
Dexamethasone-functionalized gels induce osteogenic differentiation of encapsulated hMSCs

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Abstract: Synthetic hydrogels represent highly controlled environments for three-dimensional culture of human mesenchymal stem cells (hMSCs). Encapsulated hMSCs are presented with a “blank” environment, and this environment can be closely controlled in order to elicit an osteogenic response. *In vitro*, dexamethasone is an efficient and reliable factor that leads to the osteogenic differentiation of human mesenchymal stem cells (hMSCs). The aim of this work was to develop a dexamethasone-releasing poly(ethylene glycol) (PEG)-based hydrogel scaffold to deliver dexamethasone to encapsulated cells in a sustained manner. To accomplish this goal, dexamethasone was covalently linked to a photoreactive mono-acrylated PEG molecule through a degradable lactide bond, and this molecule was covalently incorporated into the PEG hydrogel during photopolymerization. Over time, hydrolysis of the ester bonds resulted in dexametha-

some release from the gel. The biological activity of the released dexamethasone was verified in monolayer cell culture and in three-dimensional culture (i.e., in the gel) by the ability of hMSCs to express osteogenic genes, including alkaline phosphatase, osteopontin, and core binding factor alpha 1, as measured using real-time reverse transcription polymerase chain reaction (RT-PCR). These studies indicate that encapsulated hMSCs are capable of osteogenic differentiation in response to released dexamethasone. © 2005 Wiley Periodicals, Inc. *J Biomed Mater Res* 76A: 183–195, 2006

Key words: human mesenchymal stem cells; osteogenic differentiation; cell encapsulation; dexamethasone; real-time reverse transcription polymerase chain reaction

INTRODUCTION

Dexamethasone is a synthetic corticosteroid and a potent modulator for osteogenic differentiation of mesenchymal stem cells (MSCs). Dexamethasone supports osteogenic lineage differentiation^{1–8} by binding to specific regulatory proteins within the cell and activating transcription of osteoblast-specific genes. *In vitro*, continuous treatment with dexamethasone transforms the morphology of MSCs from spindle-shaped to cuboidal,⁶ increases alkaline phosphatase activity, is required for matrix mineralization,^{5,6} and

acts at multiple points in the differentiation process to stimulate osteoblastic maturation.⁹ If dexamethasone is removed from *in vitro* culture, a population of cells may regress toward a more undifferentiated state or differentiate along alternative pathways, such as the adipogenic lineage; therefore, a continued dexamethasone presence is required to achieve maximal osteogenic differentiation of MSC cultures.⁹

Many studies have shown that, while dexamethasone can induce terminal differentiation of MSCs in culture, the presence of dexamethasone is not an *absolute* requirement for *in vitro* osteogenesis in rodent MSCs.^{2,10–12} This observation is in stark contrast to human MSCs (hMSCs), in which dexamethasone was shown to be *required* for the differentiation of hMSCs *in vitro*.⁶ Bone morphogenetic proteins (BMPs) are potent effectors of MSC differentiation to the osteogenic lineage. However, the required BMP levels to regenerate bone in humans are much higher than expected from animal studies, and patients have shown significant variability in response to BMP treatment.^{13–17} For example, while mere nanogram levels of BMPs are enough to trigger bone repair when BMPs are released naturally by human cells, quantities over *six* orders of

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magnitude higher are required to produce the same effect in humans using an artificial matrix.¹⁸ The relative unresponsiveness of hMSCs to bone morphogenetic proteins (BMPs) and their inability to consistently stimulate bone formation at levels found in the body has enhanced the importance of dexamethasone for osteogenic differentiation of human MSCs.

In vitro, dexamethasone is a reliable factor for differentiating hMSCs to osteoblasts and can be easily included in the surrounding media. However, in the case of an *in vivo* injectable system to deliver hMSCs to a bone defect, dexamethasone will not be present in the required concentrations needed for osteogenic differentiation. In the clinical setting, any physically entrapped dexamethasone would quickly diffuse away from the site of delivery. Instead of relying on dexamethasone in the surrounding media to trigger osteogenic differentiation of photoencapsulated hMSCs, our approach aims to release dexamethasone from within the hydrogel in a sustainable fashion, and this released dexamethasone can act directly on encapsulated hMSCs and result in their osteogenic differentiation.

Several investigators have studied methods to release dexamethasone in a controlled fashion. For the treatment of inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, an enzyme-dependent dexamethasone-conjugated prodrug has been developed for targeting to the colon.¹⁹ Dexamethasone-loaded poly(ethylene-co-vinyl acetate) nanocomposites were developed for controlled delivery of dexamethasone for the eventual treatment of inflammatory diseases such as cerebral edema or to reduce inflammatory responses of implanted materials.²⁰ Similar to our goal of causing osteogenic differentiation of MSCs, Kim and colleagues (2003) developed a method to deliver ascorbate-2-phosphate and dexamethasone from PLGA scaffolds and found that these components were released in a sustainable manner for at least 4 weeks and had osteogenic effects on rabbit MSCs.²¹ However, the delivery of molecules from PLGA is difficult to control in a predictable manner. In contrast, hydrogels have advantages for cell encapsulation, but the sustained release of low-molecular-weight compounds, such as dexamethasone, is difficult to achieve. Other drugs have been investigated for controlled delivery from hydrogels²²⁻²⁴; in a similar manner, we aim to deliver dexamethasone from a hydrogel based on poly(ethylene glycol) (PEG).

The goal of the research presented herein was to first covalently link dexamethasone to a photoreactive PEG-based molecule through a degradable lactide linkage; this molecule could then be incorporated into the hydrogel scaffold during photopolymerization at different loading concentrations. Second, we investigate and control the release rate of dexamethasone from these gels by designing macromolecules contain-

ing different lengths of lactide spacers. Finally, we verify the biological activity of the released dexamethasone and its ability to differentiate hMSCs to the osteoblastic lineage both in two-dimensional cell culture (i.e., monolayer conditions) and three-dimensional cell culture (i.e., hMSCs photoencapsulated in PEGDA hydrogels). This type of dexamethasone delivery (i.e., covalent attachment of dexamethasone to a polymer network through hydrolytically labile lactide bonds) has several important advantages over the delivery of dexamethasone from microparticles. These advantages include the ability to easily alter the amount of dexamethasone loaded into each hydrogel, simply by altering the amount of monomer in solution prior to polymerizing the gel, and the kinetics of dexamethasone release can be easily altered by changing the number of degradable linkages attaching dexamethasone to the network. Furthermore, one may ultimately be able to spatially control the osteogenic differentiation of encapsulated hMSCs by exploiting the advantages of photoprocessing conditions, namely the ability to pattern the gel chemistry by using standard lithographic techniques.

MATERIALS AND METHODS

Synthesis of PEG526MMA-Lac

Poly(ethylene glycol) mono-methacrylate of molecular weight 526 g/mol (PEG526MMA, Aldrich, 99%), D,L-lactides (Polysciences, 98%), hydroquinone (Aldrich, 99%), and stannous octoate (Aldrich, 95%) were used as received. PEG526MMA (10.0 g, 19.0 mmol) was placed in a 50-mL round bottom flask with a stir bar and 2.74 g lactides (19.0 mmol, 1:1 lactide:PEG526MMA molar ratio), 5.48 g lactides (38.0 mmol, 2:1 lactide:PEG526MMA molar ratio), or 8.22 g lactides (57.0 mmol, 3:1 lactide:PEG526MMA molar ratio) added along with a trace amount of hydroquinone. The contents were heated to 110°C, then 30.8 μ L stannous octoate (95.0 μ mol) was added to the flask, and the contents were sealed and purged with argon for 5 min. The contents were allowed to react for 2 h at 110°C. The product of this step was PEG526MMA-Lac (Fig. 1).

