



# Cell-mediated delivery of glucocorticoids from thiol-ene hydrogels

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## ABSTRACT

Thiol-ene-based poly(ethylene glycol) (PEG) hydrogels provide a unique functional platform for the sustained and localized delivery of bioactive small molecules like glucocorticoids. As a proof of concept, the synthetic glucocorticoid Dexamethasone (Dex) was conjugated to the N-terminus of a matrix metalloproteinase (MMP)-degradable peptide, which was then easily co-polymerized into PEG gel scaffolds by a thiol-ene polymerization mechanism. The conjugated Dex was locally sequestered until released by cleavage of the MMP-degradable peptide tether triggered by cell-secreted MMPs, and was only available for uptake by local co-encapsulated cells. Elevated alkaline phosphatase (ALP) activities and calcium deposition levels were observed for human mesenchymal stem cells (hMSCs) that were encapsulated in PEG hydrogels functionalized with 10  $\mu$ M of a Dexamethasone-conjugated peptide (Dex-peptide). The cellular responses stimulated by the tethered Dex lasted for over 21 days. Using co-culture experiments, hMSCs encapsulated in hydrogels with the MMP-degradable Dex-peptides had elevated levels of ALP activity and calcium deposition, whereas no elevated cellular responses were observed in co-cultured hMSCs surrounding the gel. Moreover, modifying the peptide sequence to alter its susceptibility to cleavage and/or changing the Dex-peptide loading further regulated the hMSC response to Dex at different levels and on different time scales. Collectively, these results demonstrate a tunable system for the delivery of glucocorticoids in a localized and cell-dictated manner.

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## 1. Introduction

Glucocorticoids are used for a wide range of therapeutic applications, but their broad effects on the body, combined with poor delivery methods, create unwanted side effects and limit their efficacy. Traditionally utilized for the suppression of autoimmune disorders such as systemic lupus erythematosus and inflammatory responses [1], these potent small molecules have also been employed to alleviate chemotherapy-induced toxicity on normal tissues in cancer patients [2–4]. Unfortunately, targeting of glucocorticoids to specific cell types is difficult, and glucocorticoids are cleared in the circulation relative rapidly, resulting in limited retention at desired treatment sites [5]. To overcome these targeting issues, high doses of glucocorticoids are often administered, leading to unintended side effects in surrounding tissues [6–9]. To improve targeting and reduce systemic side effects, several glucocorticoid-delivery systems have been developed [5,10–15]. Liposomes and nano-sized micelles have been used to deliver glucocorticoid cargo directly to tumor sites or local immune cells [5,12], and polymeric systems synthesized from hyaluronan or poly(ethylene glycol) (PEG) have been developed that encapsulate glucocorticoids and slow their

release into surrounding tissues [14,16]. While these approaches to control the delivery of glucocorticoids have shown distinct promise, new engineering platforms have provided a unique opportunity to develop delivery systems that are controlled in a cell-dependent manner.

Using a photo-initiated thiol-ene chemistry [17] that allows facile synthesis of peptide-functionalized materials [18], we developed a strategy to covalently tether glucocorticoids into synthetic scaffolds. Upon cleavage of the tether by cell-secreted proteases, the glucocorticoid is released from the scaffold. Because cleavage from the network is cell-mediated, release of the glucocorticoid occurs primarily when active cells are within the immediate environment, thus increasing the likelihood of localized uptake and bioactivity. In this system, the thiol-ene polymerization chemistry is applied to crosslink norbornene-functionalized 4-Armed PEG with a matrix metalloproteinase (MMP)-degradable peptide to create a three-dimensional hydrogel system. Since most glucocorticoids have a primary hydroxyl group in their structure, these molecules can be readily conjugated to one end of a degradable peptide by generating a carbamate linkage between the primary hydroxyl of glucocorticoid and the N-terminus of the peptide. By including a cysteine at the C-terminal side of the degradable peptide sequence to provide a source of a reactive thiol, incorporation of the glucocorticoid-conjugated peptide into the thiol-ene based synthetic matrices occurs upon photo-initiated co-polymerization during matrix formation. Unlike most other traditional “controlled release” systems, which typically release glucocorticoid at a pre-engineered rate from

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the polymer to influence cells outside of the system, we sought to develop a delivery system that would be cell-mediated. In essence, we aimed to permanently immobilize the glucocorticoid in the delivery matrix, until local cells invade the scaffold such that the glucocorticoid is only available to the infiltrating cells and/or cells co-encapsulated in the scaffold. The cell-mediated, localized release system can provide advantages over traditional controlled release polymers, as the release rate is directly proportional to the local cellular response and activity, thus minimizing potential side effects of potent small molecules to local tissue. Further, the diffusion of small molecules out of hydrogels makes it difficult to sustain in cell delivery vehicles, so in situations where combined cells and small molecule delivery are desired, conjugation provides one such solution.

In the work presented herein, we use the controlled release of Dexamethasone (Dex) as a proof of concept for the cell-mediated delivery of glucocorticoid factors. Dex is a common cell culture supplement that acts to promote osteogenic differentiation of isolated human mesenchymal stem cells (hMSCs) [19–22]. By tethering Dex to our synthetic matrices with an MMP-degradable peptide linker, we clearly demonstrate that thiol-ene polymerization chemistry can be used to effectively create materials that provide enzymatic release of the glucocorticoids. In both 2D and 3D cell culture studies, Dex functionalized with an MMP-degradable peptide tether, compared to an MMP-insensitive peptide tether, successfully triggered an up-regulation in the relative alkaline phosphatase (ALP) activity and calcium deposition level of encapsulated hMSCs. This effect appeared to be localized to cells embedded in the matrices, as transwell assays demonstrated that hMSCs that were not in direct contact with the gel were not activated. Such a system has further advantages in that the loading can be readily tailored during gel fabrication, and the cell-dictated release can be tuned by modifying the relative enzyme/substrate interaction rate by modifying the peptide sequence.

## 2. Materials and methods

### 2.1. Reagents

Reagents and solvents were purchased from Sigma-Aldrich®; cell culture media and supplements were purchased from Invitrogen® unless otherwise noted.

