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A diels-alder modulated approach to control and sustain the release of dexamethasone and induce osteogenic differentiation of human mesenchymal stem cells

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Abstract

We report a new approach to controlled drug release based upon exploiting the dynamic equilibrium that exists between Diels-Alder reactants and products, demonstrating the release of a furan containing dexamethasone peptide (dex-KGPQG-furan) from a maleimide containing hydrogel. Using a reaction-diffusion model, the release kinetics were tuned to achieve sustained concentrations conducive to osteogenic differentiation of human mesenchymal stem cells (hMSCs). Efficacy was first demonstrated in a 2D culture model, in which dexamethasone release induced significant increases in alkaline phosphatase (ALP) activity and mineral deposition in hMSCs compared to a dexamethasone-free treatment. The results were similar to that observed with a soluble dexamethasone treatment. More dramatic differences were observed in 3D culture, where co-encapsulation of a dexamethasone releasing hydrogel depot within an hMSC-laden extracellular matrix mimetic poly(ethylene glycol) hydrogel resulted in a local and robust osteogenic differentiation. ALP activity reached levels that were up to six times higher than the dexamethasone free treatment. Interestingly, at 5 and 10 day time points, the ALP activity exceeded the dexamethasone positive control, suggesting a potential benefit of sustained release in 3D culture. After 21 days, substantial mineralization comparable to the positive control was also observed in the hydrogels. Collectively, these results demonstrate Diels-Alder modulated release as an effective and versatile new platform for controlled drug delivery that may prove especially beneficial for sustaining the release of low molecular weight molecules in hydrogel systems.

1. Introduction

Dexamethasone is a potent synthetic corticosteroid that has found widespread use in a variety of medical and biological applications. Clinically, dexamethasone has been used as an anti-inflammatory agent to treat conditions such as rheumatoid arthritis[1,2], cerebral edema[3] and altitude sickness[4]. Dexamethasone has also found utility in oncology, as it has been shown to alleviate treatment side effects[5,6] and bolster the efficacy of the cancer

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therapy[7]. In addition to these clinical uses, dexamethasone is known to be a powerful morphogen and is routinely used to induce the differentiation of multipotent mesenchymal stem cells (MSCs). For example, dexamethasone is a key ingredient in osteogenic differentiation medium used to differentiate MSCs into osteoblasts where typical concentrations for inducing osteogenic differentiation are on the order of 100 nM[8–10]. Dexamethasone is also used to induce adipogenic differentiation of MSCs. However, the concentration used to induce adipogenesis is an order of magnitude higher than for osteogenic differentiation[8], highlighting the dose dependent effects of dexamethasone.

To maximize therapeutic efficacy and mitigate undesired side effects^{9–11}, there is a critical need for the development of biomaterial strategies that control the time-dependent release of pharmaceuticals. To address this need, a myriad of polymeric controlled release platforms have been developed and applied to dexamethasone. Perhaps the most straightforward technique for modulating release entails simple loading of dexamethasone into a polymer substrate from which the pharmaceutical is able to diffuse and reach cells in the surrounding vicinity[11–13]. In this approach, the diffusional release can be controlled to a certain extent by varying the composition of the polymer, by changing the crosslink density or swelling, or through the inclusion of other materials such as organosilicates[14,15]. However, only limited control over the release kinetics is possible with this approach, where an initially rapid release is followed by an inability to sustain delivery of the target material for an extended period of time. This limitation is due, in part, to the low molecular weight of dexamethasone (i.e., 392 Da) and its hydrophobic nature.

An alternative approach to control the release of dexamethasone has been to attach the drug covalently to a polymer network through degradable linkages. For example, Nuttelmann *et al.* made use of hydrolytically labile lactide ester bonds to modulate the release of dexamethasone from hydrogels and showed that the length of the linker and number of ester bonds could be used to tune the release kinetics[16]. More recently, Webber, Stupp, and coworkers reported dexamethasone conjugation to self-assembling peptide amphiphile gels via hydrolysable hydrazone linkages as a means for sustained dexamethasone release[17]. A similar strategy for dexamethasone release was reported by Liu *et al.*[18]. However, the dependence on pH and susceptibility to degradation by esterases often presents a challenge, potentially resulting in deviations from predicted release kinetics. Release of covalently tethered dexamethasone has also been accomplished through the use of enzymatically degradable linkages. Yang *et al.* used this approach to release dexamethasone in response to matrix metalloproteinase (MMP) secretion by cells[19]. While this cell-mediated release mechanism offers another means to release dexamethasone, MMP expression is ubiquitous in tissue remodeling and can vary widely[20,21], making it difficult to tune the drug release kinetics predictably with this approach.