Synthesis of PEG526MMA-Lac-Suc

The PEG526MMA-Lac product was allowed to cool to 70°C and was used without further purification. Upon cooling, 50 mL chloroform, 2.40 g (24.0 mmol) of succinic anhydride (Aldrich, 97%), 1.0 g 4-dimethylaminopyridine (DMAP, Fluka, 98%), and a trace amount of hydroquinone was added to the flask. The contents were refluxed at 70°C for 1 h. Next, the chloroform was evaporated using a rotary evaporator (Buchi Rotavapor R-3000) at 30 to 35°C, the product was re-dissolved in 75 mL methylene chloride, washed

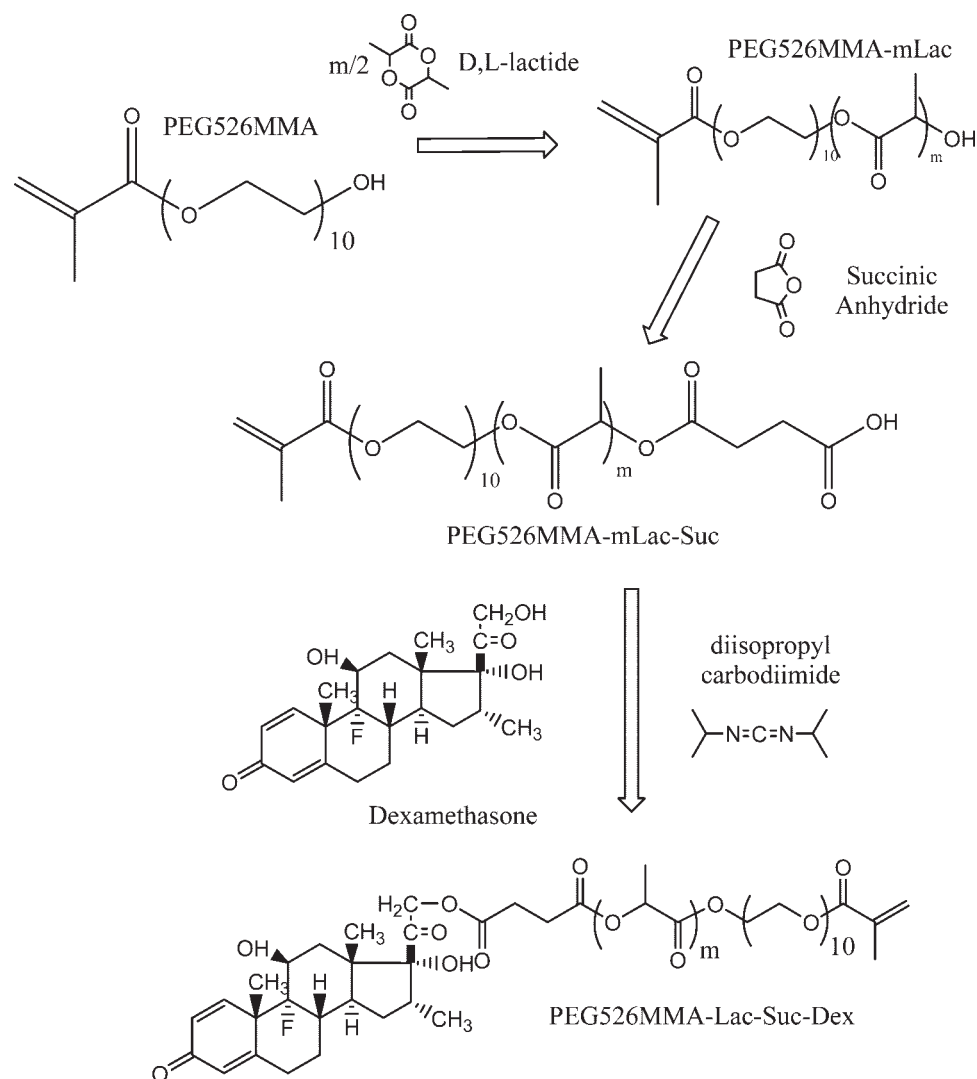


Figure 1. Synthesis scheme of PEG526MMA-Lac-Suc-Dex.

twice with 150 mL 2M hydrochloric acid, and then washed twice with 150 mL saturated NaCl solution. The organic layer was dried over sodium sulfate, filtered, and concentrated to dryness via rotary evaporation to yield PEG526MMA-Lac-Suc (see Fig. 1), which was stored at 4°C until used.

Conjugation of PEG526MMA-Lac-Suc to dexamethasone

PEG526MMA-Lac-Suc (2.04 mmol), diisopropyl carbodiimide (DIPCDI, Aldrich, 99%, 2.04 mmol), DMAP (1.64 mmol), and a trace amount of hydroquinone were dissolved in 40 mL CHCl_3 . This mixture was then added to a solution of dexamethasone (Sigma, 98%, 2.04 mmol) dissolved in 80 mL acetone, and the reaction was stirred for 24 h in the dark.

Purification of PEG526MMA-Lac-Suc-Dex

The PEG526MMA-Lac-Suc-Dex product was purified by first removing the solvent from the product by rotary evap-

oration. The product was then re-dissolved in 50 mL CH_2Cl_2 , was washed twice with 100 mL 2M hydrochloric acid, and washed twice with 100 mL saturated NaCl solution. The organic layer was dried over sodium sulfate and filtered through Whatman #40 filter paper. This PEG526MMA-Lac-Suc-Dex product solution was then loaded onto 5.0 g silica gel (Aldrich, 70-230 mesh, 60Å) and evaporated to dryness using rotary evaporation. Flash chromatography on silica gel (30.0 g) using ethyl acetate (100%) as eluent was utilized to remove any unreacted dexamethasone, as verified using thin layer chromatography (TLC). When all of the dexamethasone had been removed ($R_f = 0.7$ when TLC performed in 100% ethyl acetate), 100% acetone was eluted through the column to desorb the PEG526MMA-Lac-Suc-Dex product, and this elution was also verified using TLC. The fractions containing the product ($R_f \sim 0$ when TLC performed in 100% ethyl acetate) were combined together and rotary evaporated to concentrate the product.

Three different products were synthesized, containing either 2 (PEG526MMA-2Lac-Suc-Dex), 4 (PEG526MMA-4Lac-Suc-Dex), or 6 (PEG526MMA-6Lac-Suc-Dex) lactic acid repeat units. Proton nuclear magnetic resonance (^1H NMR) spectroscopy (500 MHz) was performed to verify the prod-

uct. The product is not a pure compound; rather, the product mixture contains unreacted PEG526MMA-Lac-Suc, as well as the dexamethasone-conjugated product, but no unreacted, free dexamethasone as verified using thin layer chromatography and high performance liquid chromatography (HPLC). ^1H NMR spectroscopy was also used to calculate the dexamethasone content.

Copolymerization of PEG526MMA-Lac-Suc-Dex with PEGDA and degradation-controlled release of dexamethasone

A solution containing 10 wt % (100 mg/mL) di-acrylated poly(ethylene glycol) (PEGDA) of molecular weight 3400 (Nektar Therapeutics) in dimethyl sulfoxide (DMSO) was made. PEG526MMA-Lac-Suc-Dex was added such that the concentration of tethered dexamethasone in the gels was 0.15 mg/gel. The amount of PEG526MMA-Lac-Suc-Dex added depended upon the dexamethasone content (i.e., mmol dexamethasone per mmol total PEG526MMA-Lac-Suc-Dex product). Photoinitiator D2959 (Ciba-Geigy) was added to 0.05 wt %, and the solution was polymerized under ultraviolet light (365 nm, ~ 5 mW/cm²) for 10 min in 1 mL syringes that had their tips cut off. This resulted in gel disks of diameter 5 mm and thickness 2 mm.

