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**14. ABSTRACT**
The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint. A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen and, is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. Several photochemically crosslinkable gels have been developed and tested in vitro and in vivo in mice including collagen, fibrin, PEG thiol-ene, and gelatin methacrylamide. All have shown satisfactory cell viability and some cartilage formation. A modification to using dynamic culture as a bioreactor has resulted in the formation of aggregates of chondrocytes called dynamic Sel-Regenerating cartilage (dSRC) that can be encapsulated in the gels. This has resulted in superior cartilage formation and has been tested in two swine in a pilot study. Long-term swine studies are underway.

**15. SUBJECT TERMS**
cartilage, articular, collagen gel, poly(ethylene)glycol gel, photochemical crosslinking

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<table>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
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# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>4</td>
</tr>
<tr>
<td>3. Overall Project Summary</td>
<td>4</td>
</tr>
<tr>
<td>4. Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>5. Conclusion</td>
<td>9</td>
</tr>
<tr>
<td>7. Inventions, Patents and Licenses</td>
<td>11</td>
</tr>
<tr>
<td>8. Reportable Outcomes</td>
<td>11</td>
</tr>
<tr>
<td>9. Other Achievements</td>
<td>11</td>
</tr>
<tr>
<td>10. References</td>
<td>11</td>
</tr>
<tr>
<td>11. Supporting Data</td>
<td>13</td>
</tr>
<tr>
<td>12. Appendices</td>
<td>21</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Injuries to the cartilage surfaces of joint are particularly problematic because, unlike bone and other vascular tissues comprising the joint, cartilage is avascular and possesses limited capacity for repair and self-regeneration. Consequently, injury to cartilage in the articulating joints from trauma results in scar formation and possible arthritic changes that can lead to pain, stiffness, and loss of structure and function [1-3]. These joint injuries not only limit physical activity and mobility of those afflicted, but the inability to move freely can cause deep psychological scars and loss of independence when individuals have to depend on family and healthcare providers for constant assistance to perform daily life functions. The level of functional capability in the injured limb and ultimate quality of life depend on the successful outcome of joint surface regeneration performed as a secondary procedure weeks or even months after the initial injury. The return of function and the probability of return to active duty rely on successful restoration of the entire joint including the articular surface, and therefore, joint function. Lesions in the joint surface are commonly treated with microfracture [4], autologous cell implantation (ACI) [5], or osteoarticular autograft transfer system (OATS) [6]. To date, however, the outcomes of many restorative procedures are very unsatisfactory and an improved method for joint repair is a clear unmet need in military medicine. **The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint.** A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. **The scope of this research is to develop regenerative medicine approaches involving biocompatible hydrogel scaffolds seeded with autologous cells that provide three-dimensional environments favorable for promoting chondrogenesis for joint surface repair.** [7-14]

2. KEYWORDS

Cartilage; articular; collagen gel; poly(ethylene)glycol gel; photochemical crosslinking

3. OVERALL PROJECT SUMMARY

In the previous annual reports covering the first 48 months of this 70 month project, we reported on generating three candidate photochemically crosslinked hydrogels for encapsulating chondrocytes or chondrocyte precursor cells. The algorithm for making and testing the gels is presented in Figure 1. If the gels perform well in making neocartilage in mice, we would then proceed to swine studies. If not, our plan would be modified to re-examine cartilage production in vitro and in mice before proceeding. Photochemically crosslinked gels were made using collagen, gelatin methacrylamide, and poly(ethylene) glycol thiol-ene gels. The photochemically crosslinked collagen gels demonstrated increased resistance to collagenase digestion over uncrosslinked gels, but had little effect on changing the bulk modulus (stiffness) of the gels. This work was published in the Journal of Biomedical Materials Research Part A (Omobono et al., J Biomed Mater Res A. 2014 Jul 7. doi: 10.1002/jbm.a.35266. [Epub ahead of print]) and the publication was attached to last year’s report. Testing of the PEG thiol-ene gels showed changes in the shear moduli were related to the weight percent of the gels. In vitro studies showed good viability of swine articular chondrocytes following the polymerization process. Modifications were made to the gel to tether TGF-β1 to the molecular backbone to promote matrix formation by encapsulated chondrocytes. This work was published in the current year in the Journal of Biomedical Materials Research Part A (Sridhar et al., J Biomed Mater Res A. 2014 Dec;102(12):4464-72.
In year 4 we developed a new strategy to grow immature cell aggregates, originally referred to as chondrons, but now termed dymanic Self-Regenerating Cartilage (dSRC). These dSRC aggregates promoted contiguous new cartilage matrix to form. A complete study of these dSRC combined with photochemically crosslinked collagen was completed in year 5 and reported below. The dSRC/collagen gel formulation was pilot tested in two swine in year 4 with favorable outcomes showing new cartilage formation in the defects using cells in gels compared to empty defects and defects with gels alone. In year 5 we began an expanded year-long study to test the ability of dSRC combined with photochemically crosslinked collagen to repair chondral defects in swine.

This annual report covering the fifth 12 months of the project reviews our progress testing the physical and chemical properties of the photochemically crosslinked PEG thiol-ene hydrogels and their ability to promote chondrogenesis. A completed study on the capacity to produce cartilage by dSRC in our nude mice model is presented. The tasks referenced below pertinent to Year 5 under Task 1 include subtasks 1.1.b, 1.1.c, 1.2.a, and under Task 2 include subtask 2.1.a. A no-cost extension was granted to continue the swine studies using favorable gel combinations.

**Task 1 Test of photochemically crosslinked gels to produce cartilage and bone using chondrocytes and osteoblasts**

**Subtask 1.1.b Perform implantation of photochemically crosslinked collagen and PEG gels with chondrocytes and osteoblasts**

All of the in vitro testing has been completed and reported in published manuscripts (Appendices 1, 2,3). Copies of the published manuscripts are attached.

**Subtask 1.1.c Evaluate cartilage and bone matrix produced in vivo in mice.**

- Cells capped with photochemically crosslinked gels implanted in mice for matrix production in vivo.

Results: In the year 4 annual report we described results on generating dSRC. The first study aimed to determine the ideal growth time of dSRC in vitro prior to implantation into mice. A cell suspension of 10x10^6 swine chondrocytes were placed in a 15mL conical tube and cultured in a horizontal motion in vitro for 7 days, 14 days, and 21 days. After the desired growth time, each dSRC was placed in the center of a devitalized ring (to simulate an actual knee cartilage defect), gel was placed on top, and the
construct was immediately implanted into nude mice. New cartilage matrix was formed in all culture
time points after 3 weeks in vivo. The 14 day culture time appeared hypercellular compared to 21 days
and showed more consistent matrix formation than 7 days so it was selected as the ideal growth time for
the dSRC in vitro. Another pilot study that year compared three different gel types – fibrin, collagen
crosslinked (collagen+hv), collagen non crosslinked (collagen-hv) – to determine if the gel type
increased the cartilage matrix production. The same method was followed and dSRC were cultured for
10 days prior to implantation into mice. After 6 and 12 weeks in vivo, each type of gel showed matrix
production and there did not appear to be significant differences in cartilage formation.

In year 5, a full study was completed using a cartilage ring model in nude mice. The dSRC were
cultured for 14 days in vitro and the fibrin, photochemically crosslinked collagen, and non-crosslinked
collagen gels and implanted for 6 and 12 weeks in vivo. Isolated chondrocytes encapsulated directly into
fibrin gel were also done as controls. Figure 2 shows the gross images of the collagen crosslinked
constructs after 6 and 12 weeks in vivo. New cartilage formation formed in vivo in mice in all groups.
Qualitative analysis of the histology images showed that all formulations permitted new cartilage matrix
formation. The control group where isolated chondrocytes were directly encapsulated in the hydrogel
showed the least amount of new matrix formation and stained poorly with Safranin O indicating less
GAG being produced. The biochemical result of this control group was consistent with the histological
results showing the least amount of total GAG among the groups (Figure 3a). The cells in this group
were not capable of making contiguous cartilage matrix. By contrast, all groups made with dSRC
produced contiguous new matrix that stained intensely with Safranin O and had higher amounts of total
GAG (Figures 2 and 3a). The biochemical results for total collagen are shown in Figure 3b, but these
results are confounded by the presence of collagen in the original gel. Nonetheless, immunostaining for
type II collagen show high amounts of collagen II. Staining for collagen type I was confined to the outer
layers of the construct. Biomechanical testing of a cohort of the constructs was performed and showed
that the compressive moduli were not significantly different across groups (Figure 4).

Additional work on the dSRC study was performed by Dr. Lawrence Bonassar’s group at Cornell
University examining the interface of the engineered cartilage with the native cartilage using confocal
reflectance microscopy and particle image velocimetry. Confocal reflectance revealed clear images at
the interface, with much variability in matrix integration between samples. Integration appeared more
prevalent near the surface, but was poorly integrated at deeper depths. To assess the interfacial
mechanics, we used particle image velocimetry to track tissue deformations via confocal strain mapping
across the interface. Shear strain applied parallel to the articular surface direction, was found to induce
two modes of deformation—sliding and peeling. Motivated by the as-of-yet unsolved issue of cartilage
integration, this study detected and characterized the local interfacial mechanics in a cartilage defect
repair model. Two distinct modes of failure were identified from this work: sliding along the
longitudinal axis and peeling along the latitudinal axis at the interface. An abstract was presented at the
2015 annual meeting of the Orthopaedic Research Society in Las Vegas, NV in March 2015 (Appendix
4).

- dSRC capped with photochemically crosslinked GelMA and PEG

Results: A pilot study was performed using two alternative gels in combination with dSRC. dSRC
prepared as above were placed in photochemically crosslinked GelMA and PEG. The dSRC were
placed into cylindrical wells and either GelMA or PEG was added and photopolymerized. Six samples
of each type of gel were prepared and placed into nude mice for 6 weeks. Histology showed that the
dSRC were capable of producing new cartilage matrix over the period studied (Figure 5). However,
there remained gaps between the dSRC aggregates at this short time period when compared to the
collagen gel study above. Plans are underway to design mice studies to improve the cartilage formation
and test the biomechanical characteristics of the new cartilage matrix

- dSRC capped with photochemically crosslinked gels placed on beta tricalcium phosphate and
implanted in mice for matrix production in vivo.

Results: After numerous unsuccessful attempts to generate bone in the hydrogels, this aspect of the
project was tabled. Osteoblasts are less tolerant to hypoxia than chondrocytes and our in vitro data
showed that osteoblasts could not survive and generate bone in the gels. A new strategy was initiated
where the engineered cartilage could be combined with bone forming substrates to address the
subchondral bone. Vitoss (Stryker, Inc.) is a beta tricalcium phosphate bone substitute and is already in
clinical use. A pilot study was performed to evaluate the cartilage formation in the portion containing
chondrocytes and compatibility with the bone substitute material. The histological results demonstrate
that the dSRC is capable of producing new cartilage matrix as shown above. Furthermore, the cartilage
was capable of integrating with the Vitoss substrate (Figure 6). Vitoss is osteoinductive and constructs
will be tested further by placing these constructs into an osteochondral defect to evaluate new bone
formation.

Subtask 1.2.a Initiate 3-month pilot study in swine with photochemically crosslinked collagen and
PEG gels with bilayer of chondrocytes and osteoblasts

- Cells were encapsulated in photochemically crosslinked gels and implanted in swine to evaluate
the survival of the cells in vivo.

Results: A pilot study reported in year 4 demonstrated that dSRC and photochemically crosslinked
collagen permitted new cartilage matrix formation and filled joint surface defects in swine. These were
either left empty, filled with gel alone, or treated with collagen gel containing dSRC. Empty defects and
those with gel alone showed clefts in the cartilage surface at the time of harvest confirming that these
treatments resulted in lesions that do not heal. Those defects treated with the dSRC and crosslinked
collagen gel showed early cartilage formation in the defects, and the matrix stained positive for GAG
production as demonstrated with Safranin O staining (Figure 6). Immunohistochemistry for collagen
types I and II were preformed in year 5. These results show that the defects filled with dSRC and gels
were entirely filled with new cartilage matrix that was predominantly collagen type II and no type I
collagen (Figure 7). Defects filled with gel alone (without dSRC) or left empty were filled with
predominantly type I collagen.

Subtask 1.2.b Initiate 6-month pilot study in swine with photochemically crosslinked collagen and
PEG gels with bilayer of chondrocytes and osteoblasts

The pilot study described in subtask 1.2.a. provided promising preliminary data on the repair of chondral
defects in swine and formed the basis for expanding into long-term studies. Thus, a long-term study
using dSRC and photochemically crosslinked collagen has been initiated in swine. This study will focus
on the repair and regeneration of 5 mm chondral defects in the trochlear groove of the swine knee
(Figure 8). In these swine two defects will be treated with dSRC and gel. Control defects are treated
with gels without cells or left entirely empty.
Task 2  Stimulation of chondrogenesis by stem cells in photochemical gels

Subtask 2.1.a Perform initial study of collagen and PEG gels with stem cells implanted in mice

- Mesenchymal stem cells grown in varying culture conditions to assess matrix production in vitro

Mesenchymal stem cells were harvested from swine bone marrow and grown in standard high glucose DMEM growth media until P3 generation. Then BMSCs were cultured in two different manners – (1) as a cell suspension in motion and (2) as a pellet. Similar to the in vitro conditions of dSRC, a cell suspension of $10^6$ cells BMSCs in 5mL of media was placed in a 15mL conical tube and cultured with reciprocating motion at 32Hz for 14 and 21 days. Half of the samples were cultured in filter top tubes to allow gas exchange, while the other half were cultured with solid caps allowing no gas exchange. dSRC samples are cultured with no gas exchange. Within these two groups, chondrogenic differentiation media and standard BMSC media were tested. In both groups, samples cultured with BMSC media showed few cells present at the 2 time points. Although cells were present in the chondrogenic differentiation media samples, they did not form clusters and begin depositing matrix like the dSRC samples (Figure 9). To examine whether swine BMSCs can undergo chondrogenic differentiation, BMSCs were grown in the standard pellet method. It is well established that human BMSCs cultured as pellets at an initial concentration of $250 \times 10^3$ cells/pellet for 21 days will undergo chondrogenic differentiation when cultured in differentiation media. However, swine BMSCs under these same culture conditions did not undergo chondrogenic differentiation (Figure 10).