As an alternative or complementary method to hydrolytically and enzymatically mediated release mechanisms, we sought to develop a biomaterial platform that enables tunable, predictable, and sustainable dexamethasone release. To achieve this goal we turned to the use of a Diels-Alder reaction. In its classical implementation, the Diels-Alder (See Figure 1b) reaction is thermally reversible: low temperatures promote the generation of the Diels-Alder adduct or product whereas elevated temperatures incite reversion to the reactant species. However, because a dynamic equilibrium exists between the product and reactants, we hypothesized that the reversibility of the Diels-Alder reaction could be exploited to tune and sustain dexamethasone release from a hydrogel without the need to apply potentially detrimental elevated temperatures. Importantly, removal of reactants, as accomplished by either diffusion out of the network or through cellular metabolism, results in reversion of the adduct to the reactants, thereby promoting sustained release. However, unlike other controlled delivery platforms in which release is diffusion controlled once hydrolytically or

enzymatically labile bonds are severed, the Diels-Alder mechanism allows for bonds to reform. Thus, the releasing species can be re-incorporated into the network, presenting a unique and powerful means for tuning drug release kinetics. To demonstrate proof of concept for Diels-Alder mediated dexamethasone release, a furan-modified dexamethasone was synthesized and incorporated into maleimide containing poly(ethylene glycol) (PEG) hydrogels. As this release platform has not been previously implemented in biological applications, the bioactivity of the furan-modified dexamethasone was first verified. Then, the ability to sustain release from a hydrogel and induce osteogenic differentiation of human MSCs (hMSCs) *in vitro* was examined and quantified in both 2D and 3D culture.

2. Materials and Methods

2.1. Materials

The following materials were acquired from their respective vendors and, unless otherwise indicated, used without further purification or modification. The main components of the hydrogel networks created as part of this investigation, a tetrafunctional maleimide and thiol-functional PEG macromers, were acquired from Laysan Bio (Arab, AL). Octa-functional PEG norbornene was synthesized from a hydroxylated PEG precursor purchased from JenKem Technology USA (Allen, TX) following a published synthetic route[22]. The di-cysteine peptide crosslinker KCGPQGIWGQCK and mono-cysteine cell adhesive peptide CRGDS were purchased from American Peptide Company (Sunnyvale, CA). The active pharmaceutical considered in the release studies, dexamethasone, was bought from Enzo Life Sciences (Farmingdale, NY). Fmoc protected amino acid residues, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate activator (HBTU), and Rink Amide MBHA resin used for solid phase peptide synthesis were purchased from ChemPep, Inc. (Wellington, FL). All other chemicals required for peptide synthesis were purchased from Sigma-Aldrich (St. Louis, MO). All cell culture reagents were purchased from Life Technologies (Grand Island, NY) except for recombinant human fibroblast growth factor-2 (FGF-2), which was purchased from Peprotech (Oak Park, CA). The alkaline phosphatase staining kit (i.e., Leukocyte Alkaline Phosphatase Kit) and alkaline phosphatase substrate solution (i.e., p-nitrophenyl phosphate) were purchased from Sigma-Aldrich. dsDNA in cell lysates was quantified using the Quant-iT™ Picogreen kit from Life Technologies. The calcium detection kit for measuring mineral deposition was purchased from Point Scientific (Canton, MI).

2.2. Synthesis of Dexamethasone Functionalized, Diels-Alder Reactive Peptide

To achieve Diels-Alder-mediated dexamethasone release, a peptide with the sequence KGPQG-furan was synthesized using standard Fmoc-mediated solid phase peptide synthesis methods on a Protein Technologies (Tucson, AZ) automated Tribute Bench Peptide Synthesizer. The 3-furoic functionality was directly incorporated during the solid phase synthesis using a synthetic Fmoc protected amino acid analogue. Details pertaining to the synthetic route to obtain the Fmoc protected 3-furoic amino acid and its inclusion into the peptide are provided elsewhere[23]. Dexamethasone NHS ester was then introduced to the resin-bound peptide sequence and allowed to react with the N-terminal primary amine overnight. Carbamate bond formation and dexamethasone conjugation were confirmed via a qualitative Kaiser test. Importantly, similar conjugation schemes have previously been reported and demonstrated no adverse effect on dexamethasone bioactivity. The reactive dexamethasone-NHS ester was prepared according to procedures previously presented[19,24].

