Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production

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Abstract

Healing articular cartilage defects remains a significant clinical challenge because of its limited capacity for self-repair. While delivery of autologous chondrocytes to cartilage defects has received growing interest, combining cell-based therapies with growth factor delivery that can locally signal cells and promote their function is often advantageous. We have previously shown that PEG thiol-ene hydrogels permit covalent attachment of growth factors. However, it is not well known if embedded chondrocytes respond to tethered signals over a long period. Here, chondrocytes were encapsulated in PEG hydrogels functionalized with transforming growth factor-beta 1 (TGF-β1) with the goal of increasing proliferation and matrix production. Tethered TGF-β1 was found to be distributed homogenously throughout the gel, and its bioactivity was confirmed with a TGF-β1 responsive reporter cell line. Relative to solubly delivered TGF-β1, chondrocytes presented with immobilized TGF-β1 showed significantly increased DNA content and GAG and collagen production over 28 days, while maintaining markers of articular cartilage. These results indicate the potential of thiol-ene chemistry to covalently conjugate TGF-β1 to PEG to locally influence chondrocyte function over 4 weeks. Scaffolds with other or multiple tethered growth factors may prove broadly useful in the design of chondrocyte delivery vehicles for cartilage tissue engineering applications.

Keywords
cartilage tissue engineering; chondrocytes; protein conjugation; hydrogels; transforming growth factor-β1

Disclosure
No benefit of any kind will be received either directly or indirectly by the authors. The content of the manuscript does not necessarily reflect the position or policy of the Government, and no official endorsement should be inferred.
1. Introduction

Healing articular cartilage defects remains a significant clinical challenge because of its limited capacity for self-repair and mechanical properties that are difficult to emulate. Articular cartilage is an avascular tissue with a sparse population of cells surrounded by an extracellular matrix (ECM) that is regulated by numerous growth factors. Therefore, tissue engineering strategies involving chondrocytes and growth factor delivery may help to improve the treatment of articular cartilage lesions.

There is growing interest in the regenerative medicine community in methods to sequester and present bioactive therapeutic proteins to chondrocytes immobilized in three-dimensional matrices. Cytokines are attractive targets for tissue engineering since, at low concentrations, they can regulate cellular functions, such as proliferation and matrix production. Many of these proteins are commonly introduced as soluble factors in culture media during in vitro experiments; however, in vivo, growth factors tend to be sequestered in the extracellular matrix, allowing local presentation to cells.

A variety of natural and synthetic materials have been examined as potential cell carriers or as therapeutic agents for cartilage repair. Hydrogel scaffolds appear to be one promising class of materials, due to their high water content which mimics native tissue microenvironments. Furthermore, poly-(ethylene glycol) (PEG) hydrogels have been used to improve microfracture cartilage regeneration outcomes in human trials.

Hydrogel systems permit sequestration of growth factors via covalent tethering, which can provide advantages compared to other forms of protein delivery. In particular, growth factors are typically cross-reactive with multiple cell types and can have short serum half-lives in vivo, limitations that often necessitate localized presentation. Since diffusion of lower molecular weight proteins in hydrogels can be quite rapid, some researchers have used microparticles for controlled release presentation of growth factors to encapsulated chondrocytes. While this approach is quite useful, the process can increase the complexity of scaffold preparation and design. Variability can result from differences in protein loading, release kinetics, as well as the size distribution of loaded microparticles. Therefore, strategies to immobilize growth factors in a bioactive, physiologically relevant context are a complementary and important step towards directing cells to regenerate cartilage tissue.

As one robust method to create protein functionalized materials, we used thiol-ene chemistry to incorporate thiolated proteins in PEG hydrogels. Previously, PEG systems have been broadly explored for cell delivery applications. Specifically, we formed PEG hydrogels through a photoinitiated step-growth polymerization, by reacting norbornene-terminated PEG macromolecules with a dithiol PEG crosslinker. This photopolymerizable system allows for precise spatial and temporal control over polymer formation, as well as facile encapsulation of cells and biologics. The resulting crosslinked PEG hydrogel has been employed to encapsulate numerous primary cells with high survival rates following photoencapsulation.