Each disk (samples performed in triplicate) was placed in 1 mL of phosphate-buffered saline solution (PBS, Gibco) under static conditions at 37°C, and the dexamethasone released into solution was measured with time. For quantifying released dexamethasone, high performance liquid chromatography (HPLC) was utilized. At each time point, the entire supernatant surrounding each disk was collected, and 300 μL was injected into the HPLC machine (Waters Delta Prep 4000 with a Waters 2487 dual wavelength ultraviolet detector). The liquid phase consisted of 65% methanol and 35% water, and the column was a Waters Nova-Pak C₁₈ column (3.9 mm \times 150 mm). Each day of HPLC analysis, known concentrations of dexamethasone in methanol were run through the machine to set up a standard curve (in triplicate). The area under the curve was obtained using Waters' Empower software. Using the standard curve, the amount of dexamethasone released by each gel because the previous measurement and the cumulative amount released could be determined. Dexamethasone-loaded hydrogels (~ 1.0 mg/gel) were also placed in sodium carbonate buffer (pH 11.5) and/or elevated temperature (60°C) to speed up the degradation process. All release measurements are results of release from gels in triplicate, and error is represented as the standard deviation of the mean.

Dexamethasone release from dexamethasone conjugates

Release supernatants collected after 165 h were allowed to incubate at 37°C for an additional several days (i.e., separate from the gel). The concentration of dexamethasone in these

supernatants was measured at several subsequent time points (97, 193.5, and 267 additional h).

Effect of released dexamethasone on hMSCs in two-dimensional culture

Samples of the released dexamethasone were obtained from supernatants surrounding the disks. Due to the lower limits of dexamethasone detection using HPLC, the concentration of released dexamethasone was, in most cases, much greater than the concentration of dexamethasone required to differentiate hMSCs. For example, the concentration of dexamethasone released from the gels was on the order of 2.5 μM (1 $\mu\text{g}/\text{mL}$), and this was on the order of the detection limit using HPLC. However, the concentration of dexamethasone required for osteogenic differentiation is on the order of 100 nM, approximately 25 times less concentrated than in the release supernatants. This required additional dilution of the release supernatants to obtain a released dexamethasone concentration of around 100 nM.

After measuring the concentration of dexamethasone in the supernatants, the concentration of released dexamethasone was adjusted to 100 nM by diluting in human mesenchymal stem cell control media (CON) [10% fetal bovine serum, 0.50 $\mu\text{g}/\text{mL}$ fungizone, 50 units/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 20 $\mu\text{g}/\text{mL}$ gentamicin in low glucose Dulbecco's Modified Eagle Media (DMEM, Invitrogen)]. In general, because the concentrations of dexamethasone in the release samples were several orders of magnitude greater than the concentration needed for *in vitro* studies (100 nM), the media composition was not altered significantly (i.e., concentration of serum and other components were affected at most by 1%).

Human mesenchymal stem cells (hMSCs) were obtained from Cambrex Bio Science (Walkersville, MD) and used as received. Human MSCs were seeded in wells of 6-well tissue culture plates in triplicate and cultured in CON media, Dex media (CON media containing 100 nM dexamethasone), and media containing released dexamethasone and media was replaced every 3 to 4 days. At various time points (2, 4, 7, 9, 11, 14, 21 days), the media was aspirated from wells and 1 mL TRI reagent (Sigma) was used to isolate total RNA according to the manufacturer's instructions. The reverse transcription polymerase chain reaction (RT-PCR) was used to analyze gene expression of cells at various time points in the various media. Real-time RT-PCR was used to analyze gene expression of selected osteogenic genes, alkaline phosphatase (ALP) and core binding factor alpha 1 (Cbfa1). In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize gene expression because GAPDH is generally proportional to cell number. Forward and reverse primer and probe sequences used are shown in Table I, and Table II shows the fluorophores and quenchers attached to each probe. Primers were designed to lie at the intron-exon boundary to eliminate amplification from contaminating DNA. All primers and probes were obtained from Integrated DNA Technologies (Coralville, IA).

Using a real-time thermal cycler (iCycler 433A, Bio-Rad), samples were heated to 95°C for 3 min followed by 45 cycles

TABLE I
Forward (FWD) and Reverse (REV) Primers for Genes Assayed Using Real Time RT-PCR

Gene	FWD primer (5'→3')	REV primer (5'→3')
ALP	GTGGAGTATGAGAGTGACGAGAA	AGATGAAGTGGGAGTGCTTGTAT
CBFA1	GGTATGTCCGCCACCACTC	TGACGAAGTGCCATAGTAGAGATA
GAPDH	GCAAGAGCACAAGAGGAAGAG	AAGGGTCTACATGGCAACT

of 30 s at 95°C and 30 s at 60°C. Finally, the samples were allowed to cool to 10°C upon completion of the PCR. The lengths of the amplified gene products were verified using standard DNA gel electrophoresis.

RT-PCR was utilized to investigate the osteogenic differentiation of encapsulated hMSCs in response to released dexamethasone.

Photoencapsulation of hMSCs in PEG3400DA hydrogels in the presence of PEG526MMA-4Lac-Suc-Dex: verification of viability

When confluent, hMSCs were trypsinized from culture, collected, and centrifuged to pellet the cells. Each cell pellet was then mixed with a 10 wt % PEGDA solution containing 2.8 mM Acryl-PEG-RGD (the synthesis described elsewhere²⁵), 0.05 wt % photoinitiator I2959 (Ciba-Geigy), and approximately 83 µg PEG526MMA-4Lac-Suc-Dex per gel (corresponds to a dexamethasone concentration of ~100 nM after 3 days of release into 2 mL media). PEG526MMA-4Lac-Suc-Dex was first dissolved in ethanol at high concentration prior to mixing with the polymer solution.

The cell/polymer suspension was mixed carefully to minimize bubble formation, and then 40 µL of the cell/polymer suspension was pipetted into 1 mL sterile syringes that had the tips cut off. The cell/polymer suspensions were subsequently photopolymerized for 10 min under ultraviolet light of intensity ~ 5 mW/cm². Upon polymerization, disks (diameter = 5 mm, thickness = 2 mm) were pushed out of the syringe using the plunger and placed in 2 mL hMSC control (CON) media (hMSC growth media without the bFGF) and cultured at 37°C and 5% CO₂. Gels without PEG526MMA-4Lac-Suc-Dex were also made and cultured in CON media and media containing 100 nM dexamethasone. Media was changed every 3 to 4 days.

Photoencapsulation of hMSCs in PEG3400DA hydrogels in the presence of PEG526MMA-4Lac-Suc-Dex: effect on osteogenic gene expression

After 1 and 2 weeks in culture, gels were removed from culture, transferred to TRI reagent, and total RNA was isolated from the gels as previously described.²⁶ Real-time

RESULTS AND DISCUSSION

Synthesis of PEG526MMA-Lac-Suc-Dex

Figure 1 shows the synthesis of PEG526MMA-Lac-Suc-Dex. In the first step, stannous octoate catalyzes the ring opening of lactides. The nucleophilic hydroxyl group of PEG526MMA reacts with the electrophilic carbonyl group of the lactide molecule, resulting in PEG526MMA-Lac. Depending on the amount of lactides present during this reaction, multiple lactide molecules can be reacted onto the PEG526MMA molecule: one molar equivalent of lactides results in two lactic acid repeat units ($m = 2$ in Fig. 1), two molar equivalents results in four lactic acid repeat units ($m = 4$), and three molar equivalents results in six lactic acid repeat units ($m = 6$). During the next step, the terminal hydroxyl group of PEG526MMA-Lac reacts with succinic anhydride to form PEG526MMA-Lac-Suc, converting the hydroxyl group into an acid group. Finally, this carboxylic acid group is coupled to the primary hydroxyl group on dexamethasone using diisopropyl carbodiimide during the next step to form the conjugate PEG526MMA-Lac-Suc-Dex. Three different products were synthesized: PEG526MMA-2Lac-Suc-Dex (containing 2 lactic acid repeat units, $m = 2$), PEG526MMA-4Lac-Suc-Dex ($m = 4$), and PEG526MMA-6Lac-Suc-Dex ($m = 6$).

