being fairly close in number (1.7 vs. 3.2), and the fact that these are average block lengths and a distribution will exist. Regardless, the release data follow the trend of increased release rate with increased number of lactic acid repeats in the linker.

The total amount of fluvastatin that was loaded into the gels of Fig. 2 was 0.5 mg/gel, which was corrected for % yield. However, less than 100% of the initial loading was actually released after 40 days (70%, 60%, and 41%, respectively, for PEG526MMA-2LA–fluvastatin, PEG526MMA-4LA–fluvastatin, and PEG526MMA-6LA–fluvastatin). Immediately after photopolymerization, the gels were rinsed with methanol for ~10 min to remove any unreacted fluvastatin. This step led to a loss of fluvastatin (all but 5% of the original monomer loaded was recovered in the release and wash steps, data not shown) and is an indication of decreased efficiency of incorporation of the more hydrophobic monomer with the greater amount of lactic acid content. In previous work utilizing the same tethering technique [14], this same phenomenon was observed.

3.3. Fluvastatin release as a function of initial loading concentration

The total release or dose of fluvastatin from the PEG526MMA-2LA–fluvastatin hydrogels scales with the initial loading of the macromer (Fig. 3). This trend agrees

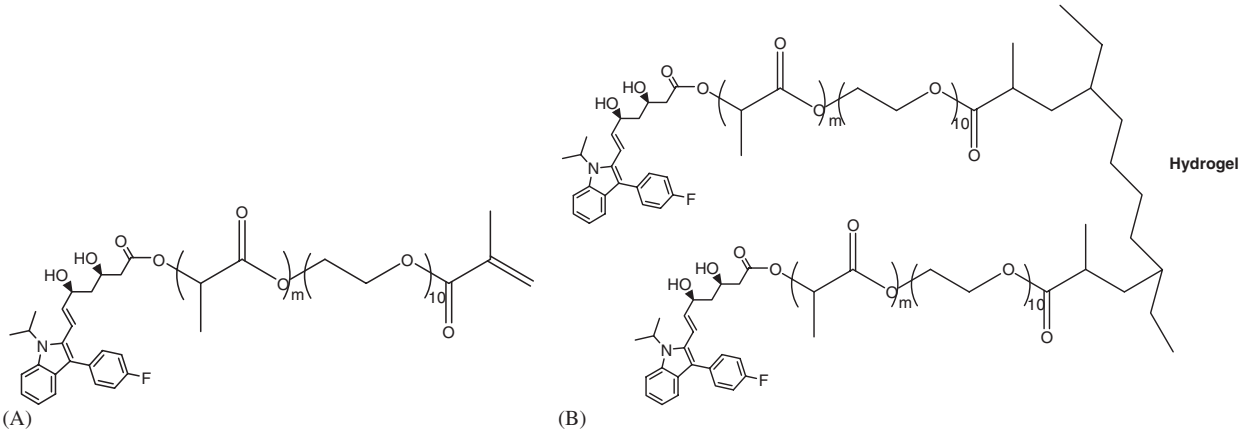

Fig. 1. Macromolecular PEG526MMA-mLA–fluvastatin macromer (A) and schematic depicting the tethering of the PEG526MMA-mLA–fluvastatin macromer throughout the bulk of a PEG-based hydrogel (B).

Table 2
Lactide repeat units per monomer (m in Fig. 1), percent yield, and amount of tethered fluvastatin per product (mg statin/mg product)

Monomer acronym	Lactic acid units per chain (m in Fig. 1)[a]	% yield[a]	mg statin/mg product[a]
PEG526MMA-2LA–fluvastatin	1.7	100	0.417
PEG526MMA-4LA–fluvastatin	3.2	91	0.357
PEG526MMA-6LA–fluvastatin	5.5	23	0.086

[a]Determined by NMR.