Previously, our group has successfully incorporated thiolated TGF-β1 in a chain-growth polymerized PEG diacrylate system and showed enhanced chondrogenesis of human...
mesenchymal stem cells (MSC). Here, we encapsulated chondrocytes in step-growth polymerize PEG thiol-ene hydrogels, and we hypothesized that local presentation of TGF-β1 would influence chondrocyte secretory properties and improve the system’s application for cartilage regeneration. Step-growth polymerization leads to more ideal network structures than chain-growth polymerization, and the thiol-ene chemistry has also been shown to be more compatible for coupling proteins and maintaining their activity. In contrast to other cell types, primary chondrocytes are a versatile cell source since they deposit a matrix more similar to articular cartilage. For example, MSC derived fibrocartilage is biomechanically inferior. Additionally, a recent comparison study revealed that encapsulating chondrocytes in a PEG thiol-ene system yielded more hyaline-like cartilage than cells encapsulated in a PEG diacrylate system.

In this work TGF-β1 was thiolated and incorporated into a PEG thiol-ene hydrogel. We selected TGF-β1 because it has been shown to increase chondrocyte proliferation and cartilage ECM production in both 3D and 2D studies. We confirmed the presence of tethered TGF-β1 in the gel by ELISA and investigated its bioactivity using a PE-25 cell reporter assay for SMAD2 signaling. We also found that tethering growth factors to a scaffold results in increased cell proliferation and ECM production in vitro. These results suggest that a step-growth PEG hydrogel system is capable of tunable control of local bioactive signals. Chondrocytes encapsulated in this system are presented with a local and sustained delivery of TGF-β1, resulting in enhanced cartilage tissue regeneration.

2. Materials and Methods

PEG monomer synthesis

8-arm polyethylene glycol (PEG) amine norbornene $M_n$ 10,000 was synthesized as previously described. Briefly, 5-norbornene-2-carboxylic acid (predominantly endo isomer, Sigma Aldrich) was first converted to a dinorbornene anhydride using N,N'-dicyclohexylcarbodiimide (0.5 molar eq. to norbornene, Sigma Aldrich) in dichloromethane. The 8-arm PEG monomer (JenKem Technology USA) was then reacted overnight with the norbornene anhydride (5 molar eq. to PEG hydroxyls) in dichloromethane. Pyridine (5 molar eq. to PEG hydroxyls) and 4-dimethylamino pyridine (0.05 molar eq. to PEG hydroxyls) were also included. The reaction was conducted at room temperature under argon. End group functionalization was verified by $^1$H NMR to be >90%. $^1$H NMR (500 MHz, CDCl$_3$) δ 6.30-5.80 (m, 16H), 4.0-3.0 (m, 1010H), 2.5-1.2 (m, 100H). The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as described. The 3.5 kDa PEG dithiol linker was purchased from JenKem Technology.

Cell harvest and expansion

Primary chondrocytes were isolated from articular cartilage of the femoral-patellar groove of 6 month old Yorkshire swine as detailed previously. Cells were grown in a culture flask in media as previously described. Briefly, cells were grown in DMEM growth medium (phenol red, high glucose DMEM supplemented with ITS+Premix 1% v/v (BD Biosciences), 50 μg/mL L-ascorbic acid 2-phosphate, 40 μg/mL L-proline, 0.1 μM dexamethasone, 110 μg/mL pyruvate, and 1% penicillin-streptomycin-fungizone with the
addition of 10 ng/mL IGF-1 (Peprotech) to maintain cells in de-differentiated state. ITS promotes formation of hyaline cartilage over serum. Cultures were maintained at 5% CO
and 37 °C.