¹H NMR was utilized to determine the dexamethasone content (i.e., milligrams dexamethasone per gram total product). Figure 2 shows a typical ¹H NMR spectrum of the product mixture and the corresponding proton peaks. The relative ratios of dexamethasone peaks (A, B, and D) to methacrylate peaks (C and

TABLE II
Probe Sequences, 5' End-Labeled Fluorophores, and 3' End-Labeled Quenchers Used in Real-Time RT-PCR

Gene	Probe Sequence	Fluorophore	Quencher
ALP	CCTGGACCTCGTTGACACCTGGAAG	Cy5	BHQ-2
CBFA1	CTACCACACCTACCTGCCACCACC	Cy5	BHQ-2
GAPDH	ACCCTCACTGCTGGGGAGTCC	6-FAM	BHQ-1

Quenchers are Black Hole Quenchers (BHQ) produced by IDT Technologies.

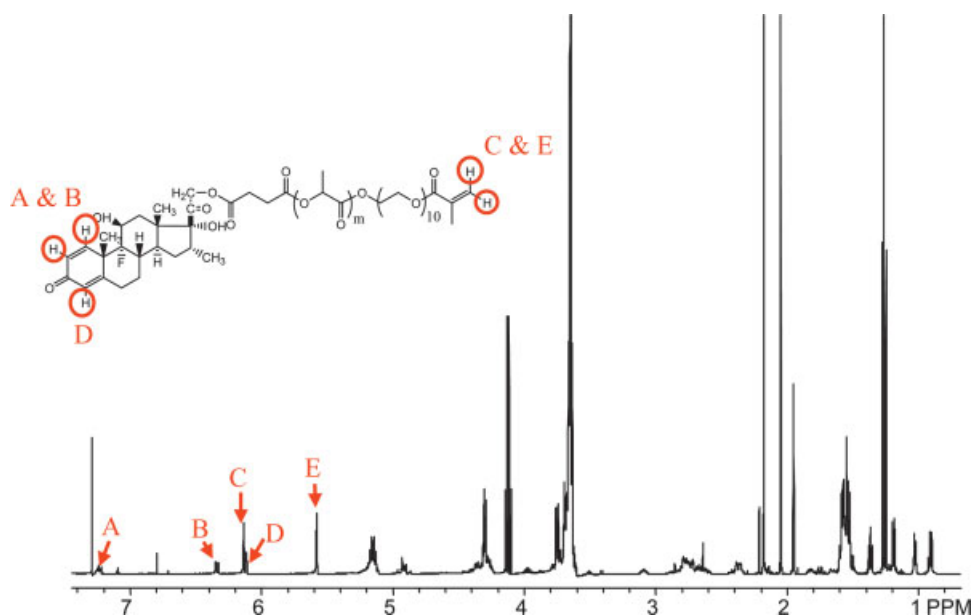


Figure 2. ^1H NMR spectrum of a typical PEG526MMA-nLac-Suc-Dex product.

E) were used to determine the milligrams of dexamethasone per gram total product [A: $\delta = 7.25, 7.23$ (doublet); B: $\delta = 6.35, 6.33$ (doublet); C: $\delta = 6.14$ (singlet); D: $\delta = 6.11$ (singlet); E: $\delta = 5.58$ (singlet)]. Table III shows the final compositions (lactide units per chain and mg dexamethasone/g product) of the three products.

Dexamethasone release as a function of pH and temperature

Through ester bond hydrolysis, dexamethasone, succinylated dexamethasone, and dexamethasone conjugates are released from gels containing PEG526MMA-Lac-Suc-Dex, as shown in Figure 3. Depending upon which ester bond hydrolyzes first, three products can result, including succinylated dexamethasone (1), succinylated dexamethasone (2), or dexamethasone conjugates (3) that are released into solution when PEG3400DA hydrogels containing PEG526MMA-Lac-Suc-Dex are placed in aqueous solution. Release supernatants were analyzed for dexamethasone content

using HPLC. Figure 4 shows the results of typical analysis from an HPLC injection analyzing these supernatants. The dual peaks on the left in Figure 4 (B,C) are thought to be a combination of dexamethasone-lactide conjugates that have not degraded yet (i.e., released from the hydrogel network but still having lactides attached, 2 and 3 in Fig. 3), as well as other dexamethasone conjugates and modifications created during the initial coupling reaction of dexamethasone to PEG526MMA-Lac-Suc. The small peak on the right (A) is dexamethasone. The concentration of dexamethasone in release supernatants can be calculated by determining the area under the dexamethasone curve.

To verify the ability of dexamethasone to be released from loaded gels in a manner consistent with ester bond hydrolysis (i.e., increased degradation under high pH and temperature), gels were incubated under conditions that accelerate ester bond hydrolysis (i.e., high pH and elevated temperature). Gels initially containing 1 mg tethered dexamethasone (PEG526MMA-2Lac-Suc-Dex) per gel were rinsed for 5 min in methanol prior to dexamethasone release to eliminate any

TABLE III
Lactic Acid Repeat Units per Monomer (m in Fig. 1), Percentage Yield, and Amount of Tethered Dexamethasone per Product (mg Dex/g product)

Monomer Acronym	Amt. PEG Reacted (g)	Lactic Acid Units per Chain* (m in Fig. 1)	Amount Dexamethasone Reacted (g)	% Yield	mg Dex/g Product
PEG526MMA-2Lac-Suc-Dex	1.568	1.8	0.80	25	150.1
PEG526MMA-4Lac-Suc-Dex	1.861	3.3	0.80	22	94.8
PEG526MMA-6Lac-Suc-Dex	2.155	5.5	0.80	27	133.6

*Determined by NMR.

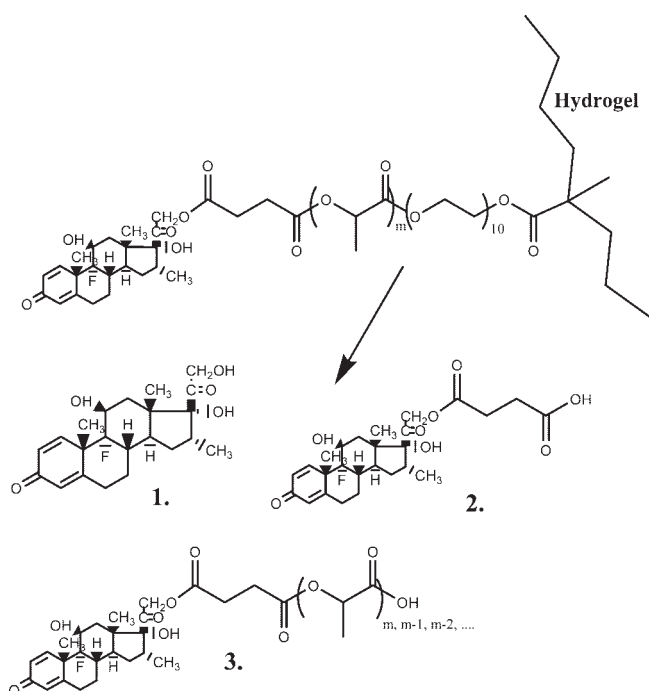


Figure 3. Schematic depicting release of dexamethasone and dexamethasone conjugates. Depending upon which ester bond hydrolyzes first, dexamethasone (1), succinylated dexamethasone (2), or dexamethasone conjugates (3) are released into solution when PEG3400DA hydrogels containing PEG526MMA-Lac-Suc-Dex are placed in aqueous solution.

unreacted PEG526MMA-2Lac-Suc-Dex. The results of these studies are shown in Figure 5. These findings indicate that dexamethasone is released in a manner consistent with ester hydrolysis. Ester bond hydrolysis is base catalyzed. By increasing the pH to 11.5, the rate of ester bond hydrolysis is increased. Furthermore, ester bond hydrolysis is increased with increasing temperature. Although these gels contained 1 mg dexamethasone initially, less than 25% of this was recovered after 250 min under these conditions. It is important to note that not every PEG526MMA-Lac-Suc-Dex molecule is incorporated in the hydrogel during photopolymerization (e.g., it may exist in the sol