After synthesis and final modification, the dexamethasone-GQPGK-3-furan peptide was cleaved from the solid support using a mixture of trifluoroacetic acid, triisopropyl silane and

water (95/2.5/2.5 v/v) and precipitated in cold diethyl ether. The crude peptide was purified by means of high performance liquid chromatography (HPLC) using a reverse-phase C-18 column on a Waters Delta Prep 4000 system and a linear gradient beginning at a ratio of 95/5 water/acetonitrile ramping over 70 minutes to pure acetonitrile. Confirmation of synthesis and isolation of the target peptide species were achieved through matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry. Prior to performing release studies, bioactivity of the dexamethasone peptide was confirmed via an *in vitro* osteogenic differentiation assay with hMSCs (see Section 2.5).

2.3 Diels-Alder Compatible Hydrogels for Dexamethasone Release

Maleimides readily participate in Diels-Alder reactions with furans. Thus, to be compatible with the desired Diels-Alder modulated release mechanism, the base hydrogels for dexamethasone release were crosslinked through the Michael addition reaction of a 10k molecular weight tetrafunctional maleimide and thiol PEG macromers (see Figure 1, Section 3.1). Prior to hydrogel polymerization, the furan modified dexamethasone peptide sequence (see Section 2.4.) was introduced to a 20 wt. % stock solution of PEG tetra-maleimide macromer, creating an initial mixture containing furans at a concentration of approximately 5mM (see Section 3.1). The resulting maleimide PEG macromer and furan dexamethasone peptide were allowed to react for at least 24 hours at ambient temperature. This process allowed for the formation of covalent Diels-Alder linkages between the furan-modified dexamethasone and the maleimide macromer. Briefly, the PEG tetra-maleimide was used at a working concentration of 7.5 wt.% (23 mM maleimide) and PEG tetra-thiol working concentration was 2.5 wt.% (17mM thiol) in phosphate buffered saline (PBS). To prevent premature polymerization, the pH of the macromer solution was adjusted to approximately 2 to inhibit the thiol-maleimide Michael addition reaction. The reaction was catalyzed by the addition of 3 μ L of 300mM triethanolamine to 30 μ L of hydrogel precursor solution. Polymerization was complete within approximately 15 minutes, as ascertained by a negative qualitative Ellman's assay. Prior to their use in cell studies, the hydrogels were stored in PBS for 24–48hrs and then washed twice with PBS to remove triethanolamine.

2.4 Cell Culture

hMSCs were isolated from the mononuclear fraction of human bone marrow specimens (purchased from Lonza) based on their adherence in culture, as previously described[25]. Cells were subcultured under standard conditions of 37°C and 5% CO₂ in medium consisting of low glucose Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum, 1% penn/strep, and 1ng/ml recombinant human FGF-2. The cells were passaged via treatment with trypsin/EDTA upon reaching approximately 70–80% confluence. Passage 3–4 cells were used for all experiments.

2.5 2D Osteogenic Differentiation

hMSCs were seeded in 24 well plates at 18,500 cells/cm² and cultured overnight in growth media (GM) consisting of low glucose DMEM supplemented with 10% FBS and 50 U/mL each penicillin/streptomycin. The media was then replaced with a base osteogenic differentiation media (OST) consisting of high glucose DMEM supplemented with 10% FBS, 50 U/mL each penicillin/streptomycin, 20 mM β -glycerophosphate, and 50 μ M ascorbic acid. Hydrogels releasing dexamethasone, which were polymerized in 1 ml syringe molds (i.e., syringes with tips removed) to have a uniform cylindrical geometry, were then suspended in the media above the plated cells using BD Falcon cell culture transwell inserts (transparent, PET membranes with 1.0 μ m pores). OST media supplemented with 0 and 100 nM dexamethasone were used as negative and positive controls, respectively. GM was also used as a negative control.