Mink lung epithelial PE-25 cells containing a stably transfected luciferase reporter gene for TGF-β1 were cultured in low glucose DMEM supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin-fungizone. Cells that were passaged three times were used in encapsulation experiments.

**PEG hydrogel polymerization and growth factor incorporation**

2-Iminothiolane (Pierce) was used to thiolate human TGF-β1 (Peprotech). Briefly, 2-Iminothiolane was reacted at a 4:1 molar ratio to TGFβ for 1 hour at RT. Thiolated TGFβ was pre-reacted at various concentrations with PEG norbornene monomer solution prior to cross-linking via photoinitiated polymerization with UV light (I_o ~3.5 mW/cm² at λ=365 nm) and 0.05 wt% LAP for 30 s. The monomer solution was then crosslinked with a 3.5 kDa PEG dithiol at a stoichiometric ratio of [40 mM dithiol]: [80 mM Norbornene] in a 10 wt% PEG solution using longwave ultraviolet light (I_o ~3.5 mW/cm² at λ=365 nm) for 30 s.

(Scheme 1)

**Quantifying growth factor incorporation**

10 wt% hydrogels were synthesized with tethered TGF-β1 at 0, 10, 50, or 90 nM and prepared for cryosectioning as previously described. Briefly, hydrogels were flash frozen in liquid nitrogen and placed in HistoPrep (Fisher Scientific) in cryomolds. 20 μm cross-sections along the plane of the construct were collected on SuperFrost® Plus Gold slides (Fisher Scientific). 40 μL disc-shaped gels (O.D. ~5 mm, thickness ~ 2 mm) without encapsulated cells and with varying concentrations of tethered growth factor were also prepared and sectioned. 20 μm sections were collected from the top, middle, and bottom of gel. To quantify the TGF-β concentration in each section, a modified ELISA was used as previously described. Briefly, sections were blocked for 1 hour at RT in 5% bovine serum albumin (BSA). Sections were washed 3x in ELISA buffer (0.01% BSA, & 0.05% Tween-20 in PBS) prior to incubation with a mouse anti-human TGF-β1 antibody (Peprotech) at 1:100 dilution overnight at 4 °C. Sections were washed again, then incubated with goat anti mouse–HRP (eBioscience) for 1 hour at RT and washed again. Sections were incubated with 100 μL of peroxidase and 3,3',5,5' tetramethylbenzidine substrate until color developed then the reaction was stopped using 100 μL 2 N sulfuric acid. The absorbance was measured at 450 nm using a Bio-Tek H1 spectrophotometer.

To calculate the theoretical loading of growth factor in each section, the volume was determined assuming the section was a thin disc with a 5 mm diameter and 20 μm height. Using \( V = \pi r^2 h \) and the molecular weight of TGF-β1 (\( M_n = 25,000 \) g/mol), the amount of growth factor per section was calculated in nanograms. For instance, a 50 nM 40 μL gel section is expected to have 0.5 ng of TGF-β1 per 20 μm section assuming ideal conditions.

Finally, a standard curve was made simultaneously by prepping 96 well high binding clear plates with known amounts of TGF-β1. The 0 nM value at 450 nm absorbance was subtracted out from all values in the curve.
TGF-β1 bioactivity and cellular signaling

PE-25 cells were encapsulated in 10 wt% gels functionalized with a 1 mM Cys-Arg-Gly-Asp-Ser (CRGDS) peptide to promote survival. Thiolated TGF-β1 was incorporated into the gel at 0, 12.5, 25, 50, or 100 nM. Additionally, cells encapsulated in PEG gels without tethered growth factor were exposed to soluble TGF-β1 at concentrations of 0, 0.2, 0.3, 1, or 2 nM. Cells were photo-encapsulated at a density of 40 million cells/mL, and cell-laden hydrogels were formed in syringe tips at a volume of 40 μL. Following encapsulation, hydrogels were placed into DMEM growth medium in 48 well plates and incubated overnight at 37 °C, 5% CO₂. Afterwards, hydrogels were incubated in Glo-Lysis buffer (Promega) for 10 min at 37 °C; the samples were centrifuged for 10 min (13,400 rpm, 4 °C), and the lysate was transferred to white 96 well plates (50 μL per well). 50 μL luciferase substrate (Promega) was added to the lysate for 5 min and luminescence was quantified between 300-700 nm.