4. KEY RESEARCH ACCOMPLISHMENTS

- dSRC capped with photochemically crosslinked collagen gel implanted in vivo in mice showed new cartilage matrix
  - dSRC were combined with fibrin, collagen crosslinked (collagen+hv), collagen non crosslinked (collagen-hv) and implanted in vivo in mice
  - The results showed reliable formation of contiguous cartilage inside the native cartilage ring
  - The new cartilage was predominantly collagen type II with small amounts of collagen type I on the periphery.

- dSRC were encapsulated in photochemically crosslinked gels and implanted in swine to evaluate the survival of the cells in vivo in swine knee cartilage defects
  - The size of the defects was 2 mm in diameter
  - Cartilage formed and filled the defect in specimens treated with dSRC and gel; not in the empty defects or those treated with gel alone
  - Cartilage formed by the dSRC was high in type II collagen, whereas the control defects had high amounts of type I collagen

- Performed a pilot study where dSRC were encapsulated in photochemically crosslinked GelMA (gelatin methacrylamide) and PEG thiol-ene gels and implanted in mice for matrix production in vivo
  - The dSRC formed new cartilage matrix, but gaps were present between individual aggregates
dSRC were encapsulated in photochemically crosslinked collagen and combined with Vitoss bone substrate and placed in vivo in mice
  o The dSRC formed cartilage matrix on the surface of the Vitoss
  o The new cartilage matrix appeared to integrate with the Vitoss

MSCs were grown under dynamic culture to form cell aggregates in different conditions
  o Small aggregates of cells were formed under dynamic conditions and placed in cartilage rings with collagen gel
  o New tissue formed, but did not have the characteristics of cartilage matrix.

5. CONCLUSIONS

At the conclusion of the fifth year of this grant award, we made modifications to the formulations of photochemically crosslinked hydrogels and the process of generating chondrocyte aggregates that will be used to deliver cells to articular cartilage defects in the swine knee joint. As we described in our testing paradigm shown in Figure 1 of this report, gels or constructs that do not perform well in vitro or in vivo in mice will be reformulated and tested before moving into large animal swine studies. With the modifications described in this report, we initiated large animal testing in year 4 and are expanding this testing in year 5.

Although the photochemically crosslinked hydrogels performed well in vitro and made cartilage in vivo in mice, some failed to produce adequate extracellular matrix to be used in our swine knee defect model. The gels demonstrate good cell viability, but the matrix that formed often had gaps between the individual cells. We explored bioreactors to address this and developed a dynamic culture method that produced collections of cells—dSRC—that performed better in making contiguous cartilage matrix in a reliable manner. This approach was tested in vitro and in vivo in mice. These results demonstrated that this might be a good solution to problems we had generating good cartilage matrix in swine. Results from a pilot study in swine showed that cells encapsulated in collagen gels regenerated the articular cartilage in the swine. A no-cost extension has been requested to complete the long-term animals in subtasks 1.2.a, 1.2.b, and 1.2.c, respectively.

Building on our results from year 4 in which we developed the protocol to make dSRC, we have begun mice studies to test the use of the photopolymerizable PEG thiol-ene hydrogel and gelatin methacrylamide to deliver the dSRC to cartilage defects. The results are encouraging, but additional studies need to be performed in mice to improve the amount of new cartilage matrix formation.

The goal of task 2 was to isolate bone marrow derived mesenchymal stem cells from swine and encapsulate these cells in the photochemical gels. Studies in years 4 and 5 reported on encapsulating BM-MSCs in the hydrogels and testing these differentiated cells in gels in vivo in mice. The results of these studies have demonstrated that photochemically crosslinkable gelatin methacrylamide and PEG can support MSC survival. However, the mesenchymal stem cells in these gels do not reliably make cartilage matrix. Another approach involving dynamic assembly of the MSC, similar to making dSRC, was attempted, but the results are inconclusive. Nodules of cells form, but new cartilage matrix is not apparent. Studies are continuing to improve matrix formation in a reliable manner with the MSC and gels.

“So What” Section:
In the past 5 years of this project our team developed several formulations of photocrosslinkable hydrogels that could be used to regenerate cartilage in defects in the articular cartilage surface of joints with the goal of restoring normal joint function. These crosslinkable hydrogels serve as biomimetic polymers that provide a favorable environment for encapsulating chondrocytes (the native cell type found in cartilage) and chondrocyte precursor cells (mesenchymal stem cells). We have successfully formulated collagen and PEG gels, and now a new gelatin hydrogel. To promote improved cartilage formation from the cells we developed a new approach where the chondrocytes are placed in dynamic reciprocating motion for two weeks to form aggregates or clusters of cells termed dynamic Self-Regenerating Cartilage. These dSRC have successfully made contiguous cartilage in both the mice and swine models. All future swine studies will employ dSRC as the implant cell source. The use of the photochemically crosslinked collagen gels have demonstrated successful cartilage growth. This combination of dSRC and collagen is already being tested in long-term swine studies. If the alternative gels show promise, they too will be tested in swine studies.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Abstracts presented:


Published manuscripts:


Manuscript drafted for submission to Tissue Engineering:

7. INVENTIONS, PATENTS AND LICENSES:
None to report

8. REPORTABLE OUTCOMES FOR YEAR 4


9. OTHER ACHIEVEMENTS:

Balaji V. Sridhar, B.S. received his Ph.D. from the University of Colorado, Boulder, CO, on December 19, 2015 for his thesis entitled “USE OF BIOFUNCTIONAL HYDROGEL MATRICES FOR CHONDROCYTE TRANSPLANTATION APPLICATIONS.”

Xing Zhao, M.D. received his Ph. D. from the University of Utrecht, Netherlands on June 15, 2015 for his thesis entitled “BIOMATERIAL AND CELL BASED CARTILAGE REPAIR STRATEGIES.”

10. REFERENCES


11. SUPPORTING DATA

**Algorithm for Testing In Vivo**

Figure 1. This is the testing paradigm proposed in the original grant application. The gels are made and tested in vitro and in vivo in mice (left box) prior to embarking on the large animal swine model (right box). If gels fail to perform in the mice they are not tested in swine. Additionally, if the gels should perform poorly in the early test phase in swine, the gels are reformulated and tested again in mice before re-embarking on the swine studies. Many changes outlined in this report describe changes to the gels and generating dynamic self-regenerating cartilage so that large animals are not used unnecessarily for gels that have not been optimized.
Figure 2. Results from dSRC implants in mice. dSRC were made for 14 days in culture and placed into cartilage rings. The dSRC were covered with crosslinked collagen gel (Top row), noncrosslinked collagen gel (second row), and fibrin gel (third row). The control was isolated chondrocyte immediately placed into fibrin gel (fourth row). The results show that the isolated chondrocytes made the least amount of new cartilage matrix that was only pericellular. All three groups containing dSRC show abundant new cartilage matrix formation filling the cartilage ring defect and intense Safranin O staining. All four types of constructs produced type II collagen with small amounts of type I collagen on the periphery. The engineered cartilage from the dSRC groups is hypercellular when compared to native cartilage (bottom row). (scale bar = 1mm except it is 100µm for the second column of Safranin O images)
Figure 3. The amount of GAG and collagen was determined from the groups in figure 2. The isolated cells in fibrin gel had the least amount of GAG and were consistent with the Safranin O staining in figure 2. The amount of GAG in the dSRC groups is approximately 50% of that in native swine articular cartilage. The total collagen across groups was not significantly different. The collagen results may be confounded by the presence of residual collagen gel in the implants.

Figure 4. There was no significant difference among the groups for compressive modulus. The new cartilage is less than one third of native cartilage, but this may be a function of few number of samples tested, the short time studied, and the placement of the constructs in a non weight bearing position in the mice. Similarly, the hydraulic permeability was not different among the groups.
Figure 5. Encapsulation of dSRC in photochemically crosslinked GelMA (top row) and PEG (middle row). The dSRC were able to form nodules of new cartilage matrix. However, there are gaps between the cartilage nodules and residual polymer shown in pink stain.

Figure 6. dSRC were combined with photochemically crosslinked collagen gel and placed on Vitoss, a beta tricalcium phosphate bone substrate. These were placed in vivo in ice for 6 weeks. The results show formation of new cartilage matrix by the dSRC and close association with the Vitoss substrate. The cartilage follows the irregular surface of the Vitoss (right).
Figure 7. The results from the swine implants shows that the dSRC were able to fill the defect in the joint surface (top row). This group produced type II collagen as shown by immunohistochemistry, and only scat amounts of type I collagen. By contrast, the defects filled with only the collagen gel only contained type I collagen (middle row). There were mixed results on the empty defects (third row). The bottom row shows osteochondral plugs placed back into the defects to replicate mosaicplasty (OATS). (scale bar = 1mm)
Figure 8. Studies initiated in year 5 show the implantation of dSRC with photochemically crosslinked collagen gel into two chondral defects in the trochlear groove of the swine knee. Two implants with dSRC are shown on the left. Top right shows a defect filled with photochemically crosslinked collagen gel only. The bottom right is an empty defect control.
Figure 9. Bone marrow mesenchymal stem cells were placed into dynamic culture in the same fashion as the dSRC. The cell aggregates were then placed into cartilage rings and covered with collagen gel. Two types of chondrogenic media were used and two types of tubes (vented for gas exchange, and closed without gas exchange). The results demonstrate tissue formation, but bears little resemblance to new cartilage matrix observed when chondrocytes are used. (All H&E images are 100x.)
Figure 10. The results from the 21-day culture of bone marrow stem cells grown pellets in either differentiation media or control bone marrow media. The pellets in the differentiation media are larger, but do not suggest that cartilage matrix has formed. (H&E)
APPENDICES


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 multiplex tethered growth factors may prove broadly useful in the design of chondrocyte delivery vehicles for cartilage tissue engineering applications. © 2014 The Authors. Journal of Biomedical Materials Research Part A Published by Wiley Periodicals, Inc.: 102A: 4464–4472, 2014.

Key Words: cartilage tissue engineering, chondrocytes, protein conjugation, hydrogels, transforming growth factor-β1


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.1 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.2 Therefore, tissue engineering strategies involving chondrocytes and growth factor delivery may help to improve the treatment of articular cartilage lesions.3,4

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.5 Cytokines are attractive targets for tissue engineering since, at low concentrations, they can regulate cellular functions, such as proliferation and matrix production.6 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.5

A variety of natural and synthetic materials have been examined as potential cell carriers or as therapeutic agents for cartilage repair.7–9 Hydrogel scaffolds appear to be one promising class of materials, due to their high water content which mimics native tissue microenvironments.10 Furthermore, poly-(ethylene glycol) (PEG) hydrogels have been used to improve microfracture cartilage regeneration outcomes in human trials.11

Hydrogel systems permit sequestration of growth factors via covalent tethering, which can provide advantages...
compared with 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.12 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.13 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.14–17 Specifically, we formed PEG hydrogels through a photoinitiated step-growth polymerization, by reacting norbornene-terminated PEG macromolecules with a dithiol PEG crosslinker.18 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 photoinactivation.19,20

Previously, our group has successfully incorporated thiolated TGF-β1 in a chain-growth polymerized PEG diacrylate system and showed enhanced chondrogenesis of human meniscal stem cells (MSC).20 Here, we encapsulated chondrocytes in step-growth polymerized 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.21 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.22 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.23

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 three-dimensional13 and two-dimensional studies.24 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.25 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.

**MATERIALS AND METHODS**

**PEG monomer synthesis**

Polyethylene glycol (PEG) (8-arm) amine norbornene $M_n$ 10,000 was synthesized as previously described.25 Briefly, 5-norbornene-2-carboxylic acid (predominantly endo isomer, Sigma Aldrich) was first converted to a dinitroborne anhydride using $N,N'$-dicyclohexylcarbodimide (0.5 molar eq. to norbornene, Sigma Aldrich) in dichloromethane. The 8-arm PEG monomer (JenKem Technology) 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$_3$) $\delta$ 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.19 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.26 Cells were grown in a culture flask in media as previously described.27 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.28 Cultures were maintained at 5% CO$_2$ 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**

Human TGF-β1 (Peprotech) was thiolated using 2-iminothiolane (Pierce). Briefly, 2-iminothiolane was reacted at a 4:1 molar ratio to TGF-β for 1 h at RT. Thiolated TGF-β was prereacted at various concentrations with PEG norborne monomer solution before cross-linking via photoinitiated polymerization with UV light ($I_o \sim 3.5$ mW/cm$^2$ at $\lambda = 365$ 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 ($I_0 \sim 3.5 \text{ mW/cm}^2$ at $\lambda = 365 \text{ nm}$) for 30 s (Scheme 1).