### 2.2. Peptide synthesis

The peptides KGPQGIAGQCK, KGPGQC and KGPGQ(d-)IAGQCK were synthesized on an Applied Biosystems 433A automated peptide synthesizer using standard Fmoc solid phase peptide synthesis protocols with Rink Amide MBHA resin as the solid support and HOBt/HBTU as coupling reagents. The N-terminus of each peptide was left exposed for Dex conjugation.

### 2.3. On-resin conjugation of synthetic peptides with Dex

Dex was modified to contain a succinimidyl reactive group and then conjugated to the N-termini of the resin-tethered peptides [23]. Briefly, 0.5 mmol Dex was reacted with 0.75 mmol N,N'-disuccinimidyl carbonate in the presence of 1.5 mmol triethylamine in dry acetone for 12 h. The reaction progress was monitored by TLC to determine when all of the Dex was reacted. The crude reaction mixture was then concentrated under reduced pressure and washed with aqueous sodium bicarbonate to remove unreacted N,N'-disuccinimidyl carbonate. The resulting crude product was then added to 0.25 mmol of the peptide-resin in dichloromethane [23] and allowed to react overnight at room temperature. The following day, a Kaiser test was used to confirm that the N-terminus of the peptide had reacted completely [24]. Cleavage of the modified peptide from the resin was then performed by stirring in a cleavage cocktail (for 0.25 mmol synthesis scale: 0.5 g dithiothreitol,

0.5 g phenol, 1.0 ml H<sub>2</sub>O, 0.2 ml triisopropylsilane and 9.0 ml trifluoroacetic acid) for 2 h at room temperature. The crude peptide-Dex conjugates were obtained by precipitation with cold diethyl ether (see Fig. S1 for synthesis procedures) followed by desiccation to remove residual ether. Peptide-Dex conjugates were purified by reverse phase HPLC (Waters Delta Prep 4000) on a C18 column using a 70-minute gradient (5–95%) of acetonitrile with water in 0.1% trifluoroacetic acid as the mobile phase. The purified fractions were over 95% desired product as determined by reverse phase HPLC. The molecular weight of the purified fraction was characterized (see Fig. S2 for spectra) using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Applied Biosystem DE Voyager). Purified peptide-Dex conjugates were dissolved in deionized water and final peptide concentrations were determined using Ellman's reagent based on each sequence containing a single cysteine [25].

### 2.4. Chemistry and characterization of the hydrogel precursor molecules

The 4-Arm poly(ethylene glycol) (PEG) ( $M_n = 20,000$  Da) monomers used in this study were end-functionalized with norbornenes as described in [18]. Briefly, norbornene acid was first converted into a dinorbornene anhydride and then reacted with the end hydroxyl groups of the PEG arm by N,N'-dicyclohexylcarbodiimide coupling in dichloromethane. To determine percent of end group functionalization, <sup>1</sup>H NMR was performed, and the ratio between the protons adjacent the ester group generated between norbornene acid and end hydroxyl (m, 4.1 ppm–4.3 ppm) and protons associated with the ethylene glycol repeat unit (m, 3.4 ppm–3.7 ppm) was calculated, indicating that roughly 95% of the end groups were modified by norbornene. Peptide crosslinker KCGPQGIAGQCK and integrin binding motif CRGDS were synthesized, purified, and characterized as described previously in Section 2.2.

### 2.5. Hydrogel solution preparation

For the hydrogel used in this study, cysteine-containing Dex-peptides, (Dex)KGPQGIAGQCK or (Dex)KGPQG(d-)IAGQCK, were photocoupled to 4-Arm PEG-norbornene monomers before hydrogel polymerization and cell encapsulations to insure complete conjugation of the Dex-peptide and minimize cellular uptake of untethered Dex during polymerization (5 min). Briefly, PEG-norbornene was dissolved in PBS, mixed with Dex-peptide (100 nM to 20 μM), and exposed to 365 nm light at 5 mW/cm<sup>2</sup> for 10 min in the presence of 1.7 mM lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP), a radical-mediated photoinitiator described in [26]. After conjugation, the PEG-norbornene solution was diluted to a final concentration of 12 mM PEG-norbornene (6 wt.-%/vol) containing 5.5 mM MMP-degradable peptide crosslinker (KCGPQGIAGQCK), 1 mM cell adhesion motif (CRGDS) [27], and 1.7 mM LAP. These concentrations provide a stoichiometric ratio of the reacting thiol (cysteines) and ene (PEG-norbornene) moieties for proper network formation during thiol-ene photopolymerization. Because the maximum concentration of the Dex-peptides was always less than two orders of magnitude lower than the peptide crosslinker and CRGDS, the network cross-linking density was not expected to be affected significantly by a reduction in the concentration of ene functional groups as a result of Dex-peptide conjugation.

### 2.6. Hydrogel polymerization conditions

To create hydrogels and encapsulate hMSCs, cells were re-suspended in the hydrogel solution described above at a density of 5 million cells per ml. 30 μl of the cell/monomer mixture were then placed into the top of a sterile 1 ml syringe (with its tip cut off) and exposed to UV light centered at 365 nm at 5 mW/cm<sup>2</sup> for 5 min at room temperature.

During this time, the hydrogel network polymerizes, effectively entrapping live cells.

### 2.7. Culture and expansion of hMSCs

hMSCs were isolated from fresh human bone marrow (Lonza Bioscience) following the protocol described in [28] and plated on tissue culture polystyrene in growth media (low glucose DMEM supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 10 µg/ml streptomycin and 0.5 µg/ml fungizone). 1 ng/ml recombinant human fibroblast growth factor-basic (FGF-2, Peprotech) was included in growth media to promote cell proliferation during cell expansion. Cells were incubated at 37 °C with 5% CO<sub>2</sub> and passaged by trypsin digestions, and hMSCs at passage 2 were used for all experiments.

When hMSCs reached ~80% confluency, cells were trypsinized, pooled and counted. For 2D studies, hMSCs were seeded on to 96-well plates at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> and incubated in 150 µl growth media. For 3D studies, hMSCs were encapsulated as described in Section 2.6. The hydrogel constructs were then transferred to 24-well plates with 1 ml growth media per well. For cell culture experiments, cell-laden hydrogel constructs were incubated in control media (growth media supplemented with 50 µM ascorbic acid and 10 mM β-glycerol phosphate to provide essential factors for osteogenic differentiation when treated with Dex). Media was changed every 3–4 days, and ALP activity and calcium deposition was measured at the indicated time points.