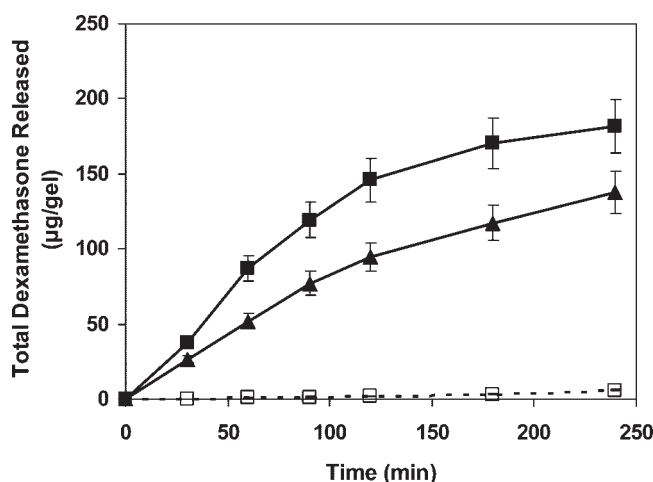


Figure 5. PEG526MMA-2Lac-Suc-Dex was copolymerized with a 10 wt % solution of PEG3400DA and the gels were rinsed with methanol and placed in PBS at 37°C (□ and dotted line), 0.1 M sodium carbonate buffer (pH 11.5) at 37°C (▲ and solid line), and 0.1 M sodium carbonate buffer (pH 11.5) at 60°C (■ and solid line). With time, released dexamethasone was measured using HPLC. Gels initially contained ~ 1.0 mg tethered dexamethasone per gel.

fraction). These gels were rinsed in methanol prior to placing in aqueous buffers, so a fraction of the product was removed during these rinsing steps. Furthermore, the dexamethasone measured and presented in Figure 5 is only *free* dexamethasone (1 in Fig. 3), not dexamethasone contained within dexamethasone conjugates (2 and 3 in Fig. 3). It is believed that a large portion of the released dexamethasone is present as dexamethasone conjugates.

Dexamethasone release as a function of number of lactic acid repeat units

Dexamethasone release should be affected by the length of the lactide spacer linking dexamethasone to the PEG3400DA hydrogel. In gels that are highly swollen, pseudo first-order degradation kinetics of the poly(lactic acid) blocks occurs, and a greater number

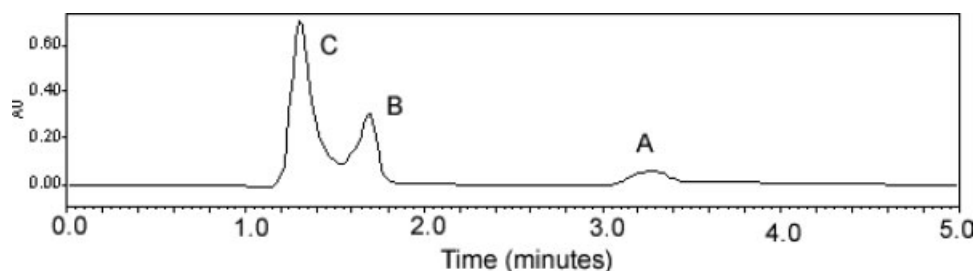


Figure 4. PEG3400DA hydrogels were loaded with PEG526MMA-4Lac-Suc-Dex, and the release of dexamethasone was measured as a function of time using HPLC. Shown here is a typical HPLC run of a release supernatant. The peak at 3.30 min is free dexamethasone (A), and the peaks at 1.25 (B) and 1.70 (C) min are dexamethasone conjugates.

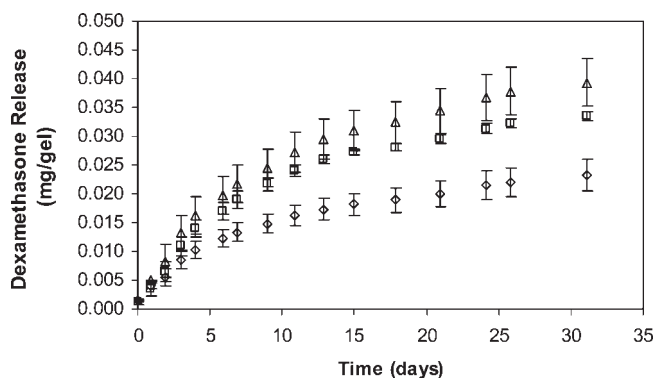


Figure 6. Cumulative release of dexamethasone from copolymers of PEG3400DA and PEG526MMA-2Lac-Suc-Dex (diamonds), PEG526MMA-4Lac-Suc-Dex (squares), and PEG526MMA-6Lac-Suc-Dex (triangles). Gels initially contained 0.15 mg tethered dexamethasone/gel.

of ester linkages increases the probability that dexamethasone and its conjugates will be released at any given time because the possible number of hydrolytically labile sites is increased. For example, it is three times more likely that one of the six ester bonds of PEG526MMA-6Lac-Suc-Dex will degrade and release dexamethasone than one of the two ester bonds of PEG526MMA-2Lac-Suc-Dex. Therefore, the rate of cleavage of the tethered dexamethasone should increase with increasing lactide content. The results of dexamethasone release, not including the conjugated forms, into PBS when these different dexamethasone-containing monomers were copolymerized with PEG3400DA are shown in Figure 6. The release rate of dexamethasone from the PEG526MMA-2Lac-Suc-Dex hydrogels is lower than both the PEG526MMA-4Lac-Suc-Dex and PEG526MMA-6Lac-Suc-Dex. This observation is consistent with the hypothesis that increasing the number of ester bonds increases the probability that dexamethasone will be released. However, there is no statistical difference in the dexamethasone release between the PEG526MMA-4Lac-Suc-Dex and PEG526MMA-6Lac-Suc-Dex hydrogels. An explanation for this may be that, due to the increase in lactide content, the PEG526MMA-6Lac-Suc-Dex hydrogels have a higher hydrophobicity than the PEG526MMA-4Lac-Suc-Dex hydrogels; any increase in lactide content past a certain point may decrease the local water concentration surrounding the pendant poly(lactic acid) chains. In addition, dexamethasone is a relatively hydrophobic molecule by itself; the hydrophobicity of the lactides is likely exacerbated in the presence of dexamethasone.

The total amount of tethered dexamethasone loaded in the gels of Figure 6 was approximately 0.15 mg/gel. However, only a fraction of this is actually released after 32 days. Immediately after photopolymerization, the gels are rinsed for 5 to 10 min in PBS, removing any of the unlinked dexamethasone contained within

the sol fraction. This would explain the loss of some of the loaded dexamethasone, but most of the dexamethasone is released as other dexamethasone conjugates (3 of Fig. 3), not actual dexamethasone *per se*.

The ability of dexamethasone to be released from these hydrogels is affected by two properties within the hydrogel: the diffusivity of free dexamethasone and the rate at which ester bonds hydrolyze to release free dexamethasone. If the time scale at which dexamethasone is released from the network through ester bond hydrolysis is much less than the time scale for dexamethasone diffusion through these hydrogels, then diffusion would play the largest role in dictating the dexamethasone release profile from these gels. In this case, there would be no expected advantages over linking dexamethasone to the network through a degradable linkage versus encapsulating free dexamethasone within these gels. In both cases, dexamethasone release would be primarily governed by diffusion mechanisms.

On the other hand, if the time scale for degradation of lactide bonds is very high compared to the time scale of dexamethasone diffusion in these gels, the rate of ester bond hydrolysis would be expected to control the rate of dexamethasone release. In this case (i.e., if diffusion is much more rapid than ester bond degradation), there would be significant advantages to using this strategy to deliver dexamethasone throughout the hydrogel because the release rate can be altered by changing the amount of lactide bonds in the degradable linkage attaching dexamethasone to the network.