Quantifying growth factor incorporation
Hydrogels (10 wt%) were synthesized with tethered TGF-$\beta$ 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 $\mu$m cross-sections along the plane of the construct were collected on SuperFrost® Plus Gold slides (Fisher Scientific).

Disc-shaped gels (40 $\mu$L) (O.D. ~5 mm, thickness ~ 2 mm) without encapsulated cells and with varying concentrations of tethered growth factor were also prepared and sectioned. Sections (20 $\mu$m) were collected from the top, middle, and bottom of gel. To quantify the TGF-$\beta$ concentration in each section, a modified ELISA was used as previously described. Briefly, sections were blocked for 1 h at RT in 5% bovine serum albumin (BSA). Sections were washed $3 \times$ in ELISA buffer (0.01% BSA, and 0.05% Tween-20 in PBS) before incubation with a mouse anti-human TGF-$\beta$1 antibody (Peprotech) at 1:100 dilution overnight at 4°C. Sections were washed again, then incubated with goat anti mouse–HRP (eBioscience) for 1 h at RT and washed again. Sections were incubated with 100 $\mu$L of peroxidase and 3,3′,5,5′-tetramethylbenzidine substrate until color developed then the reaction was stopped using 100 $\mu$L 2N sulfuric acid. The absorbance was measured at 450 nm using a BioTek 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 $\mu$m height. Using $V = \pi r^2 h$ and the molecular weight of TGF-$\beta$1 ($M_r = 25,000 \text{ g/mol}$), the amount of growth factor per section was calculated in nanograms. For instance, a 50 nM 40 $\mu$L gel section is expected to have 0.5 ng of TGF-$\beta$1 per 20 $\mu$m section assuming ideal conditions.

Finally, a standard curve was made simultaneously by prepping 96-well high binding clear plates with known amounts of TGF-$\beta$1. The 0 nM value at 450 nm absorbance was subtracted out from all values in the curve.

TGF-$\beta$1 bioactivity and cellular signaling
PE-25 cells were encapsulated in 10 wt% gels functionalized with a 1 mM Cys-Arg-Gly-Asp-Ser (CRGDS) peptide to promote survival. Thiolated TGF-$\beta$1 was incorporated into the gel at 0, 12.5, 25, 50, or 100 nM. Additionally, cells encapsulated in PEG gels without tethered growth factor were exposed to soluble TGF-$\beta$1 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 $\mu$L. Following encapsulation, hydrogels were placed into DMEM growth medium in 48-well plates and incubated overnight at 37°C, 5% CO$_2$. 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 $\mu$L per well). 50 $\mu$L luciferase substrate (Promega) was added to the lysate for 5 min and luminescence was quantified between 300 and 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-$\beta$1 at concentrations of 0 or 50 nM. 40 $\mu$L of cell-laden gels were immediately placed in 1 mL DMEM growth medium in 48 well plates and incubated overnight at 37°C, 5% CO$_2$. 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 $\mu$L per well). 50 $\mu$L luciferase substrate (Promega) was added to the lysate for 5 min and luminescence was quantified between 300 and 700 nm.

Biochemical analysis of cell-hydrogel constructs
Cell-laden hydrogels were collected at specified time points, snap frozen in LN$_2$, and stored at −70°C until analysis. Hydrogels were digested in enzyme buffer (125 $\mu$g/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.30 Sulfated glycosaminoglycan (GAG) content was assessed using a dimethyl methylene blue assay as previously described with results presented in equivalents of chondroitin sulfate.31 Collagen content in the gels was measured using a hydroxyproline assay, where hydroxyproline is assumed to make up 10% of collagen.32 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 (40× 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, Abcam). Sections were treated with appropriate enzymes for 1 h at 37°C: hyaluronidase (2080 U) for collagen II, and pepsin A (4000 U) with Retrieving 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 20× 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 (Supporting Information Fig. 1).

Statistical analyses

Data are shown as mean ± standard deviation. Two-way analysis of variance (ANOVA) with Bonferroni post-test 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.

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 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 ~20 μm 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 ± SD (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 p < 0.001. Results are presented as mean activity ± SD (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 ± SD (n = 4).
encapsulated PE-25s at 40 million cells/mL, the average bioactivity when conjugated using thiol-ene reactions.20

interestingly, when we dosed 50 nM of soluble TGF-β1 to encapsulated PE-25s at 40 million cells/mL, the average luciferase response was \(~6510\) arbitrary units \((n = 4)\), which is a threefold 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).

FIGURE 3. Increased proliferation of chondrocytes exposed to TGF-β1. (a) Live/dead staining of 50 nM 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± SD \((n = 3)\). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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 \((0.1 \text{ ng} \text{ for } 10 \text{ nM}, 0.5 \text{ ng} \text{ for } 50 \text{ nM}, \text{and } 0.9 \text{ ng} \text{ for } 90 \text{ nM})\).

Bioactivity and concentration of tethered TGF-β1 in three-dimensional culture
We investigated the bioactivity of tethered TGF-β1 in three-dimensional 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 with 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-β1 to encapsulated PE-25s at 40 million cells/mL, the average luciferase output of 50 nM gels compared with 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-β1 to encapsulated PE-25s at 40 million cells/mL, the average luciferase response was \(~6510\) arbitrary units \((n = 4)\), which is a threefold 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 [Fig. 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 with 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 [Fig. 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 with 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 [Fig. 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 [Fig. 6(a,c,e)] and that collagen II was prevalent in the growth factor treated samples [Fig. 6(d,f)] compared with the 0 nM sample [Fig. 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

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 (MACIT).

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.34–36 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 [Fig. 2(a)] for tethered TGF-β1 and 0.3 nM for soluble TGF-β1 [Fig. 2(b)]. We chose the initial concentrations of TGF-β1 for the PE-25 experiments based on previous work for promoting chondrogenesis of hMSCs.20 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.37

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 with 50 nM gels [Fig. 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.38

We chose to use human TGF-β1 with porcine chondrocytes because the PE-25 system has already been established with human TGF-β1,25 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.39

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 3(a), which suggests the cells are less likely to de-differentiate and generate hyaline-like cartilage.40 Chondrocytes also increased in cell number when cultured in PEG thiol-ene gels as shown in Figure 3(b), and especially when TGF-β1 is presented, which is known to induce proliferation.24 Porcine chondrocyte doubling time in two-dimensional culture is around $6.4 \pm 0.6$ days in serum-containing media.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 nondegradable three-dimensional scaffold which had a longer doubling time ($10.04 \pm 0.9$ days) than cells grown in 2D ($2.94 \pm 0.3$ days).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 with a tethered TGF-β study investigating MSC chondrogenesis,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 with the ones in this study.43 We expected that a degradable gel allows for greater ECM deposition as posited by various groups.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 (Fig. 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

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**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.
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.

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FIGURE 6. Collagen I versus 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.
Development of a Cellurally Degradable PEG Hydrogel to Promote Articular Cartilage Extracellular Matrix Deposition

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Healing articular cartilage remains a significant clinical challenge because of its limited self-healing capacity. While delivery of autologous chondrocytes to cartilage defects has received growing interest, combining cell-based therapies with scaffolds that capture aspects of native tissue and promote cell-mediated remodeling could improve outcomes. Currently, scaffold-based therapies with encapsulated chondrocytes permit matrix production; however, resorption of the scaffold does not match the rate of production by cells leading to generally low extracellular matrix outputs. Here, a poly (ethylene glycol) (PEG) norbornene hydrogel is functionalized with thiolated transforming growth factor (TGF-β1) and cross-linked by an MMP-degradable peptide. Chondrocytes are co-encapsulated with a smaller population of mesenchymal stem cells, with the goal of stimulating matrix production and increasing bulk mechanical properties of the scaffold. The co-encapsulated cells cleave the MMP-degradable target sequence more readily than either cell population alone. Relative to non-degradable gels, cell-degraded materials show significantly increased glycosaminoglycan and collagen deposition over just 14 d of culture, while maintaining high levels of viability and producing a more widely-distributed matrix. These results indicate the potential of an enzymatically degradable, peptide-functionalyzed PEG hydrogel to locally influence and promote cartilage matrix production over a short period. Scaffolds that permit cell-mediated remodeling may be useful in designing treatment options for cartilage tissue engineering applications.

1. Introduction

Articular cartilage has limited self-healing properties, in part due to its lack of innervation and vascularization, and cartilage repair remains a significant clinical challenge. Cartilage is composed primarily of specialized extracellular matrix (ECM) components that absorb water and maintain the structure of the tissue. Chondrocytes are the sole, differentiated resident cells found in mature articular cartilage and are responsible for the generation and maintenance of this ECM. As a result of its low cellularity and absence of stimulating growth factors provided by vasculature, cartilage exhibits a low rate of regeneration; hence, focal lesions caused by trauma or joint disorders can lead to debilitating osteoarthritis. Matrix-assisted autologous chondrocyte transplantation (MACT) involves encapsulating autologous chondrocytes into a tunable scaffold to promote increased matrix synthesis, which is then implanted into a cartilage defect of a patient. A variety of natural and synthetic materials have been examined as potential cell carriers and as therapeutic agents for cartilage repair.

Despite advances in MACT, a limitation with many of the scaffold carriers is that their resorption rates do not necessarily match the rate of matrix deposition by encapsulated cells (i.e., what is observed in healthy native tissue). In the case of hydrogel carriers, synthetic materials often limit deposition of chondrocyte-secreted matrix molecules to the space around the cell, also known as the pericellular space. In order to
overcome this issue, current synthetic hydrogels are engineered to hydrolytically degrade at physiologic pH, and while bulk degradation is readily engineered and controlled, numerous material properties are highly coupled to this degradation. For example, high extents of degradation must occur before collagen can assemble throughout hydrogel scaffolds, but this often coincides with a precipitous drop in gel mechanics.\textsuperscript{9,10} Alternatively, hydrogels derived from native matrix components (e.g., collagen, hyaluronan) can be degraded by cells, and this leads to a local degradation mechanism where the rate is dictated by the cells. However, it is often more challenging to control the degradation and mechanical properties of these materials, which can necessitate synthetic modification to these materials to control their time varying properties.\textsuperscript{11,12} As a result, recent efforts in the field have focused on hybrid synthetic ECM-mimics that can capture the tunability of synthetic scaffolds while integrating the properties of a cell-dictated local degradation.

In this work, we explored the application of a peptide- and protein-functionalized poly(ethylene glycol) (PEG) hydrogel for chondrocyte encapsulation and cartilage regeneration. PEG is a hydrophilic polymer that has been broadly explored for cell delivery applications.\textsuperscript{13–16} We formed biologically active PEG hydrogels through a photoinitiated step-growth polymerization scheme, by reacting four-arm norbornene-terminated PEG macromolecules with a non-degradable PEG dithiol linker or a bis-cysteine collagenase-sensitive peptide cross-linker, KCGPQG↓IWGQCK (where the arrow indicates cleavage site).\textsuperscript{37} This thiolene photopolymerization allows for precise spatial and temporal control over polymer formation, as well as facile encapsulation of cells and biologics.\textsuperscript{18} Multiple studies have shown the resulting cross-linked PEG hydrogel can encapsulate numerous primary cells with high survival rates (>90%) following photocapsulation.\textsuperscript{19,20}

Previous work in our group further demonstrated that chondrocyte ECM production is enhanced in the presence of locally tethered transforming growth factor (TGF-\(\beta\)) in a non-degradable PEG network; however, matrix deposition was limited to the pericellular space.\textsuperscript{21} These results motivated the experiments reported here, where we study how tethered TGF-\(\beta\) in concert with a celluлярly degradable peptide cross-linker influences cartilage ECM production and its distribution. Since degradation of collagen is a rate-limiting step in cartilage remodeling, as it is the most abundant component of the ECM,\textsuperscript{22} we selected a peptide linker derived from collagen, KCGPqG↓IWGQCK. Previously, Lutolf and Hubbell\textsuperscript{23} encapsulated chondrocytes in a PEG gel linked with this peptide and found increased gene expression of cartilage matrix molecules compared to non-degradable gels; however, matrix deposition was pericellularly restricted,\textsuperscript{24} suggesting that proper degradation did not occur to permit wide-spread ECM deposition. As chondrocytes release both MMP-8\textsuperscript{25} and MMP-13,\textsuperscript{26} which are known to cleave this sequence, they are not highly metabolically active.\textsuperscript{27} We hypothesize that when these primary cells differentiate from their stem cell origin, their low metabolic activity translates to very slow degradation of MMP-cleavable scaffolds.