### 2.8. Co-culture transwell assays

hMSCs were plated on to 24 well plates at  $2 \times 10^4$  cells/cm<sup>2</sup> density in 1 ml growth media. The following day, the media was replaced with 1 ml control media. Another batch of hMSCs was encapsulated as described in Section 2.5 with 10 µM Dex-peptide. Hydrogel constructs were cultured in 24-well format cell culture inserts (BD Falcon™, 1.0 µm pores, transparent PET membrane) with 1 ml control media. Each insert was placed on top of the plated cells in a well of the 24-well plate. Media was changed every 3–4 days. Plated and encapsulated hMSCs harvested on Day 0, Day 7, Day 14, and Day 21 were assessed for ALP activity and calcium deposition levels.

### 2.9. Alkaline phosphatase (ALP) activity

ALP activity for both the 2D and 3D studies was measured as described previously [28]. Briefly, for the ALP activity assay, cells were lysed with RIPA buffer and supernatants were reacted with p-nitrophenyl phosphate as a substrate for alkaline phosphatase. A kinetic study was conducted by measuring the change of absorbance at 405 nm of the reaction mixture over 10 min using a multi-mode, microplate reader (Synergy H1, BioTek®). Mean velocities of the change of absorbance were calculated as relative ALP activity. Care was taken to ensure that ALP activity measurements were within a linear range.

### 2.10. Calcium deposition

Calcium deposition for both the 2D and 3D studies was measured as described previously [28]. Cell samples were treated with hydrochloric acid (the 2D assay used 1 M HCl; the 3D assay used 12.4 M HCl) for at least 2 days to solubilize precipitated calcium. The resulting solution was diluted with PBS and reacted with Calcium Reagent Solution (55 µM o-cresolphthalein complexone, 8.5 mM 8-hydroxyquinoline, 488 mM 2-amino-2-methyl-1-propanol, 1 mM potassium cyanide). Absorbance of each sample at 570 nm was measured with a BioTek Synergy H1 platereader as the relative calcium deposition level. Care was taken to ensure that calcium measurements were within a linear range of concentrations.

### 2.11. Statistical analysis

Data were presented as mean ± standard error with at least 4 replicates. Unpaired t-tests were conducted to determine the statistical significance between data sets. Differences between data sets were considered statistically significant with a p value of less than 0.05.

## 3. Results

### 3.1. Enzymatic release of Dexamethasone from synthetic hydrogel scaffolds

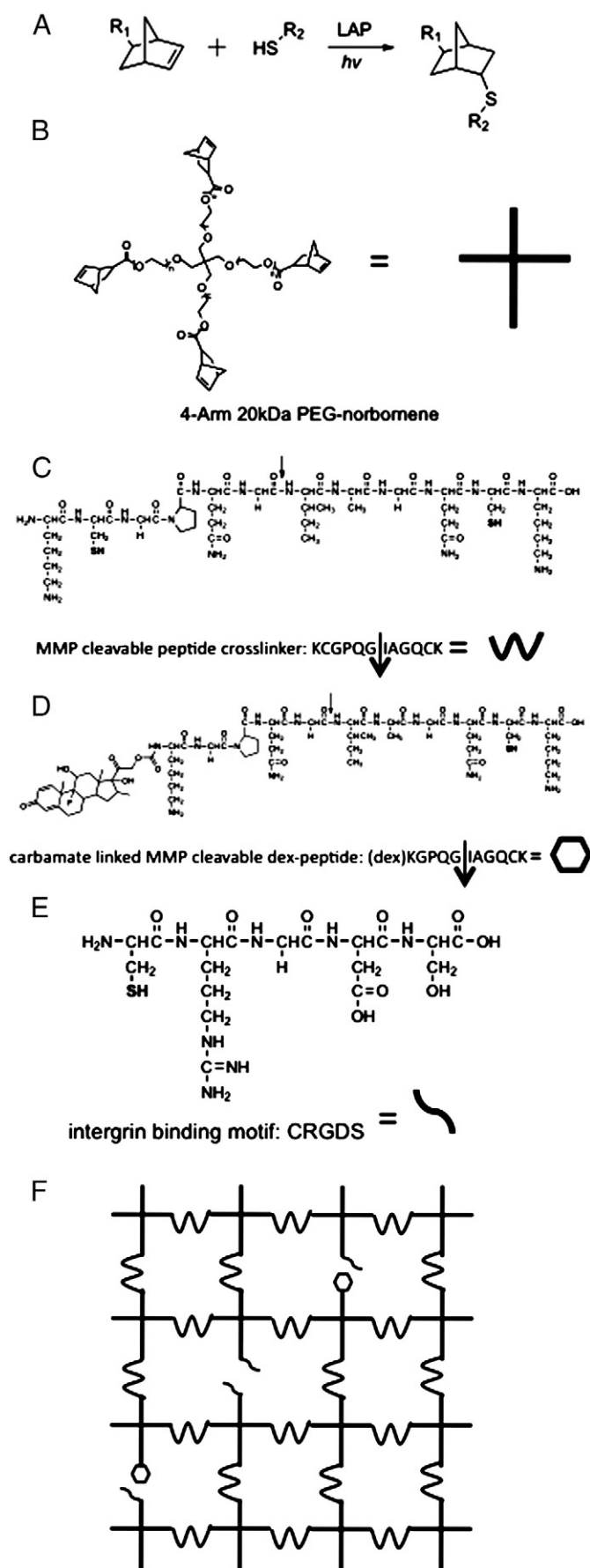
In order for Dex treatment to elicit an osteogenic response in both 2D and 3D hMSCs cultures, a sustained threshold concentration must be maintained for periods of greater than 2 weeks (see Fig. S3). As such, this material system provided an ideal model to evaluate formulations that could control the delivery of a biologically active glucocorticoid in a localized and sustained manner. Looking for a mechanism to provide a cell-mediated delivery system, we chose to covalently immobilize Dex to a PEG-based polymer matrix via an enzymatically degradable tether. As shown in Fig. 1, PEG hydrogel matrices can be easily created via a photoinitiated thiol-ene chemistry (Fig. 1A) in which multi-arm, “ene”-functionalized PEG monomers (Fig. 1B) are reacted with peptides containing a minimum of two cysteine amino acids (“thiol”-reactive groups). The use of a peptide sequence that is susceptible to enzymatic cleavage by naturally occurring MMPs (Fig. 1C), KCGPQG↓IAGQCK, provides a mechanism by which the polymer scaffold can be locally degraded by cells. Similarly, the use of an MMP-degradable peptide to tether glucocorticoids into the matrix allows for the enzymatic release of the molecule locally upon cleavage of the peptide tethering it to the scaffold (Fig. 1D). In this case, the tethering peptide includes only one cysteine located at the C-terminal side of the degradable sequence to provide a source of a reactive thiol that covalently attaches it as a pendant functional group to the PEG-norbornene scaffold backbone during photo-initiated polymerization of the matrix. Using a similar mechanism, the short extracellular matrix mimic, RGDS, can also be tethered into the scaffold, providing sites for cellular attachment to the matrix (Fig. 1E). A representation of the polymerized scaffold with MMP-degradable crosslinkers, tethered Dex, and cell adhesions motifs is depicted in Fig. 1F.