When free dexamethasone is encapsulated within PEG3400DA hydrogels, the release of dexamethasone is very rapid. Within 30 min, over 95% of encapsulated dexamethasone diffuses out of a hydrogel that is 2 mm thick (unpublished findings) due to dexamethasone's small molecular size (radius ~ 6.5 Å) when compared to the average mesh size of the hydrogel (around 50 Å). Assuming Fickian diffusion, the diffusion coefficient of dexamethasone in 10 wt % PEG3400DA gel was fit to diffusion data and was estimated to be 2.1×10^{-6} cm²/s. The timescale of diffusion can be estimated by:

$$t_{\text{dif}} = L^2/D_g \quad (1)$$

where L is the gel half-thickness (1 mm) and D_g is the diffusion coefficient of dexamethasone in the gel (2.1×10^{-6} cm²/s). Therefore, the timescale of diffusion is on the order of 80 min. The time scale of the hydrolysis reaction can be estimated by:

$$t_{\text{hyd}} = 1/k \quad (2)$$

where k is the pseudo-first order hydrolysis rate constant (estimated to be 0.02 h⁻¹, see section below). Based on this equation, $t_{\text{hyd}} = 50$ h; release of dexamethasone from the gel occurs on the order of days

(see Fig. 6). These findings indicate that the timescale of ester bond hydrolysis is much greater than the timescale of dexamethasone diffusion from the hydrogel (i.e., diffusion of dexamethasone occurs much faster than ester bond hydrolysis). As a result, ester bond hydrolysis is the limiting factor in dexamethasone release from the hydrogels; as soon as dexamethasone is released due to degradation of lactide bonds, it is able to diffuse out of the hydrogel. Based on these results, the rate of dexamethasone release from these hydrogels can truly be altered by changing the degradation properties of the dexamethasone-network linkages, and mass transfer effects can be neglected.

Dexamethasone release from dexamethasone conjugates

Dexamethasone that is released from the gel is either free (**1** in Fig. 3) or exists as succinylated dexamethasone (**2** in Fig. 3) and dexamethasone conjugates (**3** in Fig. 3). The exact distribution and composition of the dexamethasone conjugates are unknown, but they elute after 1 to 2 min during HPLC (peaks B and C in Fig. 4). It is believed that a portion of this peak is dexamethasone with lactide units still attached (i.e., one of the lactide bonds of the lactide linker has been degraded, but *not* the lactide ester bond directly adjacent to dexamethasone). Over time, these lactide bonds should degrade, releasing pure, free dexamethasone. We have successfully succinylated dexamethasone (**2** in Fig. 3), and its complete hydrolysis in aqueous solution to dexamethasone occurs within 12 h. If release supernatants are incubated longer in solution away from the gel, the concentration of dexamethasone should increase due to conversion of succinylated dexamethasone and dexamethasone-lactide conjugates to pure dexamethasone. This is shown in Figure 7. With increasing time, the concentration of dexamethasone in solution increases to a point and then levels off; there is approximately a threefold increase in dexamethasone concentration over this time period. This means that, even though dexamethasone has been released in solution, it may still be conjugated to lactide bonds. If the large dual peak in Figure 4 (peaks B and C) consisted of *only* these dexamethasone-lactide conjugates, one would expect this peak to totally disappear with extended incubation time in PBS. However, a portion of this dual peak remains present, even after the continued dexamethasone release in solution has stabilized, indicating that there are other unknown modified dexamethasone conjugates present.

Due to the fact that the lactide ester bond directly connected to dexamethasone must be hydrolyzed in order for the targeted dexamethasone molecule to be

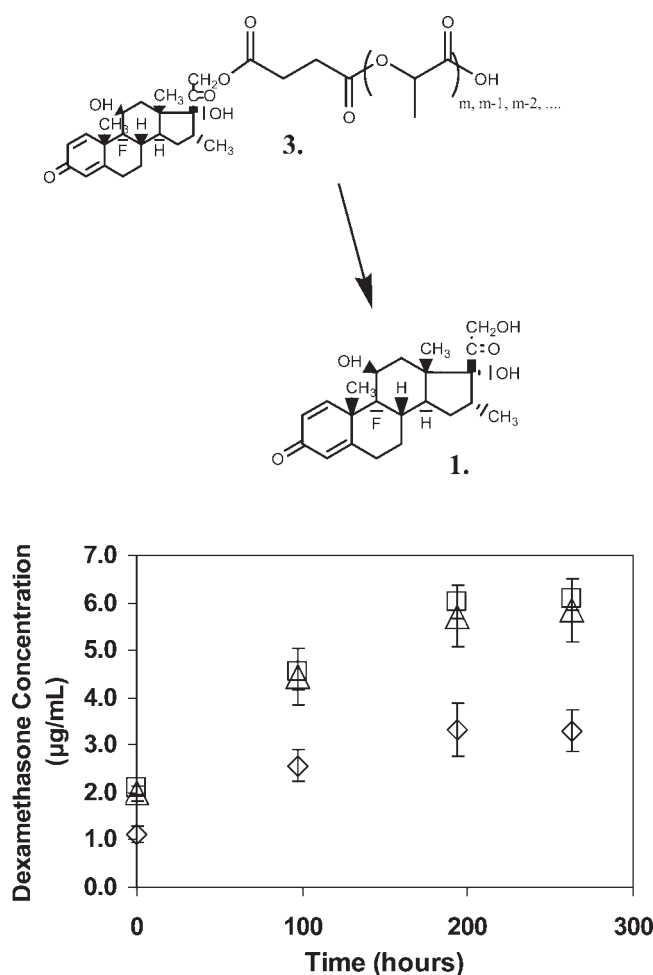


Figure 7. Dexamethasone concentration as a function of hydrolysis time. Supernatants containing released dexamethasone were cultured for additional lengths of time, up to 300 h, before analysis. Over time, the concentration of dexamethasone in solution increases due to the conversion of dexamethasone conjugates (**3**) to dexamethasone (**1**), indicating that in solution there exist other dexamethasone conjugates capable of hydrolyzing to yield pure, unconjugated dexamethasone. PEG526MMA-2Lac-Suc-Dex (diamonds), PEG526MMA-4Lac-Suc-Dex (squares), and PEG526MMA-6Lac-Suc-Dex (triangles).

released from the gel or hydrolyzed from soluble dexamethasone-lactide conjugates, one would expect the length of the lactide spacer to have no effect on dexamethasone (**1** in Fig. 7) evolution. In other words, the length of the lactide spacer simply affects the release of the tethered dexamethasone, but it is released as both dexamethasone and its conjugates. However, the generation of dexamethasone *per se* only depends on hydrolysis of the ester bond directly connected to it, and is independent of the lactide spacer length. Under this assumption, the length of the lactide spacer should not matter whatsoever.

However, our results (Fig. 6) are contradictory to this assumption; we observed an increase in the rate of dexamethasone release with increasing lactide spacer

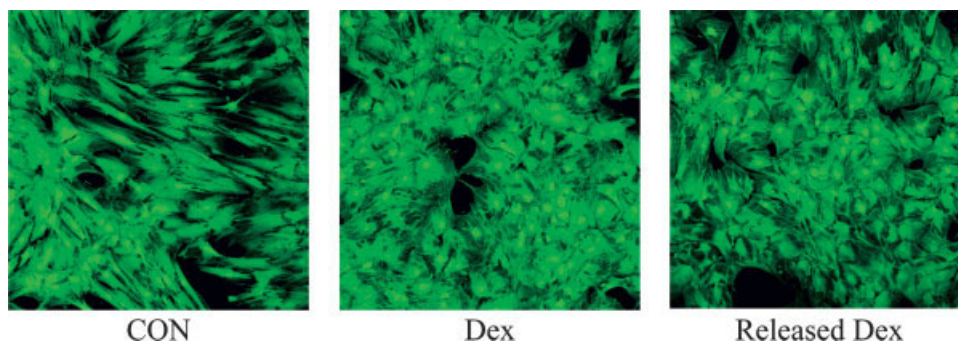


Figure 8. Human MSCs were seeded on tissue culture plastic at 5000 cells/cm² and cultured in the presence of control media (CON, left), 100 nM dexamethasone (center), and supernatant from dexamethasone that had been released from PEG-Dex hydrogels (concentration of dexamethasone adjusted to approximately 100 nM). After 11 days in culture, cells were stained with the fluorescent dye calcein AM, which fluoresces green inside living cells.

length, to a point. Siparsky and colleagues (1998) have shown that, once hydrolyzed, lactic acid groups on the ends of poly(lactic acid) chains are capable of autocatalysis²⁷; when generated, the terminal lactic acid groups are able to interact with other lactide ester bonds on the same molecule and speed up their degradation. This may explain our findings. Generation of a single acid group through hydrolysis may lead to rapid degradation of the rest of the ester bonds according to this autocatalysis mechanism. Further experimentation is needed to verify this hypothesis.