2.6 3D Osteogenic Differentiation

Dexamethasone releasing hydrogels were formed by placing a rubber gasket mold (5 mm diameter, 500 μm thickness) on a thiolated glass cover slip and filling them with the PEG macromer solution. Glass thiolation was achieved using 3-mercaptoptrimethoxysilane and a liquid phase silanization protocol, as previously described[26]. This procedure covalently attached the cylindrical hydrogel to the glass surface through a thioether linkage. hMSC laden extracellular matrix mimetic hydrogels were subsequently polymerized around the dexamethasone releasing hydrogel. These hydrogels were polymerized using photoinitiated thiol-ene chemistry, as previously described[22,25]. Briefly, 40 kDa octa-functional PEG-norbornene at 7.5 wt. % (15 mM norbornene) was combined with 7 mM KCGPQGIWGQCK crosslinker peptide, 1 mM CRGDS cell adhesive peptide, and 2 mM lithium acylphosphinate photoinitiator[27]. hMSCs suspended in PBS were added to the macromer solution so that the final cell density was 2.5×10^6 cells/mL. 100 μL of thiol-ene hydrogel solution was transferred to rubber gasket molds (10 mm diameter, 4 mm thickness) that were placed on top of the dexamethasone releasing hydrogels. The cell-laden hydrogels were then polymerized under UV light (365 nm, $\sim 4 \text{ mW/cm}^2$) for 3 min and transferred to OST media (0 mM dexamethasone) for culturing. As controls, hMSC-laden hydrogels were polymerized using an identical formulation and cultured in GM and OST media with 0 and 100 nM dexamethasone.

2.7 Alkaline Phosphatase (ALP) Activity

For the 2D osteogenic differentiation experiment, hMSC differentiation was qualitatively assessed after 14 days by staining for alkaline phosphatase (ALP). Prior to staining, the cells were fixed in neutral buffered formalin for 30 min at 4°C . The cells were then washed with PBS and stained for approximately 45 min using a commercially available histochemical staining kit for alkaline phosphatase according to the manufacturer's protocol.

For both the 2D and 3D osteogenic differentiation experiments, ALP activity was quantitatively evaluated using a colorimetric assay that is based on the conversion of p-nitrophenyl phosphate to p-nitrophenol by alkaline phosphatase. For the 2D experiment, the cells were washed with PBS and then lysed by treatment with 500 μL of 1X RIPA buffer. For the 3D experiment, the gels were washed twice for 20 min with PBS, transferred to a microtube with 300 μL of RIPA buffer, and then homogenized with a pestle. The ALP activity of all samples was determined by combining 50 μL of lysate, 50 μL of 1X RIPA buffer, and 100 μL of alkaline phosphatase substrate (i.e., p-nitrophenyl phosphate) in a clear bottom 96 well plate and monitoring the change in absorbance at 405 nm over 10 min with 1 min intervals on a BioTek Synergy H plate reader. Special care was taken to ensure that the absorbance versus time curves were linear for all samples. The mean slopes of the curves were taken to be the relative ALP activities. To account for variation in cell number between gel samples, the 3D data were normalized to the dsDNA concentration in the cell lysate. Data normalization was not performed for the 2D study. All ALP activity values are reported as fold increases relative to day 0.

2.8 Mineral Deposition

Osteogenic differentiation was also quantitatively assessed on the basis of mineral deposition. For the 2D experiment, wells were treated with 250 μL of 1 M HCl at 4°C for 72 hrs to dissolve any calcium phosphate mineral present. For the 3D experiment, the remaining volume of homogenized hydrogel samples after performing the ALP activity assay was acidified with 50 μL of 12 M HCl and stored at 4°C for 72 hrs. To quantify mineralization, the acidified solutions were diluted with phosphate buffered saline (PBS; dilutions were 1:4 and 1:10 for the 2D and 3D experiments, respectively). 50 μL of diluted solution was then combined with 100 μL of calcium reagent solution in a clear bottom 96

well plate. The absorbance values at 570 nm for each sample were then measured on a plate reader and compared to a calcium phosphate standard curve in order to determine the Ca^{2+} concentration. Special care was taken to ensure that the most concentrated samples fell within the linear response range of the assay. Final values are reported as the total mass of calcium deposited for each sample.