To catalyze this pericellular degradation process, we examine the MMP activity of chondrocytes and explore the co-encapsulation of chondrocytes with mesenchymal stem cells (MSCs) to aid in scaffold remodeling. In complementary migration experiments, MSCs have been shown to readily degrade the KCGPQG↓IWGQCK sequence when encapsulated in similar PEG gels.\textsuperscript{28} Furthermore, Bahney et al.\textsuperscript{29} incorporated a collagen-derived peptide linker into PEG hydrogels to encourage chondrogenesis of MSCs. In addition to catalyzing degradation of the target peptide linker, MSCs co-encapsulated with chondrocytes can also stimulate matrix deposition and reduce hypertrophy of chondrocytes.\textsuperscript{30} Furthermore, in clinical settings, a low density of MSCs has the potential to migrate into a PEG MACT scaffold when combined with a procedure like microfracture surgery, which stimulates MSC migration.\textsuperscript{31}

In this work, we report the development of a MMP-sensitive PEG-based hydrogel that employs co-culture of MSCs and chondrocytes to suggest that local degradation facilitates diffuse ECM deposition. This multifunctional scaffold is further engineered to present TGF-\(\beta\) to encourage matrix deposition by both chondrocytes\textsuperscript{21} and MSCs.\textsuperscript{32} MSCs are seeded at a low density to facilitate degradation of the linker, while allowing us to design experiments focused on ECM secretion by co-encapsulated chondrocytes. Other common co-culture studies utilize much higher ratios of MSCs to chondrocytes.\textsuperscript{30,33} Additionally, we demonstrate in situ degradation by encapsulated cells utilizing a fluorogenic peptide, assess construct matrix deposition both qualitatively and quantitatively, and show increased scaffold mechanical integrity over 14 d.

2. Results

2.1. Chondrocyte Cleavage of the MMP-Degradable Sequence in 3D Monoculture

We confirmed in situ degradation of the peptide linker sequence (KCGPQG↓IWGQCK) utilizing a fluorogenic peptide sensor (Dab-GGPQG↓IWGQK-Fl-AhxC)\textsuperscript{34} that was covalently tethered to the gel network. Figure 1a shows a four-arm PEG-NB hydrogel formulation, which includes tethered TGF-\(\beta\) (50 \(\times\) 10\(^{-9}\) m), and fluorogenic peptide sensor (0.5 \(\times\) 10\(^{-3}\) m), for experiments used to determine the amount of cleavage of the MMP-sensitive sequence. We chose the chondrocyte seeding density of 40 million cells mL\(^{-1}\), because chondrocytes have been studied at this density and shown to produce native-like tissue at this concentration in 3D experiments.\textsuperscript{35–37} Over a 3 d period, we found that chondrocytes seeded at this density degrade the MMP-degradable sequence at a higher rate than either a chondrocyte-laden non-degradable or acellular gel of the same formulation as shown in Figure 1b. However, when chondrocytes were encapsulated and cultured long term in this formulation, glycosaminoglycan (GAG) (Figure 1c) and collagen (Figure 1d) distribution was limited to the pericellular space in degradable, cell-laden constructs, even after 28 d. Even at a shorter culture time of 7 d, the chondrocytes alone do not significantly degrade the surrounding network, which restricts matrix deposition to the pericellular spaces (Figure S2, Supporting Information).
2.2. Utilization of Co-culture to Aid in Degradation of the MMP-Sensitive Sequence

Since chondrocytes alone could not cleave this particular MMP-degradable sequence at a rate that permitted diffuse matrix production, we investigated the use of co-culture with MSCs, as we had previous experience with high levels of degradation of this sequence over shorter time scales.\[28,38\] Figure 2a shows a four-arm 20 kDa PEG norbornene network with tethered MMP fluorescent sensor (Dab-GGPQG↓IWGQK-FIAhxC), and TGF-β1. The macromer solution, containing tethered growth factor, is combined with chondrocytes at 40 million cells mL\(^{-1}\). Resultant networks are either cross-linked by an MMP-degradable peptide sequence (KCGPQ↓IWGQCK) or non-degradable (3.5 kDa PEG dithiol) linker for in situ cleavage experiments. b) Measurement of in situ cleavage of fluorescent sensor by chondrocytes. Over 3 d, acellular and chondrocyte-laden nondegradable gels had similar normalized fluorescent activity, but in a degradable gel, chondrocytes had higher fluorescent activity (where A.U. stands for arbitrary units) suggesting cleavage of the sequence. Results are presented as mean ± SD (n = 3). c) GAG staining of sections obtained at day 28 with chondrocytes seeded in degradable gels at 40 million cells mL\(^{-1}\) with nuclei stained black and GAGs stained red. d) Collagen staining of sections obtained at day 28 with chondrocytes seeded in degradable gels at 40 million cell mL\(^{-1}\) with nuclei stained black and collagen stained blue. Scale bars represent 100 µm.

The same hydrogel formulation over a 3 d period, we found that not only do MSCs seeded at a lower density than chondrocytes degrade the sequence at a faster rate, but there also seems to be a synergistic effect between MSCs and chondrocytes to degrade the sequence at a significantly higher rate. As shown in Figure 2b, MSCs seeded at 5 million cells mL\(^{-1}\) cleaved the target sequence faster than chondrocytes seeded at 40 million cells mL\(^{-1}\) with increasing relative fluorescent activity. Interestingly, when encapsulated in co-culture with a 24:1 chondrocyte:MSC ratio, with chondrocytes held constant at a density of 40 million cells mL\(^{-1}\), the cells increased the amount of cleavage of the target sequence compared to either cell type alone. At each time point, the co-culture (8:1) gel (40 million
chondrocytes mL\(^{-1}\) + 5 million MSCs mL\(^{-1}\) MMP activity value was significantly higher than a simple additive effect (from the single-cell cultures), suggesting there is indeed a synergistic effect of the co-culture on MMP activity.

In order to determine an appropriate seeding density of MSCs to use in co-culture with chondrocytes in the matrix deposition experiments, we varied the encapsulation ratio of chondrocytes to MSCs. In Figure 2c, we show that when chondrocytes are held constant at 40 million cells mL\(^{-1}\) and the concentration of MSCs is increased in the scaffold incrementally, there is a resultant increase in cleavage of the target sequence. There is a statistically significant difference between each of the co-culture groups in Figure 2c at each time point (p < 0.05) with the 8:1 ratio generating the highest amount of degradation out of the tested conditions and was used for subsequent matrix deposition experiments. The MMP activity of each group is significantly different from each other at each timepoint (p < 0.05) with the 8:1 condition generating the highest MMP activity. Results are presented as mean ± SD (n = 3).

2.3. Viability of Cells and Morphology of MSCs in Co-culture Scaffolds

Cell viability for both non-degradable and degradable co-culture gel conditions was assessed by a live/dead membrane integrity assay at both day 1 (Figure 3a) and 14 (Figure S3, Supporting Information). Nondegradable gels had a viability of 92 ± 2% at day 1 and 95 ± 4% at day 14. Degradable gels had a viability of 93 ± 3% at day 1 and 96 ± 2% at day 14 as determined by image quantification where results are presented as mean ± SD (n = 3). Since both conditions looked very similar, only the viability results of the degradable condition are shown in this article. In addition to assessing viability, we observed the morphology of MSCs present in the scaffold to see if they maintained a rounded shape to suggest a more chondrogenic phenotype\(^{[27]}\) as opposed to an osteogenic phenotype with a more fibroblastic appearance.\(^{[38]}\) As shown in Figure 3a,
viability of both chondrocytes and MSCs was high on day 1. Furthermore, MSCs, which have been labeled with Cell Tracker Violet prior to encapsulation (blue), retained a spherical morphology in spite of being in a degradable system with integrin-binding epitopes. Moreover, DNA content was assessed at day 1, 7, and 14 (Figure 3b). There was no statistically significant difference in cellularity between degradable and non-degradable conditions, as measured by the amount of DNA present, but there was a steady increase in DNA content from day 1 to 14 in both conditions.

2.4. Effect of Local Degradation on Cartilage-Specific Matrix Production and Distribution

We assessed GAG and total collagen content of gels at day 1, 7, and 14 and further examined the distribution of these molecules throughout the network by staining sections with safranin-O (GAG) and Masson’s trichrome (collagen). Measured quantities of either non-degradable or degradable co-culture scaffolds were normalized to the wet weight (wet weight values shown in Figure S4a, Supporting Information) of the respective hydrogel formulations. In Figure 4a and Figure 5a, at day 14, GAG and collagen distribution were restricted to the pericellular space in nondegradable gels. On the other hand, in Figure 4b and Figure 5b, at day 14, GAG and collagen were widely distributed throughout the gel and connected with other molecules generated by nearby cells. Not only is the visual difference in distribution striking, but it was further confirmed by quantitative analysis. In Figure 4c and Figure 5c, the sGAG and total collagen production as a percentage of the wet weight of the gel on day 7 and 14 for the degradable construct was significantly higher than the nondegradable gel (p < 0.01). While cartilage-specific ECM production increases in both conditions over 14 d, it does so at a significantly higher amount in a locally degradable system.

2.5. Effect of Cell-Mediated, Local Degradation on the Mechanical Properties of the Scaffold

To confirm that our degradable system produced functional, cartilage-specific matrix molecules and increased its mechanical properties over time while permitting ECM expansion, we assessed the bulk compressive modulus of cell-laden non-degradable and degradable gels at day 1, 7, and 14. In Figure 6a, at day 7, and 14, the value of the compressive modulus of the degradable construct was significantly higher than of the non-degradable gel (p < 0.001). Furthermore, there was a significant increase (p < 0.001) between day 1 and 14 of the compressive modulus in degradable scaffolds while the values between day 1 and 14 were not statistically different.
(p > 0.75) with non-degradable gels even though both conditions have similar values of compressive elastic modulus initially.

2.6. Quality of Composition of ECM in Co-culture Scaffolds

To verify that the ECM produced had an articular cartilage phenotype, we qualitatively assessed the qualitative ratio of type II collagen to type I collagen on gel immunostained sections. Images revealed that at day 14 there was a scarce amount of type I collagen throughout all samples (Figure 7a,c). In contrast, type II collagen was more diffusely distributed in the degradable construct (Figure 7d) than in the nondegradable sample, where it was pericellularly restricted and less prevalent (Figure 7b). Quantification of the amount of cells that stained positive for type I and type II collagen with image analysis revealed similar conclusions. As shown in Table 1, more cells stained positive for type II collagen in the degradable than the nondegradable sample, and the number of cells staining positive for type II collagen was dramatically higher than type I positive cells for both.

2.7. Effect of Inhibition of MMP Activity on Cartilage-Specific Matrix Production and Distribution

We sought to test the effect of inhibiting MMP secretion by encapsulated cells and observe if the resulting matrix production was similar to non-degradable gels. Figure S1a, Supporting Information shows how the addition of the MMP inhibitor to a co-culture system led to a fluorescent activity level similar to non-degradable gels. Live/Dead staining of gels at day 1 and 14 showed viability greater than 90% (data not shown). Histology staining at day 14 revealed that the co-culture degradable gels treated with the MMP inhibitor had a similar appearance to non-degradable gels with pericellularly limited matrix distribution (Figure S1b,c, Supporting Information). These data show how MMP secretion specifically plays a major role in matrix deposition and remodeling within this system, even more so than TGF-β1 or the co-culture synergistic effects.

3. Discussion

Engineering a clinically viable scaffold for promotion of cartilage regeneration is challenging, partly because of the time...
required to generate a robust matrix by encapsulated cells, especially chondrocytes in monoculture. By utilizing an enzymatically degradable PEG-peptide system with localized presentation of TGF-β1 and co-culture of chondrocytes with MSCs, we have shown quantitatively and qualitatively, in vitro, that encapsulated cells generate highly distributed and elaborate cartilage-specific ECM molecules at a higher rate than in a non-degradable scaffold. This system that responds to cell-mediated cues permits cells to secrete and distribute large matrix molecules that pervade throughout the scaffold and ultimately, should lead to mechanically robust constructs. Furthermore, since the construct utilized the synergistic effects of co-culture (to promote scaffold remodeling) along with the benefits of a tethered growth factor, it expedited ECM generation by encapsulated cells relative to other common cartilage tissue engineering scaffolds.[39,40]

When chondrocytes were encapsulated in PEG gels linked with an MMP-cleavable peptide at 40 million cells mL$^{-1}$, they produced cartilage tissue that was limited to the pericellular space (Figure 1b,c). This suggests that chondrocytes alone may not sufficiently degrade this particular peptide linker. Chondrocytes have relatively low metabolic activity since they reside in a hypoxic and hyperosmotic environment.[41] This may be part of the reason that the chondrocytes were not observed to secrete MMPs at an appreciable rate in 3D culture.

On the other hand, when encapsulated alone, even at a lower seeding density of 5 million cells mL$^{-1}$, MSCs degraded the sequence at a higher rate than chondrocytes at 40 million cells mL$^{-1}$. This is likely because MSCs are more metabolically active than chondrocytes, as MSCs remodel their environments more frequently during development. There appears to be a synergistic effect between encapsulated MSCs and chondrocytes to degrade the sequence as shown in Figure 2b,c. The enhancing effect may be from paracrine signaling between cells to boost each other’s activity.[42] This may be more reflective of the native, developing cartilaginous environment, where MSCs and chondrocytes co-exist before all the MSCs differentiate into chondrocytes.[43] Furthermore, MSCs may play a role in cell number in the system, as they are known to drive chondrocyte proliferation in co-culture.[40] Future experiments

Figure 5. Collagen distribution and production in non-degradable and degradable 8:1 co-culture constructs. a) Non-degradable gel section stained for collagen at day 14. b) Degradable gel stained for collagen at day 14 with nuclei stained black or violet and collagen stained blue. Scale bars represent 100 µm. c) Total collagen content expressed as a percentage of the respective construct wet weight assessed at day 1, 7, and 14. + indicates a statistically significant difference in collagen content at day 7 between degradable and non-degradable gels (p < 0.05), and ++ indicates a statistically significant difference in collagen content at day 14 between degradable and non-degradable gels (p < 0.001). Results are presented as mean ± SD (n = 3).
could delve deeper into the signaling effects as to why there is increased MMP activity in co-culture between MSCs and chondrocytes. Additionally, studies could look for alternate ways to permit cell-mediated local degradation, which include investigating other peptide-linker sequences that are more amenable to cleavage by chondrocyte-secreted enzymes.