Chondrocyte encapsulation in PEG thiol-ene hydrogels

Chondrocytes were encapsulated at 40 million cells/mL in 10 wt% monomer solution and thiolated TGF-β1 at concentrations of 0 or 50 nM. 40 μL cell-laden gels were immediately placed in 1 mL DMEM growth medium (without phenol red) in 48 well non-treated tissue culture plates. As a positive control, a subset group of gels without tethered growth factor was exposed to 0.3 nM (7.5 ng/mL) soluble TGF-β1. Media was changed every 3 days. Samples were collected at days 1, 14, and 28 for analysis of ECM production and chondrocyte proliferation. At day 1 and 28 cell viability was assessed using a LIVE/DEAD® membrane integrity assay and confocal microscopy.

Biochemical analysis of cell-hydrogel constructs

Cell-laden hydrogels were collected at specified time points, snap frozen in LN₂, and stored at -70 °C until analysis. Hydrogels were digested in enzyme buffer (125 μg/mL papain [Worthington Biochemical], and 10 mM cysteine) and homogenized using 5 mm steel beads in a TissueLyser (Qiagen). Homogenized samples were digested overnight at 60 °C.

DNA content was measured using a Picogreen assay (Invitrogen). Cell number was determined by assuming each cell produced 7.7 pg DNA per chondrocyte. Sulfated glycosaminoglycan (GAG) content was assessed using a dimethyl methylene blue assay as previously described with results presented in equivalents of chondroitin sulfate. Collagen content in the gels was measured using a hydroxyproline assay, where hydroxyproline is assumed to make up 10% of collagen. DNA content was normalized per gel while GAG and collagen content were normalized per cell.

Histological and immunohistochemical analysis

On day 28, constructs (n=2) were fixed in 10% formalin for 30 min at RT, then snap frozen and cryosectioned. Sections were stained for safranin-O or masson's trichrome on a Leica autostainer XL and imaged in bright field (40X objective) on a Nikon inverted microscope.

For immunostaining, sections were blocked with 10% goat serum, then analyzed by anti-collagen type II (1:50, US Biologicals) and anti-collagen type I (1:50). Sections were treated
with appropriate enzymes for 1 hour at 37 °C: hyaluronidase (2080 U) for collagen II, and pepsin A (4000 U) with Retrievagen A (BD Biosciences) treatment for collagen I to help expose the antigen. Sections were probed with AlexaFluor 568-conjugated secondary antibodies and counterstained with DAPI for cell nuclei. All samples were processed at the same time to minimize sample-to-sample variation. Images were collected on a Zeiss LSM710 scanning confocal microscope with a 20X objective using the same settings and post-processing for all images. The background gain was set to negative controls on blank sections that received the same treatment. Positive controls were performed on porcine hyaline cartilage for collagen type II and porcine meniscus for collagen type I (Supplemental Figure 1).

Statistical Analyses

Data are shown as mean ± standard deviation. Two way analysis of variance (ANOVA) with Bonferroni posttest for pairwise comparisons was used to evaluate the statistical significance of data. One way ANOVA was used to assess differences within specific conditions. p < 0.05 was considered to be statistically significant.

3. Results

Distribution of thiolated TGF-β1 in PEG hydrogels

We confirmed that TGF-β1 was homogenously distributed within the gel after the thiol-ene tethering process, using a modified section ELISA.14 The results presented in Figure 1 show TGF-β1 incorporation throughout the gel, and its relatively homogeneous distribution among gel regions. We further showed that experimentally measured values were similar to theoretically calculated levels (0.1 ng for 10 nM, 0.5 ng for 50 nM, and 0.9 ng for 90 nM).