2.9 Statistical Analysis

Quantitative data for ALP activity and mineral deposition are presented as the average $\pm$ standard error based on the results of $n = 6$ samples pooled from three independent experiments. Data analysis was performed using the open source statistical software package R (available for free at www.r-project.org). At each time point, the data were analyzed by means of a one-way ANOVA to determine the effects of media treatment. Significant differences between groups were determined by post hoc Tukey comparisons ($\alpha = 0.05$).

3. Results and Discussion

3.1. Diels-Alder Chemistry Enables Sustained Release

Numerous controlled drug release strategies have been developed and are described in the literature. For dexamethasone specifically, hydrolytically mediated release and cell-mediated release from biomaterials have been achieved via covalent tethering of the drug molecule through labile ester and hydrazine bonds and enzymatically degradable peptide sequences, respectively[16,17,19]. Although effective, these approaches are limited in their ability to predictably tune the release kinetics. Ester and hydrazone hydrolysis are pH dependent. Therefore, hydrolytically mediated release is sensitive to changes in the local cellular microenvironment, which can lead to deviations from predicted release profiles. While controlled release through enzymatically degradable linkers mitigates this problem to a certain extent, this approach is still dependent on cellular activity, which can vary widely and is difficult to know *a priori*. In an effort to circumvent these problems and develop a platform capable of sustained release, we have turned to Diels-Alder chemistry. Importantly, a dynamic equilibrium exists between Diels-Alder reactants and products. This equilibrium can be exploited to control the release of covalently tethered molecules from a hydrogel biomaterial in a predictable manner[23]. Furthermore, because Diels-Alder reactive moieties are not naturally present in biological systems, this chemistry is pseudo-bioorthogonal and is insensitive to changes in the cellular microenvironment.

To achieve Diels-Alder modulated release, a suitably functionalized hydrogel platform was developed using PEG maleimide and thiol functionalized macromers, which were reacted via standard Michael addition chemistry (Figure 1A). The Michael addition between these two species is a fast and efficient reaction that has been implemented in a number of reactions including surface functionalization[28], protein crosslinking[29] and fluorescent labeling[30]. In our application, however, the maleimide and thiol groups were reacted in an off-stoichiometric manner such that thiol was the limiting reagent. Consequently, hydrogels with residual, unreacted maleimide functional groups were formed. The presence of unreacted maleimide was a key aspect of the network design, as this functionality not only possesses the ability to react with thiols to form the crosslinked network, but can also serve as the dienophile in a Diels-Alder reaction (Figure 1B). Thus, when a molecule bearing a diene functionality (e.g., furan) comes into contact with the active maleimide sites in the hydrogel, there exists the potential for the formation of a Diels-Alder adduct, thereby reversibly anchoring the species to the network through a covalent bond (Figure 1F).

To predict dexamethasone release using Diels-Alder chemistry, we employed a Diels-Alder reaction-diffusion mathematical model. The model used to forecast the dexamethasone release was developed based upon empirical data for an identical hydrogel system releasing

a peptide sequence of similar size and hydrophobicity (Data shown elsewhere)[23]. An initial loading concentration of 0.5 mM dexamethasone peptide was chosen and used to predict the release profile over 5 days (Figure 2). Analyzing the release trajectory, it is clear that an initial burst of furan dexamethasone peptide occurs over the first 24 hours, during which approximately 50% of the loaded peptide is released. This burst is most likely due to the equilibrium of the Diels-Alder reaction, whereby some of the peptide is not covalently bound to the hydrogel and rapidly leaves the system by a diffusion driven process. Importantly, the initial burst release is followed by a linear, long-term release profile that allows for a predictable concentration of the furan dexamethasone peptide to be maintained. For this condition, the predicted release rate in the linear regime was 0.5 $\mu\text{g/day}$, which can be adjusted simply by altering the number of pendant Diels-Alder reactive groups in the gel. In this study for the model predictions as well as experimental release measurements, the concentration of pendant maleimide groups was approximately 25 mM with no furan bound to the network via Diels-Alder adducts. Based on the parameters used for gel synthesis and dexamethasone loading, the model predicts that over the course of twenty-four hours, the dexamethasone concentration in approximately 1mL of media will rise to a value on the order of 100nM, which is the concentration typically used to induce hMSCs to undergo osteogenic differentiation[8]. Furthermore, according to the model, release at this rate can be maintained for an extended period (i.e., several months), illustrating that Diels-Alder modulated release is suitable for sustaining dexamethasone release.