In these experiments, a four-arm 20 kDa PEG backbone at 6 wt% was used for co-culture experiments, since this formulation was studied in the aforementioned MSC experiments to assist in degradation of the peptide linker. Other studies investigating the gel cross-link density on matrix production by encapsulated cells found that scaffolds with a lower cross-linking density, like our monomer formulation, best supported ECM deposition in hydrogels.

Extracellular matrix production data revealed that over just 14 d, the mediately degradable gels permitted greater and widely distributed matrix production than non-degradable gels as revealed in Figure 4–6. Furthermore, compressive modulus measurements confirmed that degradable constructs had superior mechanical properties relative to non-degradable gels as shown in Figure 6. There is a steadily increasing trend in modulus values over a short period of time, which suggests that the matrix macromolecules generated in the degradable construct assemble in an appropriate fashion to stiffen the mechanical properties of the scaffold. It is interesting to note that there are pockets of space around the cells in the degradable gel histology images, while only a few are present in non-degradable histology images. These pockets have a similar appearance to lacunae found in cartilage and could be due to pericellular degradation of the network. It is evident that cartilage ECM molecule distribution is widespread throughout the degradable scaffold, which likely led to the superior functional mechanical properties of the gel while the pericellularly restricted matrix in the non-degradable gels did not lead to an increased modulus.

The rate at which cartilage matrix molecules are produced and assembled in the locally degradable constructs is substantial. Compared to the bulk degradation mechanism of hydrolytically cleavable PEG-PLA gels that use chondrocytes at 75 million cells mL\(^{-1}\), our system produces greater than 2.5 fold increase in GAGs (% wet weight) after 2 weeks while also increasing modulus over time. Furthermore, when compared to a PEG/Chondroitin sulfate copolymer gel with encapsulated chondrocytes at 75 million cells mL\(^{-1}\), our system produces greater than sixfold increase in total collagen content (% wet weight) over 2 weeks. Since matrix production is relatively rapid in these constructs, long culture times may not be necessary like they are in conventional cartilage tissue engineering experiments.

There was a concern that encapsulated MSCs in the co-culture system could lead to fibrocartilage formation as they do in monoculture in scaffolds. However, past studies of co-culture scaffolds with higher amounts of MSCs have confirmed that the neotissue generated is not of fibrocartilagenous nature. Furthermore, after a day, MSCs maintain a spherical morphology in co-culture, which could indicate a chondrogenic phenotype as shown in Figure 3a. Additionally, collagen typing (high type II: type I collagen ratio by immunofluorescence) revealed an articular cartilage phenotype with type II collagen being diffusely distributed in degradable gels (Figure 7). Future studies could track the long-term fate of the MSCs in this co-culture system to ensure they maintain a chondrogenic phenotype and do not revert to generating fibrocartilage or bone.

In a potential clinical application as an MACT scaffold, this system could be advantageous due to the low amount of MSCs needed for co-culture. The subchondral bone under the cartilage defect could be stimulated by a technique like microfracture to recruit MSCs into the environment. The chondrocyte-laden PEG construct could then be implanted into the defect and the MSCs could migrate into the gel. Because only a small quantity of MSCs is required to initiate degradation of the target sequence, there is clinical potential with this technique. Furthermore, it is known that increased age of encapsulated chondrocytes can lead to increased MMP activity of the cells.

Future studies should focus on optimizing the chondrocyte: MSC seeding ratio and the monomer formulation to further tune the local degradation and enhance ECM production. Additional studies could confirm whether older chondrocytes might degrade this system without the aid of MSCs, and one could test the influence of various localized growth factors (e.g., TGF-β, insulin-like growth factor) on promoting the secretory properties and ECM deposition by aged cells. It would also be interesting to see how matrix production is affected as the length of culture time is extended, especially in an in vivo environment.

### Conclusion

A cell-mediated degradable hydrogel system based on peptide- and protein-functionalized PEG hydrogels was designed to allow local cell degradation in a manner that promotes wide-spread cartilage ECM production, which ultimately leads...
to constructs with improved mechanical properties over just 14 d. The approach exploited the synergistic effects of co-culture between MSCs and chondrocytes to facilitate degradation of a collagen-derived, MMP-degradable peptide sequence (KCGPQG↓IWGQCK) as well as to promote cartilage ECM production in the presence of tethered TGF-β1. Results confirmed that both encapsulated cell types maintained a high viability and a spherical morphology in the gels. Furthermore, the generated ECM resembles articular cartilage with respect to collagen typing by immunofluorescent staining (high type II collagen: type I collagen ratio). Local degradation seems to play a critical role in matrix elaboration with tissue engineering constructs, and non-degradable constructs of the same formulation had significantly less ECM production and lower moduli values over 14 d. This PEG hydrogel system may prove useful in applications as a scaffold for in vivo cartilage regeneration.

5. Experimental Section

**PEG Monomer Synthesis:** Four-arm PEG amine (Mn = 20 000) was modified with norbornene end groups as previously described. Briefly, 5-norbornene-2-carboxylic acid (predominantly endo isomer; Figure 7).

**Table 1.** Percentage of cells that stained positive for different types of collagen. There is a higher amount of cells that stained positive for type II collagen than type I collagen in both systems. In the degradable system, there is a significantly higher amount of cells that stained positive for type II collagen than there is in the non-degradable system (p < 0.01). Results are presented as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>Percentage of cells that stain positive for type I or type II collagen in gels at day 14</th>
<th>Non-degradable</th>
<th>Degradable</th>
</tr>
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<tbody>
<tr>
<td>Type I collagen</td>
<td>26 ± 6%</td>
<td>48 ± 6%</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>53 ± 7%</td>
<td>84 ± 4%</td>
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Sigma Aldrich) was first converted to a dinorbornene anhydride using \(N, N'-\text{dicyclohexylcarbodiimide} (0.5 \text{ molar eq. to norbornene; Sigma Aldrich)} \) in dichloromethane. Four-arm PEG amine (JenKem Technology) was then reacted overnight with the norbornene anhydride (5 molar eq. to PEG amine) in dichloromethane. Pyridine (5 molar eq. to PEG amine) and 4-dimethylamino pyridine (0.05 molar eq. to PEG amine) were also included. The reaction was conducted at room temperature under argon. End-group functionalization was verified by \( ^1\text{H} \) NMR (Varian 400 MHz) to be >90%. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as described. \(^{20}\)

Peptides were purchased from American Peptide Company, Inc., which included an MMP-degradable cross-linker (KCGPGQG↓IWGQCK) and a pendant adhesion peptide sequence derived from fibronectin (CRGD). The linear peptide was 3.5 kDa PEG diol 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. \(^{66}\) Cells were grown in a T-75 culture flask with media as previously described. \(^{46}\) Briefly, chondrocytes were grown in growth medium (high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with ITS+ Premix 1%, v/v (BD Biosciences), 50 mg mL\(^{-1}\) L-ascorbic acid 2-phosphate, 40 µg mL\(^{-1}\) L-glutamine, 100 µg mL\(^{-1}\) penicillin, and 1% penicillin-streptomycin-fungizone) with the addition of 10 ng mL\(^{-1}\) IGF-1 (Peprotech) to maintain cells in a de-differentiated state. ITS was used because it promotes formation of articular cartilage over serum. \(^{49}\)

 Cultures were maintained at 5% CO\(_2\) and 37 °C. Human mesenchymal stem cells (hMSCs) were isolated from bone marrow aspirates (Lonza) as previously described. \(^{23}\) Cells were grown in low-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-fungizone, and 1 ng mL\(^{-1}\) recombinant human fibroblast growth factor (2, Peprotech). MSCs that were passaged two times were used for encapsulation experiments.

**Hydrogel Formulation and Cell Encapsulation:** Human TGF-β1 (Peprotech) was thiolated using 2-iminothiolane (Pierce) as previously described. \(^{42}\) Briefly 2-iminothiolane was reacted at a 4:1 molar ratio of TGF-β1 for 1 h at RT. Thiolated TGF-β1 was pre-reacted with a PEG norborne monomer solution prior to cross-linking in the hydrogel formulations at a pre-determined concentration of \(50 \times 10^{-9} \text{ M} \). This concentration was selected based on previous work, demonstrating a maximal response from chondrocytes seeded at 40 million cells mL\(^{-1}\). \(^{21}\) Additionally, 1 × 10\(^{-3} \text{ M} \) CRGDs was added to promote survival of the encapsulated MSCs. \(^{30}\) RGD was not added to the chondrocyte-only system as it has previously been shown to have no impact on chondrocyte metabolic activity. \(^{51}\) Both growth factors were coupled to PEG norborne via photoinitiated thiolene polymerization with 1.7 × 10\(^{-3} \text{ M} \) LAP and light (\(I_0 = 3.5 \text{ mW cm}^{-2} \) at \(\lambda = 365 \text{ nm}\), ThrorLabs M356SL2-C2) for 30 s. Subsequently, the monomer solution was cross-linked using a degradable MMP linker (KCGPQG↓IWGQCK, MW = 1800 kDa) or 3.5 kDa PEG diol at a 1:1 (thiolene) stoichiometric ratio of (12 × 10\(^{-3} \text{ M} \) thiol in either bis-cysteine peptide, or dithio)l: (12 × 10\(^{-3} \text{ M} \) norborne) in a 6 wt% PEG solution using additional light (\(I_0 = 3.5 \text{ mW cm}^{-2} \) at \(\lambda = 365 \text{ nm}\), ThrorLabs M356SL2-C2) for 30 s. For all experiments, 40 µL cylindrical gels (O.D. = 5 mm, height = 2 mm) were formed in the cut end of a 1 mL syringe. Since the degradable MMP linker is synthesized in an acidic solution, the pH of the final solution was adjusted to 7.5 so as not to interfere with the bioactivity of the tethered TGF-β1 or the viability of encapsulated cells. Unless otherwise specified, chondrocytes were co-encapsulated at 40 million cells mL\(^{-1}\) along with MSCs at 5 million cells mL\(^{-1}\) at an 8:1 chondrocyte:MSC ratio and 5% BSA, then analyzed by anti-collagen type II (1:50, US Biologicals) and anti-collagen type I (1:50, Abcam). Sections were pretreated with appropriate enzymes for 1 h 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 555-conjugated secondary antibodies and counterstained with DAPI to reveal cell nuclei. All samples were processed at the

**In Situ Confirmation of MMP Cleavage in a 3D Microenvironment:** To determine whether the specific variant of the collagen-derived MMP degradable linker sequence used in the experiments (KCGPQG↓IWGQCK) was being cleaved by encapsulated cells, a fluorescently labeled peptide sensor of the same sequence was tethered in the gel. Leight et al. developed the fluorescent peptide substrate Dab-GCGQG↓IWGQCK-FIAhxC using solid-phase peptide synthesis (Tribute Peptide Synthesizer, Protein Technologies, Inc.) as previously described. \(^{38}\) When the MMP-sensitive sequence is cleaved, it separates the quencher dabyll (Dab) from the fluorophore, fluorescin (Fl), permitting excitation. This fluorescent peptide was tethered into the gel at 0.5 × 10\(^{-10} \text{ M} \) along with TGF-β1 (50 × 10\(^{-10} \text{ M} \) and RGD (1 × 10\(^{-10} \text{ M} \) prior to cross-linking as depicted in Figure 2a. Various ratios of co-culture seeding densities were used to determine which formulation degraded the sequence at an appreciable rate. As a control, the fluorescence of an acellular gel was measured over 4 d. For chondrocytes in monoucleur, cells were encapsulated at 40 million cells mL\(^{-1}\), and for MSCs in monoucleur, cells were seeded at 5 million cells mL\(^{-1}\). For co-culture experiments, cells were seeded at 8:1, 16:1, and 24:1 (chondrocyte:MSC) where the chondrocyte cell seeding density was held constant at 40 million cells mL\(^{-1}\). For MMP inhibitor experiments, the inhibitor GM 6001 (Millipore) was added at a concentration of 100 × 10\(^{-6} \text{ M} \) to the media for cultures gels every 3 d, and viability was assessed at day 1 and 14. All values were normalized to the fluorescence value obtained immediately after gel formation (labeled day 0). Fluorescence measurements were conducted using a Synergy H1 microplate reader (BioTek) at 494 nm excitation/521 nm emission. An area scan was performed using a 48-well plate format with a 7 × 7 matrix, and the average fluorescent intensity was calculated for the entire matrix.