Bioactivity and concentration of tethered TGF-β1 in 3D culture

We investigated the bioactivity of tethered TGF-β1 in 3D culture using a reporter cell line. Briefly, it was shown that tethered proteins typically maintain high levels of bioactivity when conjugated using thiol-ene reactions.20 We further determined concentrations of soluble and tethered TGF-β1 that yielded a maximal response in PE-25 cells at a seeding density of 40 million cells/mL. In Figure 2 a, there was a significant difference in luciferase output of 50 nM gels compared to other conditions. In Figure 2 b, 0.3 nM via soluble delivery elicited a maximal cellular response. Interestingly, when we dosed 50 nM of soluble TGF β-1 to encapsulated PE-25s at 40 million cells/mL, the average luciferase response was ~ 6,510 arbitrary units (n=4), which is a 3-fold lower response than for the same concentration of tethered TGF-β1. Based on these results, we elected to dose soluble TGF-β1 at the magnitude of 0.3 nM. Overall, these results suggest that tethered TGF-β1 is bioactive, and at 40 million cells/mL, the conditions that elicited the highest response to TGF-β1 were 0.3 nM (soluble) and 50 nM (tethered).

Proliferation of chondrocytes exposed to TGF-β1

Cell viability for all encapsulation and culture conditions was between 80%-90% assessed by live/dead membrane integrity assay at both days 1 and 28. Figure 3 a shows the rounded shape of encapsulated cells; there was significant increase in number of cells in the 50 nM
TGF-β1 tethered gels. To further quantify this proliferation, we harvested samples at day 1, 14, and 28 and assayed for DNA content (Figure 3 b). There was a statistically significant increase in DNA content, at day 28, for cells encapsulated in 50 nM TGF-β1 containing gels. Further, there was significantly more DNA in the day 28 50 nM condition than either the 0.3 nM or 0 nM gel condition (p<0.001). Combined with the viability results, these data suggest an increase in chondrocyte proliferation in response to tethered growth factor presentation.

Matrix deposition as a function of TGF-β1 presentation and culture time

We assessed glycosaminoglycan (GAG) and total collagen content of gels at day 1, 14, and 28. Encapsulated chondrocytes were either exposed to 0 nM, 0.3 nM solubly or 50 nM tethered TGF-β1. Measured quantities were normalized to cell content in the respective hydrogel formulations.

In Figure 4 a, GAG production per cell on day 28 for the tethered construct was significantly higher than non-treated groups (p<0.001). There was also a significant difference at day 28 between constructs that presented tethered TGF-β1 compared to solubly delivered TGF-β1 (p<0.05), suggesting that the tethered growth factor enhanced ECM production over soluble growth factor delivered in the media.

In Figure 4 b, total collagen production per cell was highest at day 28 from the construct with tethered TGF-β1. Further, there was a significant difference between the tethered and soluble TGF-β1 conditions (p<0.01) at day 28, and the tethered group was significantly increased from the 0 nM group (p<0.001), indicating that collagen content is highest in the tethered protein constructs.

Matrix organization

We examined the distribution and deposition of extracellular matrix molecules by histological and immunofluorescence techniques. Masson's trichrome staining (Figure 5 a,c,e) revealed collagen deposition increased in the pericellular space of encapsulated chondrocytes with both tethered and soluble TGF-β1 gels on day 28 compared to 0 nM gels. Overall, it appears that most of the pericellular collagen deposition occurs in the 50 nM gels at day 28. In a similar fashion, safranin-O (Figure 5 b,d,f) staining revealed that GAG deposition localized in the pericellular region with increased deposition per cell in the presence of TGF-β1. These results support the data that tethered TGF-β increases ECM secretion.