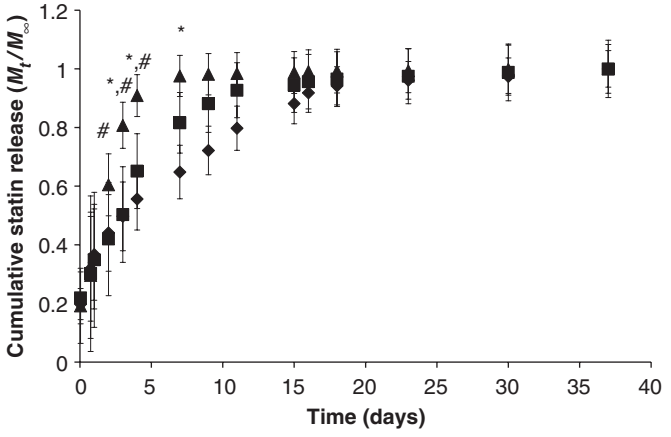

Fig. 2. Cumulative fluvastatin release (M_t/M_∞) as a function of time for hydrogels synthesized through the copolymerization of PEG526MMA-mLA–fluvastatin ($m = 2$: diamonds, $m = 4$: squares, $m = 6$: triangles) with a 10 wt% solution of PEG4600DM. Release profiles were measured in PBS at sink conditions. Gels initially contained ~500 μg of statin and were 5 mm in diameter and 2 mm in height.

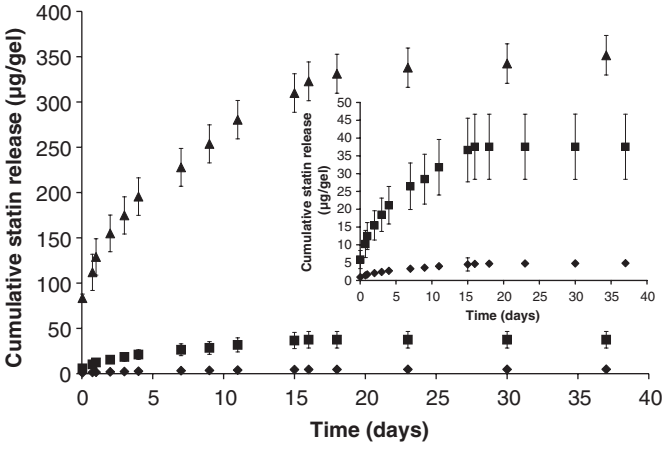

Fig. 3. Cumulative fluvastatin release as a function of time for hydrogels synthesized through the copolymerization of different amounts of PEG526MMA-2LA–fluvastatin with 10 wt% solution of PEG4600DM. Release profiles were measured in PBS at sink conditions. Gels initially contained 500 μg (triangles), 50 μg (squares), and 5 μg (diamonds) of fluvastatin, respectively, and were 5 mm in diameter and 2 mm in height.

with the hypothesis that the dose is dictated by the amount of PEG526MMA-LA–fluvastatin initially incorporated into the gel. In addition, the release profiles were not significantly affected by the variations in initial loading, demonstrating facile control over the amount of statin delivered without impacting the delivery profile.

3.4. Effect of released fluvastatin on hMSC differentiation in monolayer culture

To test if the released fluvastatin had an effect on the osteogenic differentiation of hMSCs, the gene expression of CBFA1, COL I, and ALP was examined in vitro at days

1, 7, and 14, when treated with media with 0.01 or 0.1 μM of released fluvastatin and is quantified in Fig. 4. As depicted in Fig. 4A, CBFA1 gene expression increased 7.8- and 11.3-fold with the treatment of 0.01 and 0.1 μM fluvastatin, respectively, as compared with 0 μM fluvastatin at day 7. By day 14, hMSC CBFA1 gene expression was 17.6- and 34-fold greater in the presence of 0.01 and 0.1 μM fluvastatin, respectively, than cells in the absence of fluvastatin. In addition, hMSC ALP expression (Fig. 4B) increased by 2- and 2.6-fold at day 7 and by 1.6- and 2.6-fold by day 14 in the presence of 0.01 and 0.1 μM fluvastatin, respectively, as compared to cells in the absence of fluvastatin. hMSC COL I gene expression was also increased in the presence of fluvastatin, as shown in Fig. 4C. Cells in the presence of 0.01 and 0.1 μM fluvastatin exhibited no statistical increase at day 7. However, at day 14, a 1.6- and 1.8-fold increase was found for cells in the presence of 0.01 and 0.1 μM fluvastatin, respectively. All expression was statistically the same for all genes and treatments at day 1. Gene expression profiles indicate that fluvastatin is capable of triggering hMSC osteogenic differentiation without the use of any other osteogenic molecules.