**Wet Weight, Compressive Modulus, and Biochemical Analysis:** On days 1, 7, and 14, hydrogels were removed from culture (n = 3), weighed directly on a Mettler Toledo scale to determine the wet weight, and assessed for compressive modulus. Cell-laden constructs were subjected to unconfinned compression to 15% strain at a strain rate of 0.5 mm min\(^{-1}\) to obtain stress–strain curves (MTS Synergy 100, 10 N using TestWorks 4 software). The modulus was estimated as the slope of the linear region of the stress–strain curves. Immediately afterwards, gels were snap frozen in LN\(_2\) and stored at −70 °C till biochemical analysis. Hydrogels were digested in 500 µL enzyme buffer (125 µg mL\(^{-1}\) papain (Worthington Biochemical) and 10 × 10\(^{-3} \) µM cysteine) and homogenized using 5 mm steel beads in a TissueLyser (Qiagen) that vibrates at 30 Hz for 10 min. Homogenized samples were digested overnight at 60 °C. Digested constructs were analyzed for biochemical content. DNA content was measured using a Picogreen assay (Life Technologies). Sulfated glycosaminoglycans (sGAG) content was assessed using a dimethylmethylen blue assay as previously described with results presented in equivalents of chondroitin sulfate. \(^{23}\) Collagen content in the gels was measured using a hydroxyproline assay where hydroxyproline is and cross-coupled to the respective gels. Additionally, digested acellular gels of either non-degradable or degradable formulations with tethered TGF-β1 and RGD were assessed by the colorimetric assays using the Synergy H1 microplate reader (BioTek), and the resulting values were subtracted from their respective cell-laden sample values. GAG and collagen content were expressed as a percentage of the wet weight of the respective gels.

**Histology and Immunofluorescent Analysis:** On day 14, constructs (n = 3) were fixed in 10% formalin for 30 min at RT, then snap frozen to the media-sectioned as previously described. \(^{34}\) Sections were stained for safranin-O and Masson’s trichrome on a Leica autostainer XL and imaged in brightfield (20X objective) on a Nikon (TE-2000) inverted microscope. For immunostaining, on day 14, sections were blocked with 5% BSA, then analyzed by anti-collagen type II (1:50, US Biologicals) and anti-collagen type I (1:50, Abcam). Sections were pretreated with appropriate enzymes for 1 h 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 555-conjugated secondary antibodies and counterstained with DAPI to reveal 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 (Figure S5, Supporting Information). The amount of cells that stained positive for each type of collagen was quantified by image analysis using Image J software.

Statistical Analysis: Data are shown as mean ± standard deviation. Two-way analysis of variance (ANOVA) with Bonferroni post-test for pairwise comparisons was used to evaluate the statistical significance of the data where the factors were culture time and hydrogel condition. One-way ANOVA was used to assess differences between conditions at specific time points for cases with two and three different groups. A value of p < 0.05 was considered to be statistically significant.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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A Biosynthetic Scaffold that Facilitates Chondrocyte-Mediated Degradation and Promotes Articular Cartilage Extracellular Matrix Deposition

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A Biosynthetic Scaffold that Facilitates Chondrocyte-Mediated Degradation and Promotes Articular Cartilage Extracellular Matrix Deposition

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Abstract

Articular cartilage remains a significant clinical challenge to repair because of its limited self-healing capacity. Interest has grown in the delivery of autologous chondrocytes to cartilage defects, and combining cell-based therapies with scaffolds that capture aspects of native tissue and allow cell-mediated remodeling could improve outcomes. Currently, scaffold-based therapies with encapsulated chondrocytes permit matrix production; however, resorption of the scaffold often does not match the rate of matrix production by chondrocytes, which can limit functional tissue regeneration. Here, we designed a hybrid biosynthetic system consisting of poly(ethylene glycol) (PEG) endcapped with thiols and crosslinked by norbornene-functionalized gelatin via a thiol-ene photopolymerization. The protein crosslinker was selected to facilitate chondrocyte-mediated scaffold remodeling and matrix deposition. Gelatin was functionalized with norbornene to varying degrees (∼4–17 norbornenes/gelatin), and the shear modulus of the resulting hydrogels was characterized (<0.1–0.5 kPa). Degradation of the crosslinked PEG-gelatin hydrogels by chondrocyte-secreted enzymes was confirmed by gel permeation chromatography. Finally, chondrocytes encapsulated in these biosynthetic scaffolds showed significantly increased glycosaminoglycan deposition over just 14 days of culture, while maintaining high levels of viability and producing a distributed matrix. These results indicate the potential of a hybrid PEG-gelatin hydrogel to permit chondrocyte-mediated remodeling and promote articular cartilage matrix production. Tunable scaffolds that can easily permit chondrocyte-mediated remodeling may be useful in designing treatment options for cartilage tissue engineering applications.

Lay Summary

Articular cartilage remains a significant clinical challenge to repair because of its limited self-healing capacity. In this manuscript, a biosynthetic scaffold crosslinked by both gelatin and poly(ethylene glycol) (PEG) was developed to encapsulate primary cartilage cells, also known as chondrocytes. This hybrid scaffold facilitated cartilage-specific extracellular matrix (ECM) molecule deposition by permitting cell-mediated, localized degradation of the construct so that encapsulated chondrocytes had pericellular space to generate tissue. Furthermore, the data show that the mechanical properties of this gel can be easily modified and this system can be formed in situ at a defect site via a photopolymerizable reaction. The results of this manuscript indicate the potential of this novel system in designing treatment options for cartilage tissue regeneration applications.
Keywords Cartilage tissue engineering · Chondrocytes · Gelatin · Local degradation · Hydrogel · Photopolymerization

Introduction

Articular cartilage has limited self-healing properties, which can necessitate clinical interventions to heal tissue defects. Chondrocytes, the sole, differentiated resident cells found in mature articular cartilage, are responsible for the generation and maintenance of tissue extracellular matrix (ECM) [1]. When combined with encapsulated chondrocytes, biofunctional scaffolds can facilitate cartilage ECM production and deposition. A variety of natural and synthetic materials have been examined as potential cell carriers and as therapeutic solutions for cartilage repair [2–5].

A limitation with many of the currently studied chondrocyte scaffold carriers is that their resorption rates do not match the rate of matrix deposition by encapsulated cells as found in native tissue [6]. Synthetic hydrogel carriers often limit deposition of chondrocyte-secreted matrix molecules to the space around the cell (i.e., the pericellular space) [7, 8]. To overcome this issue, hydrogels have been engineered to hydrolytically degrade at physiological pH, and while bulk degradation can be readily controlled, numerous material properties are highly coupled to this degradation. For example, high extents of degradation must occur before an ECM protein, like collagen, can assemble throughout hydrogel scaffolds, but this often coincides with a precipitous drop in gel mechanics [7, 9]. Alternatively, natural ECM proteins (e.g., collagen and hyaluronan) can form fibrillar hydrogel networks and provide numerous biological cues to guide tissue deposition by encapsulated cells [10]. These ECM proteins can also be easily degraded by encapsulated cells, which leads to a cell-mediated, local degradation mechanism [11]. However, natural protein-derived scaffolds are often mechanically weak, and it is difficult to control their reproducibility and degradation, which can necessitate synthetic modification to these materials to control their time-varying properties as well as facilitate the cell encapsulation process [12–14]. As a result, recent efforts in the field have included a focus on hybrid synthetic ECM mimics that have the potential to capture the tunability of synthetic scaffolds while integrating the properties of a cell-dictated degradation.

Previous work in our group demonstrated that cartilage cells encapsulated in poly(ethylene glycol) (PEG) hydrogels crosslinked by a collagen-derived peptide sequence (KCGPQG↓IWGQCK) generated constructs with increased, widespread articular cartilage-specific ECM compared to non-degradable gels [15]. These findings supported the hypothesis that local, cell-mediated degradation not only promotes cartilage tissue deposition but also maintains and in some cases increases scaffold mechanical integrity, in contrast to the decrease in bulk modulus typically found in hydrolytically cleavable scaffolds. However, it was found that enzymes secreted by encapsulated chondrocytes alone could not cleave the collagen-derived peptide linker with appropriate kinetics to enable a widespread distribution of matrix macromolecules. In fact, those constructs had the same pericellular matrix deposition pattern found in nondegradable scaffolds. Although previously, the Hubbell group engineered the collagen-derived peptide linker GPQG↓IWGQ (where ↓ denotes cleavage) to be more responsive to matrix metalloproteinases (MMPs) [16], chondrocytes were found to remodel synthetic scaffolds more appropriately in the presence of cartilage progenitor cells, mesenchymal stem cells (MSCs). Only when encapsulated in coculture with MSCs could the relatively metabolically inactive chondrocytes [17] readily degrade the sequence and then generate ECM throughout the scaffold. Furthermore, others have shown that cell-secreted MMPs are able to cleave a full-length protein at a greater rate than a peptide derived from that protein [18]. Collectively, these findings motivated the experiments reported herein, where we investigate whether a hybrid scaffold composed of PEG and a full-length protein, with a larger amount of collagenase-cleavable sequences per molecule than a peptide, would be responsive to chondrocyte-mediated local degradation and permit widespread matrix deposition.

Hybrid scaffolds that combine full-length proteins with synthetic linkers have been widely explored in tissue engineering applications. For example, fibrinogen [19–21] and collagen [22–24] have been chemically modified to allow covalent attachment to PEG by controlled reactions and thereby facilitate encapsulated cell development and proliferation. Collagen appears to be a good candidate material to use as a scaffold with chondrocytes since degradation of collagen is a rate-limiting step in cartilage remodeling [25]. However, collagen is resistant to most proteases and requires special collagenases for its enzyme hydrolysis. On the other hand, gelatin, a natural biomacromolecule derived from denatured collagen, is less antigenic, more cost-effective [26], and susceptible to more proteases than collagen [27]. These attributes potentially make gelatin an easier substrate to cleave by chondrocyte-secreted enzymes. Chondrocytes are also known to secrete gelatinases [28, 29], which can specifically cleave gelatin more efficiently than most proteases. Finally, gelatin has been successfully employed as a scaffold to promote articular cartilage-specific matrix production of encapsulated chondrocytes [30, 31], and hybrid PEG-gelatin networks have been developed for other tissue engineering applications with high cell viability under photopolymerization conditions [32–34].

In this work, we report the development of a gelatin network crosslinked with PEG for use with encapsulated chondrocytes and observe that the full-length protein is sensitive to local degradation cues and facilitates widespread cartilage-specific ECM deposition. Specifically, gelatin was
modified to contain pendant norbornene functionalities and reacted via a facile thiol-ene photopolymerization with PEG dithiols. The photoinitiated thiol-ene reaction is fast and highly efficient and permits precise spatial and temporal control of network formation [35].

Materials and Methods

Functionalization of Gelatin with Norbornene

Gelatin type A 300 bloom (1 wt% (w/v)) with $M_n \sim 75$ kDa [36] (Sigma-Aldrich), which contains around 21 primary amines per molecules, was dissolved in pH 8.5 sodium bicarbonate. 5-Norbornene-2-acetic acid succinimidyl ester (Sigma-Aldrich) was added to the gelatin solution (21 molar eq. to gelatin for 1:1 norbornene (NB):gelatin amine stoichiometric ratio, 10.5 molar eq. to gelatin for 0.5:1 NB:gelatin amine ratio, and 5.25 molar eq. to gelatin for 0.25:1 NB:gelatin amine ratio) and reacted with free amines on the gelatin molecule at 37 °C for 1 h. The functionalized gelatin was dialyzed against pH 8.5 sodium bicarbonate for 4 h at RT exchanging buffer every hour, using Slide-A-Lyzer™ G2 dialysis cassette MW 10 kDa (Thermo Scientific). After dialysis, solutions were frozen, lyophilized, and stored at -20 °C until use.

The degree of functionalization of gelatin with norbornene was quantified as previously described [32]. Briefly, the lysyl residue modification of gelatin was evaluated via the trinitrobenzenesulfonic acid assay (TNBSA, Thermo Scientific), which is a colorimetric assay that involves reacting the TNBSA reagent with primary amines on proteins for 2 h at 37 °C, stopping the reaction with 10 % SDS and 1 N HCl, and then reading the absorbance at $\lambda=335$ nm using a Synergy H1 microplate reader (BioTek). The functionalization efficiency was calculated using

$$\left(1 - \frac{\text{amines after gelatin modification}}{\text{amines before gelatin modification}}\right) \times 100 \%$$

Characterization of Degradation of the Functionalized Gelatin

To assess enzymatic cleavage of the norbornene-functionalized gelatin, 0.2 wt% gelatin solutions with varying extents of functionalization (1:1, 0.5:1, and 0.25:1 NB:gelatin amine molar ratios or unmodified gelatin) were dissolved in 0.1 M sodium nitrate (Sigma-Aldrich) and 0.1 M sodium dibasic phosphate (Sigma-Aldrich), so that the resulting solution could be assessed using aqueous mobile phase gel permeation chromatography (GPC) as previously described [37]. The gelatin solutions were incubated with either 20 units/mL (~0.1 mg/mL) type II collagenase (Worthington Biochemical) or chondrocyte-conditioned media from cells cultured for 3 days. The enzyme solutions were reacted with the functionalized gelatin for 1 h at 37 °C. GPC was performed using a Waters HPLC pump and refractive index detector, Polymer Standard Service Suprema columns (3000 and 100 A), and a linear PEG standard. All samples for GPC were prepared at a concentration of 0.2 wt% and filtered through a 0.4 μm filter. A mobile phase of 0.1 M sodium nitrate and 0.1 M sodium dibasic phosphate, injection volume of 25 mL, and flow rate of 0.5 mL/min were used for all samples.