As shown in Fig. 1D, we generated a carbamate linkage between the primary hydroxyl group of Dex and the N-terminus of the peptide tether. Since most glucocorticoids have a primary hydroxyl in their structures, this chemistry would be applicable to conjugate other glucocorticoids to peptides. For the work present here, we chose to use an MMP-degradable peptide sequence as a linkage between Dex and the scaffold because hMSCs are known to secrete MMPs [29,30]. Once cleaved by enzymes secreted by encapsulated cells or cells that migrated into the system from the surrounding environment, the Dex would be released and available for uptake.

### 3.2. Peptide conjugation does not abolish bioactivity of Dex

The enzymatic cleavage site of the tethering peptide, KGPQGIAGQCK, has been previously shown to occur between glycine and isoleucine [31]; therefore, a short peptide fragment, KGPQG, would be expected to remain attached to Dex immediately after cleavage from the hydrogel network (Fig. 2A). Before proceeding to release studies, we first wanted to confirm that Dex would remain bioactive with a short peptide attached to the molecule. To test this, we synthesized the KGPQGC fragment and conjugated it to Dex using the carbamate linkage chemistry described above. A cysteine was included on the C-terminus of the peptide to aid in precise quantification of the Dex-peptide conjugate for subsequent cell-based assays.

hMSCs seeded onto standard 2D tissue culture plates were treated with either 100 nM unmodified Dex, 100 nM (Dex)KGPQGC, or no Dex. As shown in Fig. 2B, hMSCs treated with the (Dex)KGPQGC peptide



had significantly elevated ALP activity, a clear indication of Dexamethasone activity in this system. Relative ALP activity was calculated at each time point by measuring absorbance at 405 nm and normalizing it to the absolute value of absorbance at Day 0. hMSCs cultured in control media did not exhibit an appreciable increase in ALP activity for up to 20 days; however, ALP activity of hMSCs treated with either 100 nM unmodified Dex media (positive control) or with the 100 nM (Dex) KGPQGC peptide showed a typical response for 2D cultures, with ALP activity increasing through Day 10 followed by a significant drop-off thereafter. hMSCs treated with (Dex)KGPQGC did not show identical ALP activities as the hMSCs treated with the same concentration of unmodified Dex, but the fact that a positive response was observed indicates that the short peptide fragment did not abolish Dex activity. Further, increases in calcium deposition were observed in hMSC cultures treated with either 100 nM unmodified Dex or 100 nM (Dex) KGPQGC peptide (Fig. 2C). Together, these data clearly demonstrate that the conjugation of a short peptide fragment to Dex does not block activity of the glucocorticoid and suggests that enzymatic cleavage of peptide-tethered Dex will produce an active form of the compound.

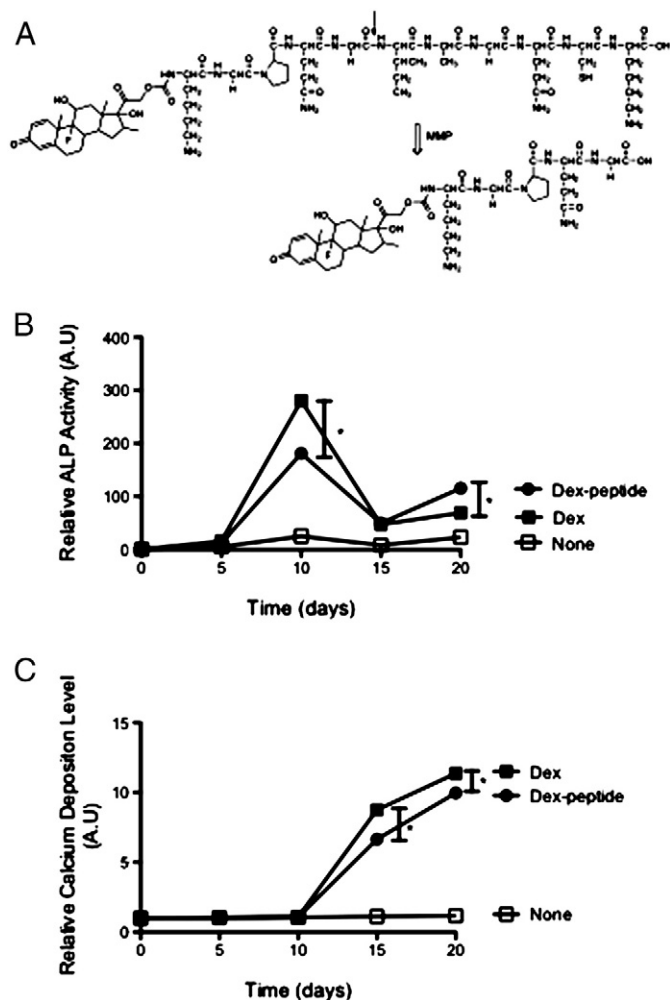
### 3.3. Peptide-specific release of Dex induces osteogenic markers in hMSCs

With the knowledge that the enzymatic cleavage product of the MMP-degradable Dex-peptide was bioactive, we synthesized a full-length Dex-peptide, (Dex)KGPQGIAGQCK, to provide a complete MMP-degradable sequence that could be covalently incorporated into thiol-ene polymer matrices (Fig. 1). We then encapsulated hMSCs into 30  $\mu$ l hydrogel constructs with 10  $\mu$ M (Dex)KGPQGIAGQCK and cultured the constructs in control media. As shown in Fig. 3A, hMSCs encapsulated in hydrogels containing the (Dex)KGPQGIAGQCK moiety showed increased levels of ALP activity starting at Day 7 and continuing through Day 21 when compared to non-functionalized hydrogels cultured under the same media conditions (none). The ALP activity of the functionalized constructs was not significantly different from positive control samples in which the media was supplemented with 100 nM soluble unmodified Dex, suggesting that this method was capable of successfully recapitulating culture conditions in which Dex is added to the media to induce osteogenesis in hMSCs. This was further corroborated by measurements of calcium deposition in which Dex-releasing hydrogels accumulated more calcium than standard gels cultured in control media, but had similar levels as positive control constructs in which soluble unmodified Dex had been added to the media (Fig. 3B).