3.5. Effect of released fluvastatin on hMSC function in monolayer culture

To verify that the released fluvastatin had the expected effect on hMSCs, BMP2 production and mineralization were analyzed. Supernatant of fluvastatin from the release experiments was diluted in media to achieve a final concentration of 0.01 and 0.1 μM and was used to treat hMSCs in vitro for 1, 7, and 14 days. Controls consisted of media without fluvastatin and media treated with unmodified fluvastatin. Fig. 5 shows the BMP2 production of hMSCs cultured in the presence of media containing PEG526MMA-LA–fluvastatin supernatant at two different concentrations and over a 14 day culture period, relative to production by cells in the absence of fluvastatin. In the presence of 0.01 μM fluvastatin, BMP2 production increased 1.7-fold at day 1 of the culture period. Increases in BMP2 production for cells in culture with 0.01 μM fluvastatin were not statistically different from controls. By increasing the fluvastatin dose to 0.1 μM, BMP2 production increased to 2.6-fold and 2.4-fold at days 1 and 7, respectively, of controls. BMP2 production decreased to

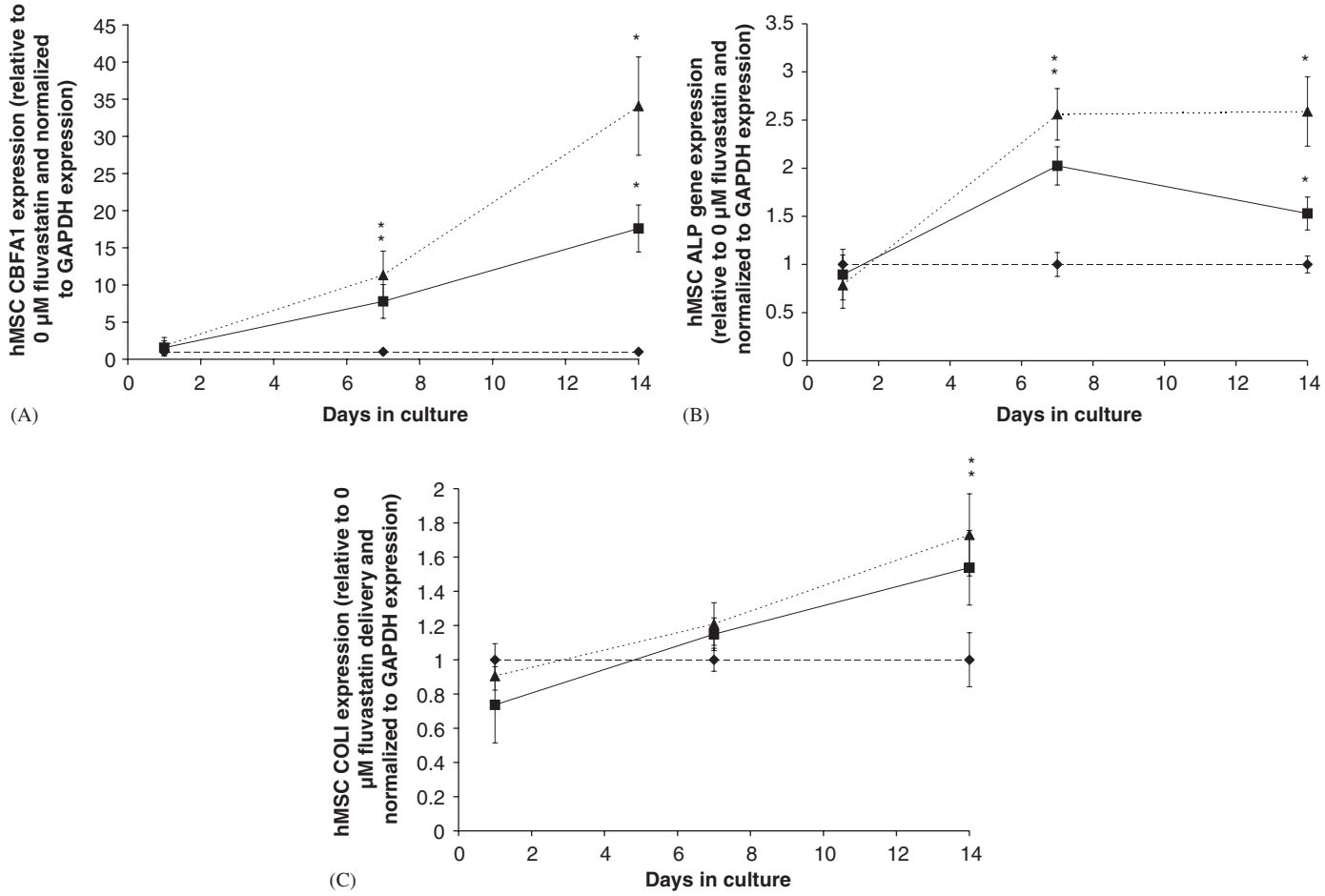