PEG-Gelatin Network Formation and Mechanical Measurements

The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as previously described [38]. Gelatin (2 wt% (w/v)) functionalized with varying amounts of norbornene was prepared in PBS. We used 2 wt% solutions since this is the critical chain overlap concentration above which gelatin can form physical gels at room temperature [39]. The gelatin macromolecules were crosslinked by 0.1 wt% (0.3 mM) 3.5 kDa PEG dithiol (JenKem Technology) via a photoinitiated thiol-ene polymerization with 0.05 wt% (1.7 mM) LAP and light ($I_0=3.5$ mW/cm², $\lambda=365$ nm) for 30 s. For all experiments, 40 μL cylindrical gels (O.D. ~5 mm, height ~2 mm) were formed in the cut end of a 1 mL syringe (BD Medical).

The various gels were placed in cell culture media to swell overnight and weighed the next day. The volume swelling ratio $Q$ was calculated by first solving for the mass swelling ratio $q$, which involved dividing the measured swollen mass by the theoretical dry mass (data not shown) and assuming that the density of the polymer was similar to that of its solvent. Parallel plate rheometry was performed on crosslinked gels that were formed with varied norbornene functionalization on the gelatin using an Ares 4400 DHR-3 shear rheometer (TA instruments) with 10 % strain frequency sweep and a 10 rad/s strain sweep. The shear modulus ($G$) of the constructs was determined when the gels were in the linear viscoelastic regime for both the frequency and the strain sweep. The final network crosslinking density $\rho_{nl}$ was calculated from rubber elastic theory [40] where $\rho_{nl} = G Q^{1/3} (RT)^{-1}$.

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 [41]. Cells were grown in a T-75 culture flask with media as previously described [42] and were used directly after expansion, P0, for all cell-based experiments. Briefly, chondrocytes were grown in growth medium (high-glucose Dulbecco’s modified Eagle’s medium [DMEM]...
supplemented with ITS+ Premix 1 % v/v (BD Biosciences), 50 mg/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 insulin-like growth factor (IGF-1) (Peprotech) to maintain cells in a dedifferentiated state. ITS was used because it can promote formation of articular cartilage from chondrocytes [43]. Cultures were maintained at 5 % CO₂ and 37 °C.

**Chondrocyte Encapsulation and Viability Assessment**

Chondrocytes were encapsulated at 40 million cells/mL in 40 μL cylindrical PEG-gelatin gels using gels formed from the 1:1 NB:gelatin amine synthesis conditions (~4.5 mM norbornene per gelatin chain). This macromolecule formulation degraded readily in response to chondrocyte-secreted enzymes. Cell-laden gels were immediately placed in 1-mL DMEM growth medium in 48-well nontreated tissue culture plates. Media was changed every 3 days. At days 1, 7, and 14, cell viability and cellularity were assessed using a LIVE/DEAD® (Life Technologies) membrane integrity assay and confocal microscopy with ImageJ (NIH) for assessment of cell circularity.

**Biochemical Analysis of Cell-Hydrogel Constructs**

On days 1, 7, and 14, gels were removed from culture (n=3), weighed directly to determine the wet weight, snap frozen in LN₂, and stored at −70 °C prior to biochemical analysis. Hydrogels were digested in 500 μL 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.

Digested constructs were analyzed for biochemical content. DNA content, as an indicator of cell number, was measured using a PicoGreen assay (Life Technologies), and the results were expressed as the amount of DNA (μg) per chondrocyte-laden gel. Sulfated glycosaminoglycan (sGAG) content was assessed using a dimethylmethylene blue assay with results presented in equivalents of chondroitin sulfate [44]. As a cell-free control, digested acellular gels were assessed by the colorimetric assays, and the resulting values were subtracted from their respective cell-laden sample values. sGAG content was expressed as a percentage of the wet weight of the respective gels.

**Histology and Immunofluorescence Analysis**

On day 14, constructs (n=3) were fixed in 10 % formalin for 30 min at RT and then snap frozen and cryosectioned to 20 μm sections as previously described [45]. At day 14, sections were stained for Safranin-O and Masson’s trichrome on a Leica autostainer XL and imaged in bright field (20x objective) on a Nikon (TE-2000) inverted microscope.

For immunostaining, on day 14, sections were blocked with 5 % BSA and then analyzed by anti-type II collagen (1:50, US Biological) and anti-type I collagen (1:50, Abcam). Sections were pretreated with appropriate enzymes for 1 h at 37 °C: hyaluronidase (2080 U) for type II collagen and Pepsin A (4000 U) with Retrievagen A (BD Biosciences) treatment for type I collagen to help expose the antigen. Sections were probed with AlexaFluor 555-conjugated secondary antibodies (1:200, Life Technologies) and counterstained with DAPI to reveal 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 a z-stack maximum intensity projection of 4×5 μm slices of the 20 μm section using the same settings and postprocessing for all the images. The background gain was set to negative controls on acellular sections that received the same treatment. Positive controls were performed on porcine hyaline cartilage for type II collagen and porcine meniscus for type I collagen (Supplementary Fig. 2a, b).

**Statistical Analysis**

Data are shown as mean ± standard deviation. One-way ANOVA with a two-tailed Bonferroni’s multiple comparison test was used to assess differences in experimental outputs at different culture time points, since means of three samples are being compared simultaneously. α=0.05 was used for all analysis. Statistical calculations were performed on GraphPad Prism®. p<0.05 was considered to be statistically significant.

**Results**

**Modifying PEG-Gelatin Network Properties**

By varying the extent of norbornene functionalization of gelatin, we aimed to tune the crosslinking density of the final network and therefore the macroscopic properties of the resulting hydrogel. Figure 1a contains a schematic of gelatin functionalization with norbornene, along with the estimated amount of norbornenes attached to each gelatin molecule (based on calculations). Figure 1b depicts the photopolymerization between 2 wt% NB-functionalized gelatin with varying amounts of norbornene and 0.1 wt% PEG dithiol along with the concentrations of norbornene and thiol.

We characterized the efficiency of lysine modification on gelatin by the 5-norbornene-2-acetic acid succinimidyl ester reaction using the TNBSA assay, with data shown in Table 1 (n=5). The 1:1 norbornene:gelatin amine condition led to 75±...
6 % functionalization efficiency, which is similar to that observed with other studies using similar amine-modifying techniques [32, 33]. Lower stoichiometric ratios (0.5:1 and 0.25:1 norbornene/gelatin amine) led to corresponding decreases in the functionalization efficiency, 36±5 % and 19±5 %, respectively. The unmodified gelatin was measured to have ∼21 amine groups per molecule, which was also confirmed by other studies [32, 36], and suggests that the synthesized gelatin macromolecules have on average ∼17 (75 %), 8 (36 %), and 4 (19 %) norbornene pendant groups per molecule for crosslinking. The swollen shear modulus of the resulting acellular constructs was measured, and the crosslinking density of the scaffold was calculated. Consistent with the expected crosslinking reaction, both values increased with increasing amount of norbornene functionalization as shown in Table 1 (n=5).

**Table 1** Material properties of acellular hybrid scaffolds (n=5)

<table>
<thead>
<tr>
<th>Molar ratio of norbornene succinimidyl ester reacted with gelatin amines</th>
<th>Norbornene functionalization efficiency (%)</th>
<th>Calculated amount of norbornene on gelatin chain (mM)</th>
<th>Thiol concentration (mM)</th>
<th>Swollen shear modulus G (kPa)</th>
<th>Crosslinking density $\rho_{xL}$ (mM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25:1 Norbornene:gelatin amines</td>
<td>19±5</td>
<td>∼1.1</td>
<td>0.6</td>
<td>&lt;0.1</td>
<td>&lt;0.18</td>
</tr>
<tr>
<td>0.5:1 Norbornene:gelatin amines</td>
<td>36±5</td>
<td>∼2.2</td>
<td>0.6</td>
<td>0.2±0.1</td>
<td>0.36±0.04</td>
</tr>
<tr>
<td>1:1 Norbornene:gelatin amines</td>
<td>75±6</td>
<td>∼4.5</td>
<td>0.6</td>
<td>0.5±0.1</td>
<td>0.90±0.05</td>
</tr>
</tbody>
</table>

*a* Norbornene functionalization efficiency was calculated using $\left(1-\frac{[\text{amines}\text{ after modification}]}{[\text{amines}\text{ before modification}]\times100}\%$.

*b* Norbornene concentration per gelatin molecule was calculated by accounting for the amount of amines on type A gelatin (∼21) and the norbornene functionalization efficiency for each condition assuming that only norbornenes replaced the gelatin amines.

*c* Crosslinking density was calculated from the rubber elastic theory: $\rho_{xL} = \frac{GQ^{1/3}}{(RT)^{-1}}$.

**Verification of Degradation of Modified Gelatin by Chondrocyte-Secreted Enzymes**

Modification of proteins can alter their structure and change their susceptibility to enzymatic degradation since altered lysines may affect the way that an enzyme binds to its site to initiate cleavage. To verify that the modified gelatin could degrade in the response to chondrocyte-secreted enzymes, we performed a solution-based assay and tested the degradability of various modified gelatin macromolecules when exposed to either collagenase or chondrocyte-conditioned media. We chose chondrocyte-conditioned medium that was collected after 3 days of culture, since we have previously observed that the secreted enzymes can cleave a collagen-derived peptide sequence during that time frame [15]. After incubation of the modified gelatin with enzymes, GPC was
used to monitor the degradation of both unmodified and norbornene-substituted gelatin. The unmodified gelatin peak elutes at 18 min and is clearly distinguished from a large system peak at 20 min that occurs as a consequence of a small dissimilarity in composition, and thus a refractive index difference, between the injected buffer and the mobile phase. A substitution of 0.25:1 or 0.5:1 NB:gelatin amine resulted in no significant change in retention time compared to the unmodified gelatin, while the 1:1 NB:gelatin amine showed a distinguishable yet slightly shifted and significantly smaller peak. We hypothesize that this may be due to the formation of a slightly more hydrophobic macromolecule that may be more susceptible to interaction with the column stationary phase than the unmodified gelatin. When collagenase was added to each gelatin sample, the peak at 18 min completely disappears, indicating degradation of gelatin into smaller molecular weight fragments. A lower MW shoulder appears on the system peak, which corresponds to the gelatin degradation products. Finally, chondrocyte-conditioned medium was added to each gelatin sample to assess degradation in the presence of cell-secreted enzymes. Similar to treatment with collagenase, the gelatin peak was shifted to the low-molecular-weight shoulder, indicating that the norbornene-functionalized gelatin can be degraded by chondrocyte-secreted enzymes. Results are summarized in Supplementary Fig. 1, while Fig. 2 shows the effect of adding collagenase or chondrocyte-secreted enzymes to the most highly substituted gelatin (i.e., reacted at 1:1 NB:gelatin amines).

**Chondrocyte Viability, Cellularity, and Morphology**

Gelatin amines that were reacted at a 1:1 molar ratio with norbornene were used in all subsequent cell encapsulation experiments, since this formulation contained a higher amount of covalent crosslinks and thus could retain a gel structure for a longer period of time for the slow-growing chondrocytes. We chose a chondrocyte seeding density of 40 million cells/mL, as chondrocytes have been studied at this density for cartilage tissue engineering experiments using 3D scaffolds and shown to secrete an elaborate ECM at this concentration [46-48]. After chondrocytes were encapsulated, their viability, cellularity, and morphology were assessed at days 1, 7, and 14. In Fig. 3a, the chondrocytes retained spherical morphology at day 1 and begin to spread as observed on days 7 and 14, which suggests that the chondrocytes are able to locally remodel the environment. Image processing reveals that the circularity of the chondrocytes decreases from day 1 to day 7 and day 14 as shown in Fig. 3b, further corroborating the results seen in Fig. 3a.

Additionally, the cells retain high viability (calculated to be >95%) at all the time points and appear to proliferate over time, as there are many more cells in the constructs at day 14 than there are at day 1. This increase in cellularity was quantified by the PicoGreen analysis that measures DNA content and shows a significant increase in cell number between day 1 and day 14 (p=0.01) (Fig. 3c). Collectively, these results suggest that chondrocytes can thrive, proliferate, and remodel their environment in this scaffold system.

**ECM Production and Deposition in Scaffolds**

GAG content was assessed biochemically at days 1, 7, and 14, and the distribution of secreted matrix molecules was examined by staining sections with Safranin-O (GAG) and Masson’s trichrome (collagen). Measured quantities of the matrix molecules were normalized to the wet weight of each construct. As Fig. 4a shows, at day 14, GAGs (red) were extensively distributed throughout the gel. Staining of an acellular construct revealed low background staining (Fig. 4b), indicating that staining was primarily from macromolecules secreted by the resident chondrocytes. Furthermore, quantitative analysis, as shown in Fig. 4c, supports the conclusion that the amount of sGAG deposition increased significantly from day 1 to day 14 (p=0.0004). A similar distribution pattern to what was seen with GAGs is observed with collagen (dark blue) as seen in Fig. 5a, b. Figure 5c depicts the scaffold as optically opaque, with a cartilaginous appearance after 14 days of culture, much more than either the acellular (Fig. 5c) or day 1 time point (Fig. 5d), further suggesting widespread ECM distribution in the scaffold [49]. This versatile PEG-crosslinked gelatin system appears to provide a scaffold that is readily degraded by chondrocytes and can support matrix deposition over time.
Fig. 3 Viability, morphology, and cellularity of 1:1 NB:gelatin amine hybrid scaffold. 