In order to demonstrate that the Dex-mediated induction of ALP activity and calcium deposition was dependent on cleavage of the peptide tether, a non-degradable Dex-peptide, (Dex)KGPQGI<sup>d</sup>AGQCK, was tested in parallel experiments. The (Dex)KGPQGI<sup>d</sup>AGQCK peptide substitutes the natural isoleucine with the unnatural D isoform, I<sup>d</sup>, making the peptide unsusceptible to MMP-mediated cleavage [32]. As shown in Fig. 3A & B, no significant increase in ALP activity or calcium deposition was observed in these constructs. These data suggest that activity of Dex in this system is dependent on the ability of the tethering peptide to be degraded and that this system is functioning as designed.

Of note, activity profiles of ALP are markedly different in 3D hydrogel cultures than in 2D tissue culture plates. This may be a result of negative feedback loops that are not initiated in 3D cultures due to the increased space available for cell growth and extracellular matrix production. The fact that the hydrogel constructs functionalized with degradable Dex tethers appear to behave in a similar manner to cultures treated with soluble unmodified Dex, suggests that biologically relevant

**Fig. 1.** Hydrogel network for delivering Dexamethasone. Hydrogels were formed via a photoinitiated thiol-ene chemistry (A) by reacting a 4-Armed 20K PEG-norbornene (B) with an MMP degradable peptide crosslinker KCGPQGIAGQCK (C). The network was further modified with various concentrations of Dex-peptide to promote osteogenic differentiation (D) and 1 mM CRGDS to promote cell attachment (E). The general network structure is depicted in (F).



**Fig. 2.** (A): The chemical structures of the MMP-degradable Dex-peptide and its corresponding enzymatic cleavage product. (B): Results of ALP activity assay and (C): calcium deposition assay for a 2D study to test the bioactivity of the corresponding enzymatic cleavage product of the MMP-degradable Dex-peptide. hMSCs were seeded on to 96-well plates at a density of 20,000 cells/cm<sup>2</sup>. Condition None: hMSCs were cultured in CON media without Dex addition; Condition Dex: hMSCs were cultured in CON media with 100 nM unmodified Dex; Condition Dex-peptide: hMSCs were cultured in CON media with 100 nM Dex-peptide (Dex)KGPQGC. \*Indicated statistically significant with  $p < 0.05$ .

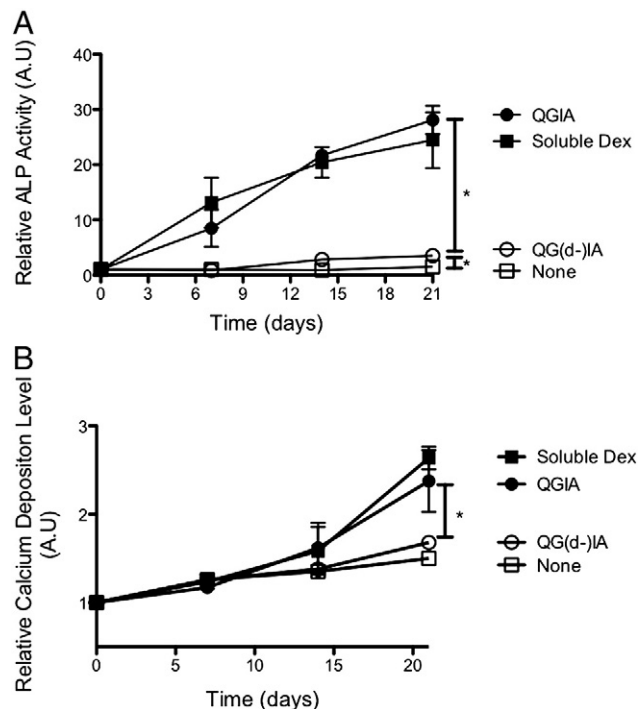
doses of Dex are delivered to hMSCs encapsulated within these matrices.

#### 3.4. Dose response threshold observed in Dex-functionalized hydrogel constructs

Dosing studies were performed to identify the optimal loading concentrations for this Dex-functionalized hydrogel system. As shown in Fig. 4A & B, there is no significant increase in either ALP activity or calcium deposition in constructs that incorporated 0.1  $\mu$ M or 1  $\mu$ M of the tethered degradable Dex-peptide. Only when a concentration of 10  $\mu$ M was used did we observe a significant increase in these markers. Moreover, no observable difference was seen between 10  $\mu$ M and 20  $\mu$ M loadings of the degradable Dex-peptide, implying that 10  $\mu$ M loading of the Dex-peptide achieves a maximum cellular response at the encapsulated cell density.

#### 3.5. Cellular response to enzymatically released Dex remains local

An obvious advantage to enzymatically controlled delivery of glucocorticoids is that it has the potential to limit distal effects of the