Fig. 4. Core binding factor α1 (CBFA1) (A), alkaline phosphatase (ALP) (B), and collagen type I (COL I) (C) gene expression normalized to levels of GAPDH of hMSCs cultured in the absence of fluvastatin (diamonds with dashed lines), in the presence of 0.01 μM fluvastatin (squares with solid lines), and in the presence of 0.1 μM fluvastatin (triangles with dashed lines). Data are reported relative to 0 μM fluvastatin delivery. $n = 4$ samples per condition. *$p < 0.05$ of sample vs. control (0 μM fluvastatin) at that time point.

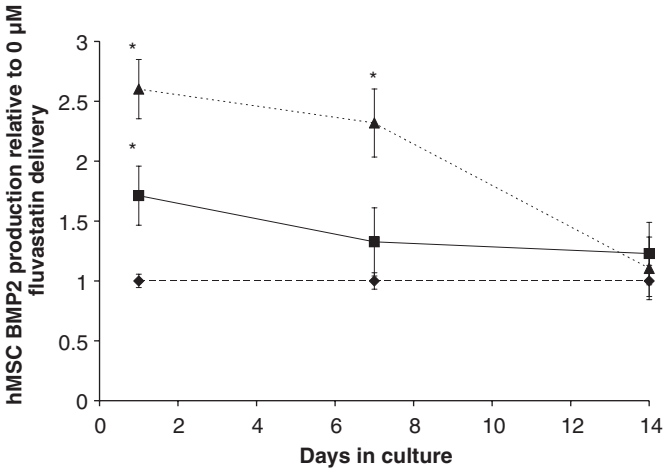

Fig. 5. BMP2 production of hMSCs cultured in the absence of fluvastatin (diamonds with dashed lines), in the presence of 0.01 μM fluvastatin (squares with solid lines), and in the presence of 0.1 μM fluvastatin (triangles with dashed lines) over 14 days of culture (error bars designate standard deviation). Data are reported relative to 0 μM fluvastatin delivery. $n = 4$ samples per condition. *$p < 0.05$ compared with 0 μM fluvastatin at that time point.

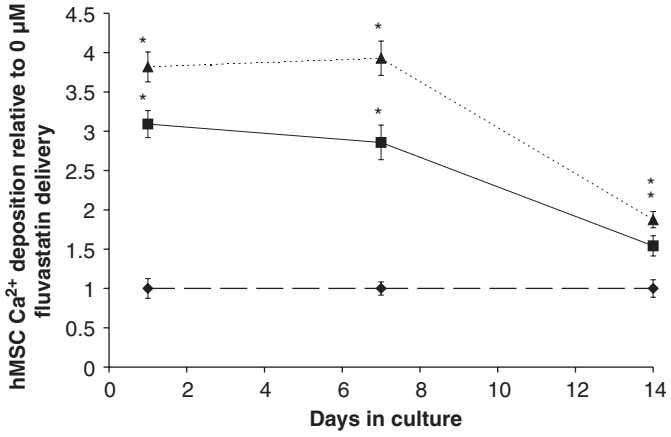

Fig. 6. Calcium deposition of hMSCs cultured in the absence of fluvastatin (diamonds with dashed lines), in the presence of 0.01 μM fluvastatin (squares with solid lines), and in the presence of 0.1 μM fluvastatin (triangles with dashed lines) over 14 days of culture (error bars designate standard deviation). Data are reported relative to 0 μM fluvastatin delivery. $n = 4$ samples per condition. *$p < 0.05$ compared with 0 μM fluvastatin at that time point.