Immunofluorescence staining revealed that by day 28, there was a scarce amount of collagen I throughout all samples (Figure 6 a,c,e) and that collagen II was prevalent in the growth factor treated samples (Figure 6 d,f) compared to the 0 nM sample (Figure 6 b). A high collagen II and low collagen I signal is indicative of articular cartilage, and the constructs maintained that phenotype over 28 days of culture.33

4. Discussion

Engineering a clinically viable scaffold for chondrocyte delivery and promotion of cartilage regeneration is challenging, partly because of the time required for chondrocytes to generate
a robust matrix. By encapsulating chondrocytes in a PEG thiol-ene system with localized presentation of a growth factor, we have shown quantitatively and qualitatively, in vitro, that cells survive, proliferate, and generate cartilage specific ECM molecules at a higher rate than without the growth factor. Tethering growth factors into a synthetic material scaffold integrates the promoting effects of a protein cross-linked gel without gel to gel variability. A cell delivery system with such properties can provide certain advantages for clinical applications in techniques such as matrix assisted autologous chondrocyte transplantation (MACT).

There are many advantages to tethering growth factors into a gel system for tissue engineering purposes. Localized presentation precludes growth factors from activating unnecessary cell targets in an in vivo setting. Additionally, it requires a lower amount of growth factor. In this 28 day study, TGF-β1 is dosed in 1 mL media every 3 days at 0.3 nM that results in ~ 70 ng of protein delivered to the cell-laden gel. For the same time period and experimental conditions, a 50 nM tethered gel corresponds to ~50 ng of TGF-β1/gel, yet led to higher matrix production and DNA content at day 28. When using an expensive and/or potent growth factor to promote tissue regeneration, a tethered system can potentially provide a more efficient and effective delivery system for long time periods appropriate for clinical settings.

In these studies, we chose to look specifically at chondrocytes encapsulated at 40 million cells/mL, since this cell density has been previously shown to be an optimal choice for in vivo studies with hydrogel delivery systems. We used a cellular assay, based on PE-25 cells as a reporter system with a luciferase output, to determine that an effective concentration of growth factor to deliver to cells was 50 nM (Figure 2 a) for tethered TGF-β1 and 0.3 nM for soluble TGF-β1 (Figure 2 b). We chose the initial concentrations of TGF-β1 for the PE-25 experiments based on previous work for promoting chondrogenesis of hMSCs. We hypothesized that encapsulated cells may not respond as well to higher concentrations of soluble TGF-β1 than tethered TGF-β1, because PE-25s may internalize the factor, and seeding at high density may reduce the cellular response. Related studies with Mv1Lu cells showed that they internalized TGF-β1, so it is reasonable to consider this explanation for the PE-25 experiments.

We speculate that for gels presenting 100 nM of tethered TGF-β1, the PE-25s encapsulated at 40 million cells/mL showed less activity compared to 50 nM gels (Figure 2 a) because growth factors can have pleiotropic effects that may lead to a negative feedback loop. Additionally, since TGF-β binds to a dimer receptor, which requires two receptor subtypes to join to initiate the signaling cascade, it is possible that the orientation of growth factors around the cell prevents complete binding since both subtype receptors may be occupied by separate ligands when only one is required for signaling activation.

We chose to use human TGF-β1 with porcine chondrocytes because the PE-25 system has already been established with human TGF-β1, and porcine chondrocytes will be used in future pre-clinical animal studies. We believe that this is unlikely to affect the outcomes of our studies, since mature TGF-β1 is known to be highly conserved (>99% amino acid sequence identity) throughout mammalian species.
The data presented in this study suggest that the PEG thiol-ene platform with tethered TGF-β represents a bioactive scaffold with potential tissue engineering applications for chondrocyte delivery. Chondrocytes maintained a spherical morphology, similar to native chondrocytes, in the gel over a 28 day period, as shown in Figure 3a, which suggests the cells are less likely to de-differentiate and generate hyaline-like cartilage.\textsuperscript{40} Chondrocytes also increased in cell number when cultured in PEG thiol-ene gels as shown in Figure 3b, and especially when TGF-β1 is presented, which is known to induce proliferation.\textsuperscript{24} Porcine chondrocyte doubling time in 2D culture is around 6.4 ± 0.3 days in serum-containing media.\textsuperscript{41} We speculate that part of the reason the cells did not double at a similar rate when encapsulated in the PEG gels is that the selected gel formulations are non-degradable. Thus, the polymer network limited the amount of space available for chondrocytes to grow, and the media did not contain serum. This result was confirmed by a study with rat chondrocytes grown in a non-degradable 3D scaffold which had a longer doubling time (10.04 ± 0.9 days) than cells grown in 2D (2.94 ± 0.3 days).\textsuperscript{42}