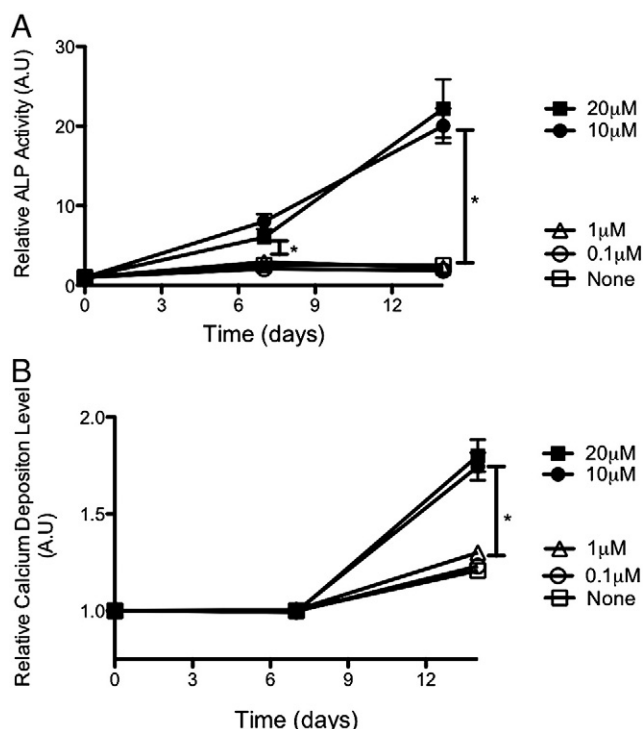


**Fig. 3.** (A): Results of ALP activity assay and (B): calcium deposition assay as a function of time in a 3D cell encapsulation study to test the cellular response of hMSCs to the tethered MMP-degradable Dex-peptide. Condition None: cell-laden hydrogel constructs were incubated in control media without Dex addition; Condition Soluble Dex: cell-laden hydrogel constructs were incubated in control media with addition of 100 nM soluble Dex in media; Condition QGIA and QG(d-IA): hMSCs were encapsulated in hydrogels with 10  $\mu$ M tethered Dex-peptide (Dex)KGPQGIAGQCK and (Dex)KQGQG(d-IA)AGQCK respectively, cell-laden hydrogel constructs were incubated in CON media. Cells were encapsulated at a density of 5 million cells/ml. \*Indicated statistically significant with  $p < 0.05$ .

glucocorticoids. To determine whether this delivery system would reduce undesired distal effects, a transwell co-culture experiment was performed in which hMSCs encapsulated in Dex-functionalized hydrogels were placed in transwells above hMSCs that were plated below. A cultured monolayer of hMSCs in control media supplemented with 0 nM, 0.1 nM, 1 nM, 10 nM or 100 nM soluble unmodified Dex served as a control reference to better quantify the potential range of concentration of Dex escaping from the hydrogel. ALP activity of hMSCs exposed to 0 nM to 100 nM Dex is depicted in Fig. 5A, indicating that at least 10 nM soluble Dex is required to trigger a significant cellular response. As shown in Fig. 5B, the ALP activity of plated hMSCs co-cultured with encapsulated hMSCs in the presence of 10  $\mu$ M degradable Dex-peptide (condition denoted as co-culture in figure) closely matched those observed by culturing cells with 1 nM soluble Dex. In addition, the result of measuring calcium deposition at Day 21 (Fig. 5C) indicated that the hMSCs co-cultured outside of the gel had significantly lower mineralization levels than culturing cells with 10 nM or 100 nM Dex. Overall, plated hMSCs outside of the hydrogel constructs were not affected by Dex released from MMP-degradable peptides inside of the scaffold, and thus, the MMP-degradable Dex-peptide appeared to function primarily in a localized manner.

#### 4. Discussion

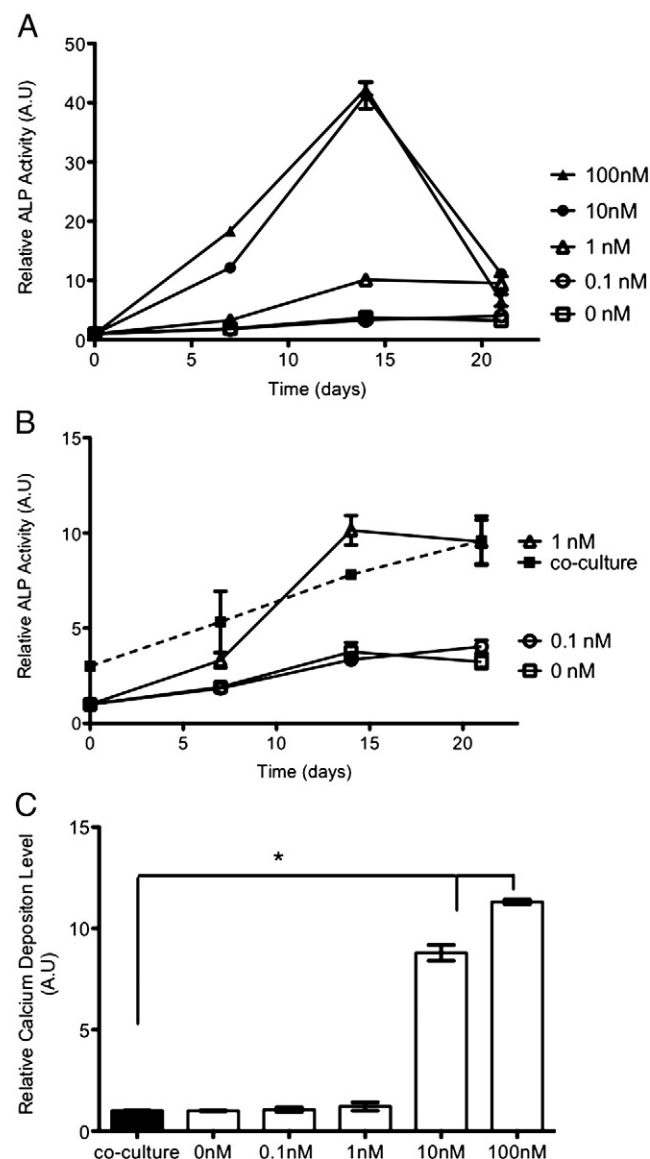
Targeting the release of glucocorticoids to specific tissues in the body is pertinent to a broad range of medical applications. However, current delivery systems, which depend on controlled-diffusion processes, have an inherent limitation in localizing the effect of the glucocorticoids to the desired site. As a result, achieving sustainable and therapeutic doses requires initial concentration of the glucocorticoids to be several orders of magnitude higher than physiological doses. This high loading



**Fig. 4.** hMSCs respond to different concentrations of the Dex-peptide in a 3D environment. (A): Results of an ALP activity assay and (B): calcium deposition assay of hMSCs exposed to 0 μM, 0.1 μM, 1 μM, 10 μM and 20 μM loadings of Dex-peptide.

could lead to severe side effects, especially in the initial burst stage. In the work presented here, a glucocorticoid was covalently immobilized into a hydrogel via an enzymatically degradable peptide tether to allow its release upon cell-dictated peptide cleavage. This system permits the controlled delivery of the glucocorticoid at different rates by designing peptide tethers of variable sequences. While such strategies do not allow one to achieve high loadings, this approach can be quite useful for delivering potent molecules in a spatiotemporally regulated manner. Here, a thiol-ene chemistry was used to incorporate MMP-degradable Dex-conjugated peptide into the PEG-norbornene system along with the encapsulated hMSCs, whose osteogenic responses were monitored via relative ALP activity and calcium deposition. Experimental results indicated that enzyme-responsive degradation of the peptide successfully delivered Dex in a sustained and physiologically relevant manner. Moreover, the biological activity of the Dex was localized to hMSCs embedded within the scaffold in that hMSCs plated on nearby surfaces outside of the hydrogel constructs did not show signs of osteogenic differentiation.