nearly control levels over the 14-day study, likely due to an increase of confluence and subsequent contact-inhibited protein down-regulation.

Using an assay specific for calcium, mineralization was determined for the cells cultured in the absence of fluvastatin, and media containing two different concentrations of PEG526MMA-LA–fluvastatin supernatant at days 1, 7, and 14. Results are shown in Fig. 6. The treatment of fluvastatin increased calcium deposition at all time points. Calcium deposition was increased 3.0-fold at day 1, 3.0-fold at day 7, and 1.6-fold at day 14 for cells treated with 0.01 μM fluvastatin. For cells in the presence of 0.1 μM fluvastatin, calcium deposition increased by 3.8-fold at day 1, 4.0-fold at day 7, and 1.7-fold at day 14. Like BMP2 production, the overall deposition decreased over time, nearing the level of calcium deposition of cells in the absence of fluvastatin. Again, this is likely due to a confluent cell layer and subsequent contact-inhibited down-regulation of protein production, thus fewer mineralization sites. This, along with the BMP2 production results, provides support that the synthesis involved in tethering fluvastatin into the hydrogel network does not compromise its biological activity.

4. Discussion

Statins are a family of small molecules routinely prescribed to lower blood cholesterol levels and have been shown to increase the production of BMP2 by stimulating its promoter [7,8]. However, statins are not used clinically to increase bone formation due to lack of a suitable delivery system. Here, we aimed to design a system for fluvastatin delivery to cells with a controlled dose and localized release profile to exploit outside-in signaling to affect cell functions. Similar to a dexamethasone-releasing hydrogel [14], a fluvastatin-releasing macromer was synthesized where the mechanism for release is through the hydrolytic degradation of the ester bonds of the lactic acid units (Fig. 1B). During this release, two products can result, free fluvastatin and fluvastatin–lactic acid conjugates. According to research performed by Siparsky et al. (1998) on PLA, the increased rate of ester bond hydrolysis over time is attributed to the high reactivity of the terminal ester and the kinetics of autocatalysis [19]. As hydrolysis occurs, the carboxylic acid end-group concentration increases and autocatalysis occurs, effectively increasing the rate of complete degradation of that particular segment. Also, cells produce enzymes such as esterases and lipases capable of cleaving any residual lactic acid units present on fluvastatin after it enters the cell [20]. These studies along with results presented here would suggest that if fluvastatin is released in any form including residual lactic acid segments, the lactic acid units are quickly degraded through either autocatalysis or enzyme catalysis, resulting in free fluvastatin available to the cell.

In this particular delivery system, two governing transport phenomena could control the eventual availability of fluvastatin: kinetics of ester bond hydrolysis to release fluvastatin and diffusion of fluvastatin within the gel. Since fluvastatin is a small molecule (radius ~10 Å), free fluvastatin within hydrogels (mesh size ~100 Å) is released very rapidly. Within 0.5 h, over 95% of encapsulated fluvastatin diffuses out of a pseudo one-dimensional hydrogel that is 2 mm thick (data not shown). Assuming Fickian diffusion, the diffusion coefficient of fluvastatin was fit to diffusion data and estimated to be $\sim 8.1 \times 10^{-7}$ cm^2/s, which is only about 30% less than when the coefficient is estimated by the Stokes–Einstein equation for

the molecule in water. The timescale for diffusion can be estimated by

$$t_d = \frac{L^2}{D_g}, \quad (1)$$

where L is the gel half-thickness (1 mm) and D_g is the diffusion coefficient of the fluvastatin in the gel. Therefore, the characteristic timescale for diffusion from a hydrogel with these dimensions is about 3.5 h. The timescale of the hydrolysis reaction can be estimated by

$$t_h = \frac{1}{k'}, \quad (2)$$

where k' is the pseudo-first-order kinetic constant for hydrolysis of the block in a highly swollen gel. The degradation of lactic acid bonds in hydrogel environments similar to those present in PEG526MMA-LA–fluvastatin has been thoroughly characterized [21,22]. The degradation of an ester bond within the PLA block can be assumed to be pseudo-first-order, assuming a large excess of water surrounding the PLA blocks.