The proportion of released dexamethasone existing as free dexamethasone (**1** in Fig. 7) versus dexamethasone conjugates (**2** and **3** in Fig. 7) is thought to have important clinical implications. Dexamethasone conjugates that are not taken up by cells will eventually diffuse away from the site of delivery. Later on, these conjugates are expected to hydrolyze to free dexamethasone, as shown in the bottom of Fig. 7. If sufficient time has passed that these dexamethasone conjugates have diffused away from the site of delivery (i.e., the hydrogel), they may later hydrolyze to free dexamethasone in other areas of the body and perhaps lead to an undesired response. As a result, this would not be considered a local delivery strategy for dexamethasone. As such, it may be more important to use a shorter length lactide spacer so that a greater portion of released dexamethasone existed as free dexamethasone. However, it is quite possible that these dexamethasone conjugates do have some biologic activity and can be taken up by cells. Further studies are necessary to determine the biologic activity of these dexamethasone conjugates under *in vitro* conditions.

The degradation of lactide bonds similar to those present in PEG526MMA-Lac-Suc-Dex have been thoroughly characterized.^{28,29} Assuming a large excess of water surrounding the lactide bonds, the degradation of a single ester bond can be assumed to be pseudo first order. Based on the data from Figure 7 and assuming pseudo first-order degradation kinetics, we have calculated an approximate rate constant for the

degradation of lactide bonds to be approximately 0.02 h⁻¹.

Effect of released dexamethasone on hMSCs in two-dimensional culture

To determine whether released dexamethasone had an effect on the differentiation of hMSCs, supernatants of dexamethasone were used for *in vitro* experiments. Using calculated concentrations of free dexamethasone from HPLC runs, supernatants were diluted in hMSC control media to a final concentration of 100 nM. Human MSCs were then cultured in media containing this released dexamethasone from the three PEG526MMA-Lac-Suc-Dex products to verify their osteogenic differentiation. Figure 8 shows the morphology of hMSCs that were cultured in control media (CON), media containing 100 nM dexamethasone (Dex), and media containing PEG526MMA-6Lac-Suc-Dex supernatant (Released Dex, adjusted to 100 nM). Control cells (left) exhibit the classic elongated, spindle morphology of MSCs, whereas cells in cultures containing dexamethasone (center) and released dexamethasone (right) have the flattened, cuboidal morphology typical of osteoblasts.

Figure 9 shows the relative gene expression of hMSCs, cultured under the same conditions, with respect to the osteogenic genes ALP and Cbfa1. As can be seen, there is greater ALP and Cbfa1 gene expression by hMSCs that were cultured in dexamethasone and released dexamethasone as compared to control media (containing no dexamethasone). The maximum ALP levels in hMSCs cultured in the presence of released dexamethasone and 100 nM dexamethasone were 28-fold and 13-fold the maximum ALP expression of hMSCs cultured in control media, respectively. In addition, the maximum Cbfa1 levels in hMSCs cultured in the presence of released dexamethasone and 100 nM dexamethasone were 2.9-fold and 2.3-fold

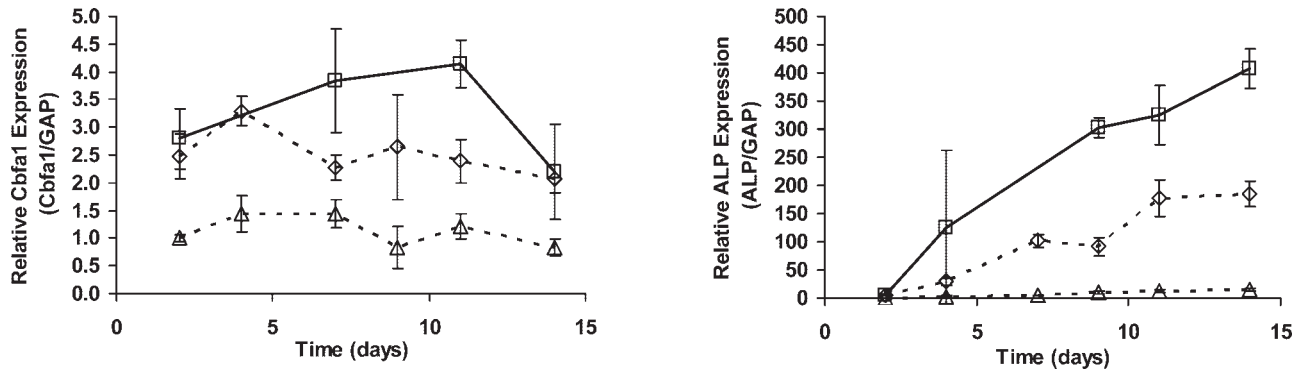


Figure 9. Human MSCs were cultured in the presence of control media (triangles and dotted line), 100 nM dexamethasone in control media (diamonds and dotted line), and control media containing released dexamethasone (squares and dotted line) for up to 2 weeks. The gene expression of Cbfa1 (left) and ALP (right) were elevated in the presence of both dexamethasone and the released dexamethasone, indicating that dexamethasone released from the gel is biologically active (i.e., leads to osteogenic differentiation). Maximum ALP levels in hMSCs cultured in the presence of released dexamethasone and 100 nM dexamethasone were 28-fold and 13-fold the maximum ALP expression of hMSCs cultured in control media, respectively; maximum Cbfa1 levels in hMSCs cultured in the presence of released dexamethasone and 100 nM dexamethasone were 2.9-fold and 2.3-fold the maximum Cbfa1 expression of hMSCs cultured in control media.

the maximum Cbfa1 expression of hMSCs cultured in control media. These results indicate that released dexamethasone is biologically active (i.e., has osteogenic properties).

It is difficult to determine the relative bioactivities of the other dexamethasone conjugates (i.e., products 2 and 3 in Fig. 3) as compared to pure dexamethasone. Based on measured dexamethasone amounts in the release supernatants, the concentration of released dexamethasone was adjusted to 100 nM. These release supernatants also contained dexamethasone conjugates, but it is not known if these dexamethasone conjugates have biological activity. However, it appears that the level of Cbfa1 gene expression is slightly higher when hMSCs were cultured in the presence of released dexamethasone than media containing 100 nM dexamethasone. Therefore, these dexamethasone conjugates may have an effect on the osteogenic differentiation of hMSCs. Alternatively, because these dexamethasone conjugates continue to hydrolyze over time (see Fig. 7), the concentration of dexamethasone in the media surrounding the cells increases over time, from 100 nM to approximately 300 nM after 10 days in culture, although the media is completely replaced every 3 to 4 days. This increase in dexamethasone concentration may lead to an increase in Cbfa1 expression, although it is unknown whether hMSCs respond to dexamethasone in a concentration-dependent manner.