Our initial concern was that this system requires that Dex released from the matrices would retain a peptide fragment that would reduce its biological activity. Covalent conjugation of Dex to the peptide tether might potentially impair its biological function according to a previous study, which discusses the potential for loss of activity of proteins after modifying its structure [33]. If the peptide fragment that remained attached to the Dex prevented the interaction between Dex and the intracellular glucocorticoid receptor, the Dex would not be biologically active. However, a Dex-peptide fragment that represented the theoretical enzymatic cleavage product of the MMP-degradable product appeared to be equally as active as unmodified Dex. These positive results indicated that the bioactivity of Dex would be preserved when released from our matrices. Moreover, Dex delivered from the MMP-degradable peptide in the 3D hydrogel system was able to promote cellular ALP activity and calcium deposition of encapsulated hMSCs (see Fig. 3). However, it is a limitation of conjugation approaches that the activity of the drug or biologic can be compromised and must be evaluated carefully on a case-by-case basis.



**Fig. 5.** Co-culture of encapsulated hMSCs with Dex-peptide conjugated in a hydrogel with monolayer hMSCs. (A) hMSCs were plated into 24 well plates in 1 ml growth media with 0 nM to 100 nM Dex. The cellular responses of hMSCs to 0 nM to 100 nM Dex concentrations in the media were measured by following ALP activity; (B): Comparison of ALP activity of plated cells in the same culture system as the Dex-peptide conjugated hydrogel construct to that of culturing cells with 0 nM to 1 nM Dex. (C): Comparison of calcium deposition level of plated cells in the same culture system as Dex-peptide conjugated hydrogel constructs to that of culturing cells with 0 nM to 100 nM Dex at Day 21. \*Indicated statistically significant with  $p < 0.05$ .

Distinct cellular responses were observed between matrices created with the (Dex)KGPQGIAGQCK versus the (Dex)KGPQGI<sup>d</sup>AGQCK moieties. The only difference between these two Dex-peptides is the chirality of the isoleucine at the cleavage site. The non-native D isoform greatly reduces the rate of MMP-mediated cleavage of this peptide sequence, preventing substantial release of Dex from these matrices. Because glucocorticoid activity is generally mediated by cellular uptake of the molecule, Dex tethered to the hydrogel matrix should remain biologically unavailable. The possibility that different cellular responses are caused by different bioactivities of the product is unlikely because the corresponding enzymatic cleavage products of (Dex)KGPQGIAGQCK and (Dex)KGPQGI<sup>d</sup>AGQCK would be the same.

Our group had also tested an ester-linked MMP degradable peptide for tethering Dex to these thiol-ene matrices, but we observed limited

control of biological activity (data not shown). In these studies, conjugation of Dex to the MMP-degradable tethering peptide left an ester bond between the primary hydroxyl of Dex and a glutamic acid that was incorporated into the N terminus of the MMP degradable peptide. In contrast to the enzymatic specific responses observed in experiments using carbamate-linked Dex-peptide, a non-specific delivery of Dex was observed, in which changing the sequence of the tethered peptide did not affect the cellular responses (see Fig. S4). We believe that this was likely due to ester linkage hydrolysis, which rendered the cleavage of the ester-linked Dex-peptide hydrolytically controlled rather than enzymatically controlled.

Another special feature of this system is that the small molecule immobilized in the scaffold will only be delivered to local embedded cells. For these experiments, Dex-mediated osteogenic differentiation was only observed in encapsulated hMSCs, but not hMSCs in close proximity to the gel. A transwell assay (see Fig. 5) supports this statement, as cells outside of the scaffold did not exhibit elevated levels of ALP activity or calcium deposition. These results suggested that local cells uptake the released Dex and limit its diffusion out of the scaffold, a critical consideration for glucocorticoid delivery devices.

## 5. Conclusion

We demonstrated that peptide functionalized PEG hydrogels formed via a thiol-ene chemistry could produce biologically active materials to deliver Dex in a cell-dictated and localized manner. The delivery of immobilized Dex upon MMP secretion was modified by the sequence of the tethered peptide and the loading of the Dex-peptide to control different cellular responses of hMSCs. This approach can be readily utilized to deliver other glucocorticoids. Similarly, by tailoring the peptide tether to be specifically susceptible to other cell-secreted enzymes, this system can be easily modified to respond to distinct cell types that would utilize other peptidases. Overall, we presented a versatile delivery system for glucocorticoids that functions in an enzyme responsive and localized manner.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2012.08.002>.