$$\frac{dn_{PLA}}{dt} = k' n_{PLA}. \quad (3)$$

Here n_{PLA} is the number of degradable PLA blocks in the gel, t is the degradation time, and k' is the pseudo-first-order kinetic constant for hydrolysis of the block. Based on data from Fig. 2 and applying Eq. (3), hydrolysis rate constants for the release of fluvastatin were calculated as 0.005, 0.009, and 0.022/h, respectively, for monomers with PLA blocks containing 2, 4, and 6 ester linkers. Alternatively, the average hydrolysis of the ester bonds within each block can be calculated by replacing k' with mk_{ester} where m is the number of lactic acid repeat units in the PLA block. From this analysis k_{ester} is calculated to be ~0.003–0.004/h, which is similar to rate constants reported in the literature for PLA hydrolysis, ranging from 0.0001 to 0.131/h [23,24]. Based on Eq. (2), $t_h = 202$, 110, and 44.6 h; release of fluvastatin from the gel can be sustained over several days at a rate dictated by the macromer chemistry. In addition, when the initial dose of PEG526MMA-2LA–fluvastatin is altered, the hydrolysis rate constants are almost identical regardless of starting concentration ($k'_{0.5\,mg} = 0.005$/h, $k'_{0.05\,mg} = 0.005$/h, and $k'_{0.005\,mg} = 0.006$/h), again indicating that the length of spacer dictates the rate.

By culturing hMSCs with fluvastatin-treated media, induction of osteogenic differentiation of hMSCs, as measured by CBFA1, COL I, and ALP gene expression, was found without the use of any other osteogenic molecule. In general, osteogenic differentiation begins with an increase in CBFA1 expression, continues with augmented protein levels, a cascade that starts with an increase in ALP, bone sialoprotein, and osteopontin, followed by a heightened production of COL I which continues at a high level until mineralization proceeds [18]. Also, diluted, delivered fluvastatin increased BMP2 production and calcium deposition at all time points tested. Differentiation is likely due to an increase of BMP2 production caused by statin, as supported by data in Fig. 5. BMP2 is known to singly induce de novo bone formation at orthotopic and ectopic sites [2]. In addition, during fracture healing, BMP2 acts locally as a growth and differentiation factor that binds to receptors on the surface of MSCs and causes them to differentiate into osteoblasts [1,6]. Specifically, BMP2 can induce bony trabeculae and bone marrow, which can shorten the time required for osteogenesis and increase the amount of bone formed [3]. While this work demonstrates a specific approach to immobilize and locally deliver statins to influence hMSCs osteogenic differentiation and function, an interesting area for further exploration would be to examine the role of statin release profile and its effects on BMP2 levels and hMSC osteogenesis. Further, gels that could locally sequester the cell secreted BMP2 and serve as a reservoir during healing would be a powerful platform to influence local bone regeneration. In sum, through the proper design of a delivery system for fluvastatin, an in situ forming hydrogel capable of cell encapsulation can be utilized to initiate hMSC osteogenic differentiation and promote their osteoblast phenotypic function.

5. Conclusions

The synthesis of macromolecular, fluvastatin-releasing monomer was achieved and this monomer was successfully incorporated into poly(ethylene glycol)-based hydrogels and released at different rates based on the number of hydrolytically degradable lactic acid bonds incorporated in the linkage. The dose of fluvastatin was controlled by the starting macromer concentration in the gels. Delivered fluvastatin caused a dramatic increase on hMSC CBFA1, ALP, and COL I gene expression, indicating fluvastatin's effect on osteogenic differentiation. In addition, released fluvastatin had an effect on hMSCs, resulting in an increase in BMP2 production and mineralization. In summary, fluvastatin-releasing hydrogels may be useful in bone tissue engineering applications by initiating hMSC osteogenic differentiation and modulating their function.