- Violin (green)/dead (red) stains of encapsulated chondrocytes. Viability is calculated to be greater than 95% at all the time points. Insets highlight single cells changing morphology from rounded to more spread over time. Scale bars represent 50 μm.

- Degree of circularity between 0 (not rounded) and 1 (rounded) of the encapsulated cells. A number sign with a line indicates a statistically significant difference in circularity value between day 1 and day 7 (p=0.02). Results are presented as mean±SD (n=3).

DNA content (μg of DNA/gel) as measured by Picogreen assay over 14 days. An asterisk with a line indicates a statistically significant difference between day 1 and day 14 in DNA content (p=0.01). Results are presented as mean±SD (n=3).

Fig. 4 Glycosaminoglycan distribution and production in a 1:1 NB:gelatin amine hybrid scaffold. 

- Section with encapsulated chondrocytes at day 14 stained for GAGs with nuclei stained black and GAGs stained red. Scale bars represent 100 μm.

- Acoustic section stained for GAGs at day 14. Scale bars represent 100 μm.

- Total GAG content expressed as a percentage of the respective construct wet weight assessed at days 1, 7, and 14. A plus sign with a line indicates a statistically significant difference in GAG content between day 1 and day 14 (p=0.0004), and two plus signs with a line indicates a statistically significant difference in GAG content between day 7 and day 14 (p=0.001). Results are presented as mean±SD (n=3).
Quality of Collagen Generated by Chondrocytes in the Scaffold

To verify that the collagen generated by the chondrocytes had an articular cartilage phenotype, we qualitatively assessed the ratio of type II collagen to type I collagen on gel immunostained sections. Images revealed that at day 14, negligible type I collagen (red) was observed, but robust type II collagen (red) was observed throughout the scaffold as seen in Fig. 6.

Discussion

Engineering a clinically viable scaffold to promote cartilage regeneration is challenging. By using a novel, tunable hybrid biological-synthetic system, we have shown quantitatively and qualitatively, in vitro, that encapsulated chondrocytes generate highly distributed cartilage-specific ECM molecules. We showed that chondrocyte-secreted enzymes can degrade norbornene-functionalized gelatin and further demonstrated that cells are viable when embedded in the hydrogel formulation. The chondrocytes appear to spread throughout the scaffold, suggesting that encapsulated cells are locally remodeling their environment and facilitate the eventual deposition of matrix throughout the scaffold. Future investigations with this scaffold could prove useful in designing a cell carrier system to promote cartilage regeneration in vivo.

Modulus studies confirmed that these PEG-gelatin scaffolds could be tuned to adjust their crosslinking density and macroscopic properties that depend on this parameter (Table 1). In general, it can be more difficult to change material properties of pure protein-based gels, so covalent crosslinking can lead to higher mechanical properties [22]. If the crosslinking density can be adjusted by the user, it can also alter the amount of tissue deposition [9, 50]. Gel properties,
degradation, and ECM deposition might be further tuned by changing the network connectivity using different molecular weight PEG crosslinkers or multifunctional PEG crosslinkers depending on whether a more tightly or loosely crosslinked network is desired.

We confirmed that chondrocyte-secreted enzymes could degrade norbornene-functionalized gelatin by visualizing the degradation products with GPC (Fig. 2). GPC data suggest that norbornene-functionalized gelatin is degraded to smaller molecular weight products in the presence of chondrocyte-conditioned media. This confirms that norbornene-functionalized gelatin is a viable platform for the formation of chondrocyte-specific, cellulosically degradable hydrogels. We demonstrated these results in a solution-based assay, but a complementary study has shown that functionalized and crosslinked gelatin can be degraded by cell-secreted enzymes as well [51]. Here, we observe this directly by changes in the chondrocyte morphology (Fig. 3a); however, there are alternate ways to design systems that might respond to chondrocyte-secreted enzymes. One idea would be to include an aggrecanase-based cleavable peptide linker [52]. We selected the full-length protein gelatin for this study, as we hypothesized that its multiple MMP-cleavable sites would lead to more facile cleavage by chondrocytes than small peptide sequences as suggested by other groups [18]. In fact, the gelatin molecule contains about six MMP-sensitive sequences per molecule [27], which appears to be readily cleaved by chondrocyte-secreted enzymes at the cell density studied.

Viability results revealed that chondrocytes thrive and proliferate in the hybrid network. Cellularity in the gel quantitatively increased, which was confirmed by PicoGreen, and the cells spread over time (Fig. 3). Part of the reason for this change in cell morphology is likely because the chondrocytes are binding to adhesion factors present on the protein [26]. Another explanation is that the cells are degrading the network by a cell-mediated mechanism and spreading [51], which could facilitate the observed widespread matrix deposition. Future studies could focus on improving methods to verify that local degradation is occurring, which could include monitoring the cell-based degradation using microrheological techniques, such as microparticle tracking [53].

Cartilage-specific matrix produced by chondrocytes was distributed throughout the entire scaffold as it was in other cell-medicated degradable scaffolds [15] (Figs. 4a and 5a). Additionally, the GAG production from this scaffold was comparable to what was seen in a cellulosically degradable cartilage tissue engineering scaffold with a similar cell seeding density [15]; however, this system did not need to tether a growth factor, such as TGF-β, to the network in order to elicit a response from chondrocytes. A possible explanation for the robust secretory properties might relate to the fact that gelatin can bind to growth factors released by cells and perhaps present them to embedded chondrocytes in a local and sustained manner [1].

The encapsulated chondrocytes have a lower circularity at day 14, which can be suggestive of a hypertrophic phenotype that generate higher amounts of type I collagen and functionally inferior cartilage tissue [54]. Interestingly, the collagen produced by the encapsulated cells maintained a higher quality articular cartilage phenotype, as indicated by the collagen typing result with a high type II collagen:type I collagen ratio (Fig. 6).

In a potential clinical application as a scaffold, this system could be advantageous since it is easy to tune formation of this network. Currently, collagen-based materials are used as scaffolds in the clinic and have yielded variable success, partly due to high variations in network formation [55]. The hybrid system presented in this manuscript can be easily tuned and permits widespread elaboration of cartilage-specific ECM molecules over a short period of time. It would be interesting to see how matrix production is affected as the length of culture time is extended, especially in an in vivo environment where the presence of macrophages could help stimulate chondrocyte gelatinase secretion [28].

Conclusions

In summary, a novel hydrogel system based on crosslinking a full-length protein, gelatin, by PEG was designed to increase control over network formation and permit local chondrocyte-mediated degradation for cartilage tissue engineering applications. The hydrogel increases chondrocyte cellularity and facilitates cartilage ECM production via cell-mediated degradation in a manner that promotes widespread collagen deposition in just 14 days. The materials’ approach was to modify gelatin with norbornene functionalities that were crosslinked with PEG dithiols via a photoinduced thiol-ene reaction. Results confirmed that the final PEG-gelatin gel properties could be altered by modifying the amount of norbornene functionalization, while maintaining susceptibility to enzymatic degradation. Culture of chondrocyte-laden hydrogel scaffolds led to ECM molecules distributed throughout the construct and resembled articular cartilage with respect to gross appearance of the generated matrix molecules and collagen typing (high type II collagen:type I collagen ratio). This biosynthetic system may prove useful in clinical applications as a scaffold to promote cartilage regeneration.

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Detection and Characterization of Local Interfacial Mechanics in a Cartilage Defect Repair Model

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Introduction: A major challenge in engineering cartilage repair is the integration of the tissue-engineered constructs with the adjacent native cartilage. The lack of lateral anchorage and integration of repair or graft tissue is more susceptible to mechanical failure that could lead to poor prognosis. This problem has motivated a great deal of research into methods of enhancing the integration of repair tissue with the surrounding host cartilage; however, the biomechanical characterization along the interface is poorly understood. Quantitative in vitro articular cartilage studies have measured integration strength with failure test [1] and push out model test [2]; while, in vivo studies have relied solely on histological methods for analysis of integration[3]. As such, an appropriate biomechanical assessment of repair-host tissue retrieved in vivo is much needed. The lack of knowledge is in part due to the fact that a higher mechanical resolution method for detecting local mechanics at the interface has not yet been established. Recently, our lab has developed a novel system for measuring the microscale mechanics of native and repaired on the length scale of 20µm [4]. This approach could provide new information of the mechanical environment at the interface of repaired and host native cartilage. The objective of this study was to detect and characterize the local interfacial mechanics in a murine cartilage defect repair model.

Methods: 10 million cells were placed into 15 cc of polypropylene and subjected to a horizontal back and forth motion for 14 days. Cell clusters or chondrons formed over 14 days. The chondrons were placed in middle of previously frozen cartilage disk with 6 mm cylindrical hole in middle. Type 1 collagen gel was added to the hole and cross-linked with riboflavin and blue light. Disks were implanted subcutaneously in female nude mice and harvested at 6 weeks (n=4). The center of the cartilage disk was removed using a 3mm biopsy punch leaving a ring of engineered and native matrix (Fig.1A). Rings were cut into ~2mm cubes (Fig. 1B) and were prepared for confocal reflectance microscopy (CRM) and confocal strain mapping. Top and cross sectional views were imaged on a Zeiss 710 at 25x magnification (Fig. 1C). Localized strains were measured using particle image velocimetry (PIV) as described previously [5]. Samples were placed into a 2 µg/mL 5-DTAF solution for 30 min to fluorescently stain chondrocytes within the tissue. The deep zone of the cubes were glued to a tissue deformation imaging stage (TDIS) and compressed to 7% axial strain. The TDIS was mounted on an inverted Zeiss LSM 510 confocal microscope and imaged using a 488 nm laser. Shear deformation was induced by displacing the moveable plate in the direction parallel to the articular surface at 1% shear strain. After each increment of shear strain, multiple snapshots were taken throughout the sample and pieced together in order to obtain an image spanning the entire tissue. The horizontal and vertical displacement field of cells in 512×512 µm2 windows was calculated by performing particle image velocimetry analysis on confocal
images before and after application of shear. Cell positions and displacements were obtained via PIV graphical user interface, and used to calculate shear strain \( \gamma_{xy} \) \((du/dy)\) and axial strain \( \varepsilon_{yy} \) \((dv/dy)\) at the interface.

**Results:** Confocal reflectance revealed clear images at the interface, with much variability in matrix integration between samples. Integration appeared more prevalent near the surface, but was poorly integrated at deeper depths (Fig 1C). To assess the interfacial mechanics, we used particle image velocimetry to track tissue deformations via confocal strain mapping across the interface (Fig 2A, B). Shear strain applied parallel to the articular surface direction, was found to induce two modes of deformation, sliding \((\gamma_{xy})\) and peeling \((\varepsilon_{yy})\). Two-dimensional vector maps and plots of \(\gamma_{xy}\) and \(\varepsilon_{yy}\) exhibited large strains at the interface (Fig. 2C, D). \(|\gamma_{xy}|\) values were lower near the articular surface (100-400 \(\mu m\)), and higher in deeper depths (~800\(\mu m\)). In contrast, \(|\varepsilon_{yy}|\) values were higher near the surface and remained consistently high throughout the entire depth of the tissue (Fig. 3).

**Discussion:** Understanding the mechanical properties at the interface of tissue-engineered cartilage are important for improving integrative repair of articular cartilage. This study revealed the interfacial mechanics of articular cartilage exhibit a complex and heterogeneous response to shear loading. This response has two distinct modes of failure at the interface: the sliding along the longitudinal axis and peeling along the latitudinal axis. There was minimal sliding at the surface, where tissue appeared to be well integrated. Contrary, significant sliding occurred in deeper depths along the interface. This high compliance (lower strains) region could be in part due to the lack of anchorage with host tissue as observed in CRM (Fig 1C). Additionally, there was a high degree of peeling along the interface, which could infer a new probable delaminating mechanism. In this study, detection and mechanical characterization of repair-host tissue at the interface provides us with a powerful tool for studying cartilage integration. These results shed more light on the necessary mechanical properties for strategic engineered constructs to withstand physiological loads and promote integration in cartilage repair.

**Significance:** Motivated by the as-of-yet unsolved issue of cartilage integration, this study detected and characterized the local interfacial mechanics in a cartilage defect repair model. Two distinct modes of failure were identified from this work: sliding along the longitudinal axis and peeling along the latitudinal axis at the interface.
Figure 1. Articular cartilaginous ring of engineered and native matrix (A) Rings were cut into ~2mm cubes (B) CRM micrographs of top and cross sectional views of interface (C)

Figure 2. Schematic representation of sample loaded in TDT (A) Confocal micrograph taken at the interface of a sample (B) TV vector maps (C) and measurements of strain fields (C) across entire tissue, including interface.
Figure 3. Average $|\gamma_{xy}|$ (dx/dy) and $|e_{xy}|$ (dy/dy) curves with shaded error bars for interface curves. (n=5)