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## References

- [1] P.J. Barnes, Corticosteroid effects on cell signalling, *Eur. Respir. J.* 27 (2) (2006) 413–426.
- [2] I. Herr, E. Ucur, K. Herzer, S. Okouyo, R.d. Ridder, P.H. Krammer, M. von Knebel Doeberitz, K.-M. Debatin, Glucocorticoid cotreatment induces apoptosis resistance toward cancer therapy in carcinomas, *Cancer Res.* 63 (12) (2003) 3112–3120.
- [3] C.-H. Pui, W.E. Evans, Treatment of acute lymphoblastic leukemia, *N. Engl. J. Med.* 354 (2) (2006) 166–178.
- [4] I. Herr, J. Pfizenmaier, Glucocorticoid use in prostate cancer and other solid tumours: implications for effectiveness of cytotoxic treatment and metastases, *Lancet Oncol.* 7 (5) (2006) 425–430.
- [5] M. Banciu, M.H. Fens, G. Storm, R.M. Schiffelers, Antitumor activity and tumor localization of liposomal glucocorticoids in B16 melanoma-bearing mice, *J. Control. Release* 127 (2) (2008) 131–136.
- [6] T. Rhen, J.A. Cidlowski, Antiinflammatory action of glucocorticoids – new mechanisms for old drugs, *N. Engl. J. Med.* 353 (16) (2005) 1711–1723.
- [7] R.S. Weinstein, Glucocorticoid-induced bone disease, *N. Engl. J. Med.* 365 (1) (2011) 62–70.
- [8] S. Walsh, G.R. Jordan, C. Jefferiss, K. Stewart, J.N. Beresford, High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors in vitro: relevance to glucocorticoid-induced osteoporosis, *Rheumatology* 40 (1) (2001) 74–83.
- [9] J.N. Clore, L. Thurby-Hay, Glucocorticoid-induced hyperglycemia, *Endocr. Pract.* 15 (5) (2009) 469–474.
- [10] R. Schaffnit, P. Wicklandt, F. Moll, Controlled iontophoretic release of glucocorticoids through epithelial cell monolayers, *J. Control. Release* 54 (1) (1998) 9–14.
- [11] C. Marianecchi, D. Paolino, C. Celia, M. Fresta, M. Carafa, F. Alhaique, Non-ionic surfactant vesicles in pulmonary glucocorticoid delivery: characterization and interaction with human lung fibroblasts, *J. Control. Release* 147 (1) (2010) 127–135.
- [12] K.Y. Dane, C. Nembrini, A.A. Tomei, J.K. Eby, C.P. O'Neil, D. Velluto, M.A. Swartz, L. Inverardi, J.A. Hubbell, Nano-sized drug-loaded micelles deliver payload to lymph node immune cells and prolong allograft survival, *J. Control. Release* 156 (2) (2011) 154–160.
- [13] L.W. Norton, J. Park, J.E. Babensee, Biomaterial adjuvant effect is attenuated by anti-inflammatory drug delivery or material selection, *J. Control. Release* 146 (3) (2010) 341–348.
- [14] Y. Yeo, M. Adil, E. Bellas, A. Astashkina, N. Chaudhary, D.S. Kohane, Prevention of peritoneal adhesions with an in situ cross-linkable hyaluronan hydrogel delivering budesonide, *J. Control. Release* 120 (3) (2007) 178–185.
- [15] M.L.R. Carla, S. Reinhard, W. Mark Saltzman, John Hilton, Henry Brem, Polymeric controlled release of dexamethasone in normal rat brain, *J. Control. Release* 16 (3) (1991) 331–339.
- [16] C.R. Nuttelman, M.C. Tripodi, K.S. Anseth, Dexamethasone-functionalized gels induce osteogenic differentiation of encapsulated hMSCs, *J. Biomed. Mater. Res. A* 76 (1) (2006) 183–195.
- [17] C.E. Hoyle, C.N. Bowman, Thiol-Ene Click Chemistry, *Angew. Chem. Int. Ed.* 49 (9) (2010) 1540–1573.
- [18] B.D. Fairbanks, M.P. Schwartz, A.E. Halevi, C.R. Nuttelman, C.N. Bowman, K.S. Anseth, A versatile synthetic extracellular matrix mimic via thiol-norbornene photopolymerization, *Adv. Mater.* 21 (48) (2009) 5005–5010.
- [19] H.A. Awad, Y.D. Halvorsen, J.M. Gimble, F. Guilak, Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells, *Tissue Eng.* 9 (6) (2003) 1301–1312.
- [20] F.M. Gregoire, C.M. Smas, H.S. Sul, Understanding adipocyte differentiation, *Physiol. Rev.* 78 (3) (1998) 783–809.
- [21] S.E. Bae, D.H. Choi, D.K. Han, K. Park, Effect of temporally controlled release of dexamethasone on in vivo chondrogenic differentiation of mesenchymal stromal cells, *J. Control. Release* 143 (1) (2010) 23–30.
- [22] H. Atmani, C. Audrain, L. Mercier, D. Chappard, M.F. Basle, Phenotypic effects of continuous or discontinuous treatment with dexamethasone and/or calcitriol on osteoblasts differentiated from rat bone marrow stromal cells, *J. Cell. Biochem.* 85 (3) (2002) 640–650.
- [23] T.T.D. Arun, K. Ghosh, Sean P. McKee, Wayne J. Thompson, N,N'-dissuccinimidyl carbonate: a useful reagent for alkoxy-carbonylation of amines, *Tetrahedron Lett.* 33 (20) (1992) 4.
- [24] E. Kaiser, R.L. Colescott, C.D. Bossinger, P.I. Cook, Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides, *Anal. Biochem.* 34 (2) (1970) 595–598.
- [25] G.L. Ellman, Tissue sulfhydryl groups, *Arch. Biochem. Biophys.* 82 (1) (1959) 7.
- [26] B.D. Fairbanks, M.P. Schwartz, C.N. Bowman, K.S. Anseth, Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphine: polymerization rate and cytocompatibility, *Biomaterials* 30 (35) (2009) 6702–6707.
- [27] M.D. Pierschbacher, E. Ruoslahti, Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule, *Nature* 309 (5963) (1984) 30–33.
- [28] P.D. Mariner, E. Johannesen, K.S. Anseth, Manipulation of miRNA activity accelerates osteogenic differentiation of hMSCs in engineered 3D scaffolds, *J. Tissue Eng. Regen. Med.* 6 (4) (2012) 314–324.
- [29] F. Mannello, G.A. Tonti, G.P. Bagnara, S. Papa, Role and function of matrix metalloproteinases in the differentiation and biological characterization of mesenchymal stem cells, *Stem Cells* 24 (3) (2006) 475–481.
- [30] M. Egeblad, Z. Werb, New functions for the matrix metalloproteinases in cancer progression, *Nat. Rev. Cancer* 2 (3) (2002) 161–174.
- [31] H. Nagase, G.B. Fields, Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides, *Pept. Sci.* 40 (4) (1996) 399–416.
- [32] K.A. Neil, J. Clendeninn, Matrix metalloproteinase inhibitors in cancer therapy, Humana press, 2001.
- [33] C.C. Lin, K.S. Anseth, Controlling affinity binding with peptide-functionalized poly(ethylene glycol) hydrogels, *Adv. Funct. Mater.* 19 (14) (2009) 2325.