3.2. Diels-Alder Reactive Dexamethasone is Bioactive

The Diels-Alder reactive dexamethasone peptide designed in this study was synthesized via coupling of the primary hydroxyl functionality of dexamethasone to the N-terminus of a synthetic peptide (Figure 3A). Multiple studies[16,19] have reported that dexamethasone modification at this location is not detrimental to bioactivity. Nevertheless, prior to testing the efficacy of the Diels-Alder modulated release platform, it was first necessary to verify the bioactivity of our dexamethasone peptide. In addition to the peptide modification, which is similar to that used by Yang *et al.*[19], the inclusion of the 3-furoic functionality was of particular concern, as this functionality has not previously been utilized in cellular applications. Importantly, after 7 days in 2D culture, hMSCs treated with osteogenic media containing 100 nM dexamethasone-KGPQG-furan were viable and appeared morphologically identical to control cells (data not shown). Furthermore, the dexamethasone peptide treatment significantly increased hMSC ALP activity compared to negative controls, and the effect was similar but slightly higher compared to that observed for hMSCs treated with 100 nM dexamethasone (Figure 3B). As ALP activity is a key indicator of hMSC osteogenic differentiation[31], these results indicate that the peptide modification and furan functionalization did not significantly impair the molecule's bioactivity.

3.3 Diels-Alder Modulated Dexamethasone Release Induces Osteogenic Differentiation of hMSCs in 2D and 3D Culture

To test the efficacy of the Diels-Alder modulated release platform to alter cell activity, transwell inserts were used to suspend dexamethasone peptide releasing hydrogels above hMSCs in 2D culture. To avoid the elevated concentrations associated with the initial burst release and operate the Diels-Alder release solely in the linear region, furan dexamethasone peptide loaded hydrogels were placed in PBS buffer solution for approximately 36 hours prior to introduction to cell culture. As can be noted by the vertical line in Figure 2, the release is well into the linear phase after 36 hours. Thus, introduction of the hydrogels at this time allowed for the release to gradually increase the amount of dexamethasone present in the media over time. Given the results forecast by the model, the sustained release achieved using the Diels-Alder platform results in hMSCs being continually supplied with dexamethasone at levels conducive to osteogenic differentiation.

Indeed, robust hMSC osteogenic differentiation in standard 2D culture conditions was achieved using Diels-Alder modulated dexamethasone release from hydrogels. Osteogenic differentiation was first observed qualitatively by staining for ALP after 14 days in culture (Figure 4A). As expected, minimal staining was observed for cells cultured in GM, as well as OST media lacking dexamethasone. However, intense blue/purple staining was observed in cells cultured in OST media and exposed to dexamethasone releasing hydrogels. Similar staining was observed for hMSC cultured in OST media supplemented with 100 nM dexamethasone. Quantitative analysis of ALP activity further supported the efficacy of the Diels-Alder modulated platform to release active and physiologically relevant dexamethasone concentrations. While hMSCs cultured in GM and OST minus dexamethasone only showed marginal increases in ALP activity from baseline levels over the 14 day time course, exposure to Diels-Alder modulated dexamethasone peptide releasing hydrogels resulted in a significant increases in ALP activity at 7 and 14 days, similar to what was observed for the positive control (Figure 4B). Significantly increased mineral deposition by the cells cultured with dexamethasone releasing hydrogels was also observed at 14 days. However, at 21 days the extent of mineralization was lower than that seen for cells cultured OST with 100 nM dexamethasone (Figure 4C).

While the results of the 2D study support the ability of the Diels-Alder reaction to controllably release and sustain the levels of a bioactive material from a suitably functionalized network, we also sought to demonstrate efficacy in 3D cell culture. Unfortunately, despite published work on maleimide-thiol Michael addition hydrogels[32], this crosslinking chemistry was not suitable for direct encapsulation of cells for 3D culture in our work, as we noted poor cytocompatibility, presumably due to the unreacted maleimides in our hydrogel formulation. However, the gels can be readily processed as a delivery depot residing within a cell-laden matrix to influence resident cells in a local and three-dimensional manner. To demonstrate this concept, we fabricated a Diels-Alder delivery gel, containing the dexamethasone peptide, within an hMSC-laden hydrogel (Figure 5A). The hMSC-laden hydrogels, which consisted of PEG as well as ECM mimetic synthetic peptides to allow for cell adhesion and cell-mediated degradation, were photocrosslinked with a cytocompatible thiol-ene chemistry[22]. This approach eliminates cellular exposure to unreacted maleimides and enabled us to test the effects of Diels-Alder modulated dexamethasone release in 3D. The dexamethasone releasing hydrogels were fabricated using the same formulation as for the 2D experiments and were pre-treated similarly in PBS to maintain a steady dexamethasone release rate at osteoinductive concentrations.