Extracellular matrix production data revealed that over 28 days, the tethered-protein gel stimulated chondrocytes to produce more GAGs and collagen, as quantified in Figure 4. The cells maintained a high rate of ECM production even though matrix proteins accumulate around the cell after 28 days. This phenomenon implies that TGF-β1 may maintain activity and interact with the chondrocytes, despite the increased pericellular matrix. Furthermore, when compared to a tethered TGFβ study investigating MSC chondrogenesis,\textsuperscript{20} chondrocytes maintained a similar level of GAG production and also express collagen type II on a similar time scale.

A study with juvenile and adult chondrocytes encapsulated in degradable gels had higher GAG and collagen outputs per cell over a 28 day period compared to the ones in this study.\textsuperscript{43} We expected that a degradable gel allows for greater ECM deposition as posited by various groups.\textsuperscript{44,45} Additionally, histology and immunofluorescence staining confirmed that matrix was primarily deposited pericellularly in all conditions, but at a higher level in gels with tethered TGF-β1. While the secreted matrix was primarily confined to the pericellular region, there were some areas where the ECM molecules, especially GAGs, were more dispersed between cells (Figure 5). These data suggest the need for tethering TGF-β1 to a degradable PEG thiol-ene system to enhance ECM production and elaboration, with the potential to better capture biochemical and biomechanical properties of native hyaline tissue.

**Conclusion**

We confirmed that thiol-ene reactions allow conjugation of TGF-β1 into PEG gels, while maintaining bioactivity and signaling to encapsulated cells. We showed that tethered TGF-β1 increased the proliferation rate and ECM production of chondrocytes over a 28 day period, at levels exceeding that of cells in gels where TGF-β1 was dosed in the culture medium or those that were untreated. The tethered TGF-β hydrogels utilized a lower total protein dosage while still promoting high levels of proliferation and matrix production of chondrocytes. Furthermore, chondrocytes maintained a spherical morphology in the thiol-ene PEG gels with high viability and a phenotype that resembles articular cartilage (i.e. high
collagen II and low collagen I levels). Collectively, these results demonstrate the feasibility of delivering bioactive protein signals in a 3D culture platform to enhance matrix production of chondrocytes. This platform may have further applications as a scaffold for in vivo cartilage regeneration.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

The authors would like to acknowledge Dr. Xuedong Liu for the PE-25 cells. We would also like to acknowledge Dr. William Wan, Dr. Huan Wang, Dr. Justine Roberts, and Stacey Skaalure for assistance on experimental design, as well as Dr. Malar Azagarsamy for help with NMR characterization of the macromolecules. This research was sponsored by the Howard Hughes Medical Institute and Department of Defense award number W81XWH-10-1-0791. The US Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office.