Photoencapsulation of hMSCs in PEG3400DA hydrogels in the presence of PEG526MMA-4Lac-Suc-Dex: viability and osteogenic differentiation

Human MSCs were photoencapsulated in PEG3400DA hydrogels containing 2.8 mM Acryl-PEG-RGD and

PEG526MMA-4Lac-Suc-Dex. Our previous results have shown that integrinlike moieties, such as the cell-adhesive RGD sequence and a charged environment created by the photoreactive phosphate-containing molecule ethylene glycol methacrylate phosphate (EGMP), are required within the PEG3400DA hydrogel in order for photoencapsulated hMSCs to maintain their viability.²⁵ Our results (not shown) indicate that EGMP-containing hydrogels lead to a slight osteogenic response, especially with respect to Cbfa1 expression, in the absence of dexamethasone in the media. Therefore, we wanted to isolate the effects of released dexamethasone on encapsulated hMSCs in a cell-permissive environment without confounding effects, and RGD-containing gels are suitable for this purpose. To verify that photoencapsulated cells were viable upon photoencapsulation in the presence of PEG526MMA-4Lac-Suc-Dex, the LIVE/DEAD assay, in which living cells fluoresce green and dead cells fluoresce red, was utilized. Figure 10 shows an example of the viability of encapsulated hMSCs in these gels. The viability of hMSCs encapsulated in dexamethasone-releasing gels is excellent; greater than 97% of the cells are viable after 1 week in culture.

The ability of tethered dexamethasone to be released from the cell-gel constructs and lead to osteogenic differentiation of the photoencapsulated hMSCs was assessed using real-time RT-PCR. Figure 11 shows the gene expression of core binding factor alpha 1 (Cbfa1) in two-dimensional monolayer culture (2D, essentially the 14-day Cbfa1 results from Fig. 9) and inside the dexamethasone-releasing PEG3400DA hydrogel (3D). Cells were cultured in control media (CON), 100 nM dexamethasone (Dex), and released dexamethasone (adjusted to 100 nM for monolayer experiments). Cell/polymer constructs were cultured in either CON or Dex media (without copolymerized

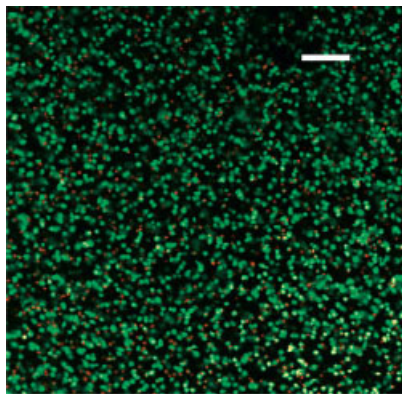


Figure 10. Human MSCs were photoencapsulated in PEG3400DA hydrogels containing 2.8 mM Acryl-PEG3400-RGD and 83 μ g PEG526MMA-4Lac-Suc-Dex per gel. After 1 week, gels were sectioned with a vibratome and the LIVE/DEAD assay was performed to visualize distribution of living and dead cells. Greater than 97% of the encapsulated cells were viable. (Bar = 200 μ m).

PEG526MMA-4Lac-Suc-Dex) or in CON media (with copolymerized PEG526MMA-4Lac-Suc-Dex, “Released Dex”). The cell/polymer constructs were fed every 3 to 4 days. Based on the initial loading of PEG526MMA-4Lac-Suc-Dex (83 μ g product/gel), the concentration of dexamethasone in the surrounding media was expected to be approximately 100 nM after every 3 to 4 days during the initial time points of culture (i.e., the linear release range occurring up to approximately 10 days). The results in Figure 11 show that hMSCs that were photoencapsulated in copolymer hydrogels containing PEG3400DA and PEG526MMA-4Lac-Suc-Dex differentiate to the osteogenic lineage, as shown by an increase in gene expression of Cbfa1. This gene expression is similar to when the same cells were photoencapsulated in the same gels without PEG526MMA-4Lac-Suc-Dex but cultured in media containing 100 nM free dexamethasone. It is also interesting to point out that hMSCs photoencapsulated in hydrogels and cultured in CON media expressed significantly elevated Cbfa1 levels than the same cells in monolayer culture in the presence of CON media. This may indicate that the three-dimensional hydrogel environment by itself causes a slight osteogenic response when compared to cells in monolayer culture.

CONCLUSIONS

In this article, the synthesis of a dexamethasone-containing, macromolecular monomer with a degradable lactide spacer is described. In combination with a di-acrylated poly(ethylene glycol) solution and under photopolymerization conditions (i.e., ultraviolet light and in the presence of a photoinitiating molecule), this dexamethasone-containing molecule was covalently

incorporated into hydrogel networks. Over time, the hydrolysis of lactide ester bonds resulted in the release of dexamethasone from the hydrogel into the surrounding solution. By altering the length of the lactide spacer, the rate at which dexamethasone was released from the gel could be controlled. Sustained release of dexamethasone occurred over about a month.

Next, we showed that released dexamethasone had an osteogenic effect on monolayer hMSCs *in vitro*. Gene expression levels of two common markers for osteogenic differentiation, alkaline phosphatase (ALP) and core binding factor alpha 1 (Cbfa1), were elevated in cells cultured in media containing released dexamethasone, indicating that dexamethasone released by PEG526MMA-Lac-Suc-Dex is biologically active. The maximum Cbfa1 and ALP gene expression levels by hMSCs cultured in the presence of released dexamethasone reached 2.8 and 28 times, respectively, that of hMSCs cultured in the absence of released dexamethasone.

Finally, we showed that hMSCs are viable up to at least a week *in vitro* upon photoencapsulation in PEG3400DA hydrogels containing 2.8 mM Acryl-PEG-RGD and PEG526MMA-Lac-Suc-Dex. After 1 week, greater than 97% of the encapsulated hMSCs were viable, indicating that this hydrogel environment is suitable for the delivery of hMSCs. The osteogenic differentiation of hMSCs inside the gel was demonstrated by measuring a 2.3-fold elevation in Cbfa1 gene expression as opposed to gels containing hMSCs and no PEG526MMA-Lac-Suc-Dex. These results indi-

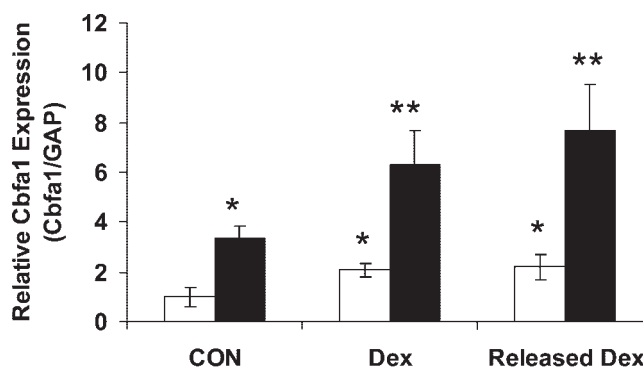


Figure 11. Human MSCs were cultured in monolayer culture (empty bars) in the presence of control media (CON), 100 nM dexamethasone (Dex), and released Dex (adjusted for monolayer experiments to 100 nM). In addition, hMSCs were photoencapsulated in PEG3400DA hydrogels (solid bars) containing 2.8 mM Acryl-PEG-RGD and cultured in CON media or Dex media. Cells were also encapsulated in PEG3400DA hydrogels in the presence of PEG526MMA-4Lac-Suc-Dex and cultured in CON media (solid bar, “Released Dex”). After 2 weeks in culture, total mRNA was isolated and gene expression of core binding factor alpha (Cbfa1) was assessed using real-time RT-PCR. A single asterisk denotes that results are significant when compared to 2D CON results, and a double asterisk denotes that results are significant when compared to 3D CON results ($p < 0.05$).

cate that encapsulated hMSCs are capable of differentiating to the osteoblast lineage in response to hydrolytically released dexamethasone.

In summary, we have developed a cytocompatible system for the sustained and localized delivery of dexamethasone to hMSCs photoencapsulated in PEG hydrogels. The work presented here represents the first study regarding the osteogenic differentiation of hMSCs in an injectable, PEG-based hydrogel where the chemical functionality controls cell differentiation.

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