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References

[1] Govender S, Csimma C, Genant HK, Valentin-Opran A. Recombinant human bone morphogenetic protein-2 for treatment of open

tibial fractures—a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg 2002;84A:2123–34.
[2] Groeneveld EHJ, Burger EH. Bone morphogenetic proteins in human bone regeneration. Eur J Endocrinol 2000;142:9–21.
[3] Lane JM. BMPs: why are they not in everyday use? J Bone Joint Surg 2001;83A:S161–3.
[4] Farnier M, Davignon J. Current and future treatment of hyperlipidemia: the role of statins. Am J Cardiol 1998;82:3J–10J.
[5] Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochem Biophys Res Comm 2001;280:874–7.
[6] Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, et al. Stimulation of bone formation in vitro and in rodents by statins. Science 1999;286:1946–9.
[7] Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. Biochem Biophys Res Comm 2000;271:688–92.
[8] Cummings SR, Bauer DC. Do statins prevent both cardiovascular disease and fracture? J Am Med Assoc 2000;283:3255–7.
[9] Meier CR, Schlienger RG, Kraenzlin ME, Schlegel B, Jick H. HMG-CoA reductase inhibitors and the risk of fractures. J Am Med Assoc 2000;283:3205–10.
[10] Wang PS, Solomon DH, Mogun H, Avorn J. HMG-CoA reductase inhibitors and the risk of hip fractures in elderly patients. J Am Med Assoc 2000;283:3211–6.
[11] Whang KM, McDonald J, Khan A, Satsangi N. A novel osteotropic biomaterial OG-PLG: synthesis and in vitro release. J Biomed Mater Res A 2005;74A:237–46.
[12] Service RF. Tissue engineers build new bone. Science 2000;289: 1498–500.
[13] Haberstadt C, Anderson P, Bartel R, Cohen R, Naughton G. Physiological cultured skin substitutes for wound healing. Mater Res Soc Symp Proc 1992;252:323–30.
[14] Nuttelman CR, Tripodi MT, Anseth KS. Dexamethasone-functionalized gels induce osteogenic differentiation of encapsulated hMSCs. J Biomed Mater Res A 2006;76A:183–95.
[15] Kim H, Kim HW, Suh H. Sustained release of ascorbate-2-phosphate and dexamethasone from porous PLGA scaffolds for bone tissue engineering using mesenchymal stem cells. Biomaterials 2003;24: 4671–9.
[16] Sawhney AS, Pathak CP, Hubbell JA. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-*co*-poly(alpha-hydroxy acid) diacrylate macromers. Macromolecules 1993;26: 581–7.
[17] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997; 89:747–54.
[18] Stein GS, Lian JB. Molecular mechanism mediating proliferation, defferentiation interrelationships during progressing development of the osteoblast phenotype. Endocrine Rev 1993;14:424–42.
[19] Siparsky GL, Voorhees KJ, Miao FD. Hydrolysis of polylactic acid (PLA) and polycaprolactone (PCL) in aqueous acetonitrile solutions: autocatalysis. J Environ Polymer Deg 1998;6:31–41.
[20] Carvalheira AF, Pearse AGE. Comparative cytochemistry of C cell esterases in the mammalian thyroid–parathyroid complex. Histochem Cell Biol 1967;8:175–82.
[21] Metters AT, Anseth KS, Bowman CN. Fundamental studies of a novel, biodegradable PEG-*b*-PLA hydrogel. Polymer 2000;41: 3993–4004.
[22] Bryant SJ, Anseth KS. Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. J Biomed Mater Res A 2003;64A:70–9.
[23] Shih C. Chain-end scission in acid-catalyzed hydrolysis of poly (D,L-lactic acid units) in solution. J Controlled Rel 1995;34:9–15.
[24] Hu DSG, Liu HJ. Effect of soft segment on degradation kinetics in polyethylene glycol/poly(L-lactic acid units) block-copolymers. Polym Bullet 1993;30:669–76.