As in the 2D study, Diels-Alder modulated and sustained release of dexamethasone efficiently induced osteogenic differentiation of hMSCs in 3D culture over the time course of 14 days. Measurement of ALP activity, which was normalized to dsDNA content to account for any differences in cell number within the hydrogel samples, showed significant increases in differentiation compared to the negative controls (i.e., GM and OST media without dexamethasone) at 5, 10, and 14 days (Figure 5B). Interestingly, the ALP activity values for the Diels-Alder modulated dexamethasone release exceeded those of the OST plus 100 nM dexamethasone treatment at the 5 and 10 day time points, suggesting enhanced efficacy in 3D culture. This difference could possibly be due to enhanced cellular uptake resulting from the peptide modification. When present in solution, the dexamethasone peptide slightly increased ALP activity at 7 days compared to an equal concentration of unmodified dexamethasone. Alternatively, the increased ALP activity in the 3D study could also be the result of proximity and increased bioavailability in the 3D system, since dexamethasone was continually diffusing out from the inner gel depot to the encapsulated hMSCs. In addition to the increased ALP activity, substantial mineralization was also observed by 21 days. Mineralization in the OST minus dexamethasone treatment was also

observed, most likely due to the presence of β -glycerophosphate in the media. However, the extent of mineral deposition in response to the Diels-Alder mediated dexamethasone release was comparable to the positive control, and these two groups were not significantly different. Collectively, the results of both the 2D and 3D studies demonstrate that the Diels-Alder modulated release platform is an effective strategy for controlling dexamethasone release and inducing hMSC osteogenic differentiation.

This work has demonstrated the ability to make use of a maleimide/furan Diels-Alder reaction to release dexamethasone controllably from a suitably functionalized polymeric platform. The results portrayed in this work demonstrate one potential implementation of the Diels-Alder mediated release to controllably and sustainably deliver bioactive materials. Based on the results presented, the dexamethasone release platform that we developed could, in addition to bone tissue engineering applications, easily be tuned for other applications in which sustained dexamethasone release is required, such as suppressing chronic inflammation. Furthermore Diels-Alder moieties could also be incorporated into other bioactive molecules, such as antibiotics or other therapeutic compounds, to control their delivery.

In addition the release of other molecules, there exists the possibility to further tune the drug release profile. One method to adjust the release profile relies on altering the number of Diels-Alder reactive sites in the polymer network. Previous work has demonstrated that varying the number of Diels-Alder reactive sites in the network impacts the release profile of Diels-Alder functionalized molecules; a smaller number of reactive sites corresponds to a faster release and vice versa[23]. Another technique that could be invoked to exercise control over the release kinetics revolves around the use of other diene/dienophile species that participate in Diels-Alder and retro-Diels-Alder reactions. Previous work[33] has demonstrated that the diene/dienophile pair selected dictate the kinetic and thermodynamic properties of the Diels-Alder reaction, over a wide range of possible values. For example placement of an electron withdrawing group near the dienophile typically enhances the adduct formation and would be expected to elicit a slower release than a Diels-Alder pair without this attribute. Conversely, the ability to increase the rate of release also exists as the placement of electron withdrawing groups on the diene (i.e. furan) generally results in a diminished propensity for the forward Diels-Alder reaction, favoring instead the reverse, retro-Diels-Alder reaction.