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Scheme 1. Pre polymerization scheme with thiolated TGF β1
Initially thiolated TGF-β1 is phototethered into the 8 arm 10 kDa PEG norbornene network, then the 3.5 kDa dithiol crosslinker is added in with chondrocytes to complete the encapsulation process. Growth factor is not drawn to scale. In featured experiments, there is a lower amount of growth factor attached to the monomer end. Chondrocytes seeded at 40 million cells/mL retain a rounded morphology similar to cells in native tissue. Scale bar represents 50 μm. 190x225mm (300 x 300 DPI)
Figure 1. TGF-β1 is homogenously distributed throughout the PEG hydrogel
Section ELISA of tethered gels without cells show detection of TGF-β at similar levels to theoretical values with graphic on top depicting slice areas. Each section ~ 20μm thickness. Theoretical values indicated by dashed lines (0.1 ng for 10 nM, 0.5 ng for 50 nM, and 0.9 ng for 90 nM gels). 0 nM value is subtracted out of all conditions. Results are presented as mean activity ± s.d. (n=2). Solid lines indicate p values with one way ANOVA analysis to confirm sections of each gel are not statistically different from each other.
Figure 2. Determining TGF β1 concentration that yields maximal response
(a) PE 25s were encapsulated at 40 million cells/mL with varying concentrations of tethered TGF β and 50 nM yielded a maximal response. * indicates statistically significant difference between 50 nM and the other concentrations with<0.001. Results are presented as mean activity ± s.d. (n=4). (b) PE 25 cells encapsulated at 40 million cells/mL were transiently exposed to varying concentrations of TGF β in the media. The 0.3 nM output is higher on average than the other concentrations. Results are presented as mean activity ± s.d. (n=4).
Figure 3. Increased proliferation of chondrocytes exposed to TGF β1
(a) Luve/Dead staining of 50nM gels seeded at 40 million cells/mL on day 1, and day 28 shows chondrocytes retain a spherical morphology, have high viability, and increase in number. Scale bars represent 50 μm. (b) DNA content of chondrocytes encapsulated at 40 million cells/mL that were exposed to 0 nM, 0.3 nM which was delivered through the media, or 50 nM which was tethered into the gel. Over a 28 day period, the cells in the 50 nM condition show a steady rate of increase of DNA content. + indicates significant difference between the 0.3 nM and 0 nM case (p<0.001), ++ indicates significant difference between 50 nM and 0 nM case (p<0.001), * indicates significant difference between 0.3 nM and 0 nM (p<0.001), ** indicates significant difference between 50 nM and 0.3 nM case at day 28 (p<0.001), and *** indicates significant difference between 50 nM and 0 nM for day 28 (p<0.001). Results are presented as mean± s.d. (n=3).
Figure 4. Enhanced matrix production of encapsulated chondrocytes exposed to TGF β
(a) GAG production was normalized per cell. * indicates significant difference between 50 nM and 0.3 nM condition at day 28 (p<0.05), ** indicates significant difference between 50 nM and 0 nM at day 28 (p<0.001). Data presented as mean ± s.d. (n=3). (b) Collagen production was normalized per cell. + indicates significant difference between 50 nM and 0.3 nM at day 28 (p<0.01) and ++ indicates significant difference between 50 nM and 0 nM at day 28 (p<0.001). Data presented as mean ± s.d. (n=3).
Figure 5. Matrix protein distribution in gels
At day 28, gels seeded with chondrocytes at 40 million cells/mL were sectioned and stained for matrix distribution. (a) 0 nM gel stained for collagen, (b) 0 nM gel stained for GAG, (c) 0.3 nM (soluble) gel stained for collagen, (d) 0.3 nM (soluble) gel stained for GAG, (e) 50 nM (tethered) gel stained for collagen, (f) 50 nM (tethered) gel stained for GAG. Blue indicates collagen and red indicates GAG. Scale bars represent 100 μm.
Figure 6. Collagen I vs. collagen II distribution in constructs
Gels seeded with chondrocytes at 40 million cells/mL were cryosectioned at day 28.
Immunohistochemistry analysis reveals collagen type distribution in scaffolds. (a) 0 nM
with collagen I, (b) 0 nM with collagen II, (c) 0.3 nM (soluble) with collagen I, (d) 0.3 nM
(soluble) with collagen II, (e) 50 nM (tethered) with collagen I, (f) 50 nM (tethered) with
collagen II. Sections were stained red for both anti-collagen I and anti-collagen II antibodies
and were counterstained with DAPI (blue) for cell nuclei. Scale bars represent 50 µm.