4. Conclusions

The dynamic nature of the Diels-Alder reaction can be exploited to controllably release covalently tethered bioactive molecules in a manner uncharacteristic of traditional platforms. The ability to make use of a maleimide and furan diene/dienophile pair as a means to controllably release dexamethasone to hMSCs from a suitably fabricated hydrogel network was demonstrated in this study. Here, we demonstrated proof of concept for Diels-Alder modulated controlled release using dexamethasone. Specifically, we conjugated dexamethasone to a furan containing synthetic peptide to achieve sustained release from a maleimide containing hydrogel. Using a predictive model, we were able to identify a dexamethasone release and achieve concentrations suitable for inducing osteogenic differentiation of hMSCs. In 2D culture, Diels-Alder mediated dexamethasone release stimulated hMSCs to undergo osteogenic differentiation, as indicated by increased ALP activity and mineral deposition. Robust osteogenic differentiation was also achieved in 3D culture when a dexamethasone releasing hydrogel was encapsulated within an hMSC-laden hydrogel. ALP activity was significantly increased, even compared to the positive control at 5 and 10 days. Substantial mineralization was also observed after 21 days. The results portrayed in this work demonstrate one potential implementation of the Diels-Alder

mediated release to controllably and sustainably deliver bioactive materials. Based on these results, the dexamethasone release platform developed here could be useful for bone tissue engineering applications. Importantly, opportunities beyond dexamethasone also exist, as a similar Diels-Alder strategy could feasibly be expanded upon and applied to control the release of other drug molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design and development of a hydrogel compatible with Diels-Alder modulated release. **A.** The Michael addition reaction between thiol and maleimide functionalities that is used to form the initial network. **B.** The Diels-Alder reaction between a generic furan (diene) and maleimide (dienophile) species. **C.** A dexamethasone labeled peptide sequence equipped with a Diels-Alder reactive furan functionality. **D** and **F.** Tetrafunctional maleimide and thiol PEG macromers. Reaction of these species in an off stoichiometric ratio, where the thiol is the limiting reactant, allowed for the creation of sites at which the Diels-Alder reaction could occur. **E.** Schematic representation of the network created as a result of a Michael addition between (D) and (E) and including (C) as a releasable species.

Figure 2.

Simulated results predicting the release profile of the furan dexamethasone peptide sequence from the maleimide-thiol hydrogel platform. The red dashed line indicates the time at which the furan dexamethasone loaded hydrogels were introduced to two and three dimensionally cultured hMSCs after being introduced to PBS buffer. This time point was selected to avoid the large initial burst of dexamethasone and operate in the linear release regime. A. The dexamethasone release rate in the linear regime was calculated to be approximately $0.5\mu\text{g/day}$.

Figure 3. Diels-Alder reactive dexamethasone peptide induces hMSC osteogenic differentiation. A. Structure of the furan functionalized dexamethasone peptide (dex-GQPGK-furan). B. Fold increase in ALP activity after hMSCs were treated for 7 days in growth media (GM), osteogenic media without dexamethasone (OST(-dex)), osteogenic media (OST (+dex)), and osteogenic media with the dexamethasone replaced by dex-GQPGK-furan. The concentration of dex-GQPGK-furan was 100 nM. * indicates statistical significance compared to the other experimental groups (n = 5; p < 0.05).

Figure 4.

Diels-Alder modulated dexamethasone release induces hMSC osteogenic differentiation in 2D culture. A. Representative images from ALP stain after 14 days (scalebars = 500 μ m). B. Fold increase in ALP activity after hMSCs were treated for 4, 7, and 14 days. C. Mineral deposition after hMSCs were treated for 7, 14, and 21 days. Note: DA = Diels-Alder; * indicates significance compared to the GM treatment; # indicates significance compared to the OST (- dex) treatment; ** indicates significance compared to all other treatments (n = 6 samples pooled from three independent experiments; p < 0.05).

Figure 5. Diels-Alder modulated dexamethasone release induces hMSC osteogenic differentiation in 3D culture. A. Schematic of the experimental setup. Dexamethasone releasing hydrogels (30 μ L gel containing an initial dexamethasone concentration of 0.5mM) were encapsulated within an hMSC laden, peptide functionalized PEG hydrogel. B. Fold increase in ALP activity after hMSCs were treated for 5, 10, 14, and 21 days. ALP activity was normalized to dsDNA content to account for differences in cell seeding number. C. Mineral deposition in the hydrogels after hMSCs were treated for 10, 14, and 21 days. Note: DA = Diels-Alder; * indicates significance compared to the GM treatment; # indicates significance compared to the OST(- dex) treatment; ** indicates significance compared to all other treatments (n = 6 samples pooled from three independent experiments; p < 0.05).