Published in final edited form as:

Biotechnol Bioeng. 2017 September ; 114(9): 2096–2108. doi:10.1002/bit.26320.

Characterization of the chondrocyte secretome in photoclickable poly(ethylene glycol) hydrogels

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Abstract

Poly(ethylene glycol) (PEG) hydrogels are highly tunable platforms that are promising cell delivery vehicles for chondrocytes and cartilage tissue engineering. In addition to characterizing the type of extracellular matrix (ECM) that forms, understanding the types of proteins that are secreted by encapsulated cells may be important. Thus, the objectives for this study were to characterize the secretome of chondrocytes encapsulated in PEG hydrogels and determine whether the secretome varies as a function of hydrogel stiffness and culture condition. Bovine chondrocytes were encapsulated in photoclickable PEG hydrogels with a compressive modulus of 8 and 46 kPa and cultured under free swelling or dynamic compressive loading conditions. Cartilage ECM deposition was assessed by biochemical assays and immunohistochemistry. The conditioned medium was analyzed by liquid chromatography-tandem mass spectrometry. Chondrocytes maintained their phenotype within the hydrogels and deposited cartilage-specific ECM that increased over time and included aggrecan and collagens II and VI. Analysis of the secretome revealed a total of 64 proteins, which were largely similar among all experimental conditions. The identified proteins have diverse functions such as biological regulation, response to stress, and collagen fibril organization. Notably, many of the proteins important to the assembly of a collagen-rich cartilage ECM were identified and included collagen types II($\alpha 1$), VI ($\alpha 1$, $\alpha 2$, and $\alpha 3$), IX ($\alpha 1$), XI ($\alpha 1$ and $\alpha 2$) and biglycan. In addition, many of the other identified proteins have been reported to be present within cell-secreted exosomes. In summary, chondrocytes encapsulated within photoclickable PEG hydrogels secrete many types of proteins that diffuse out of the hydrogel and which have diverse functions, but which are largely preserved across different hydrogel culture environments.

Graphical Abstract

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Conflict of interest: All authors declare no conflict of interest

Keywords

hydrogel; chondrocyte; cartilage tissue engineering; secretome; poly(ethylene glycol)

Introduction

Poly(ethylene glycol) (PEG) hydrogels are promising *in situ* forming hydrogels for cell delivery and cartilage tissue engineering. PEG hydrogels have been shown to retain the chondrocyte phenotype, maintain high cell viability after encapsulation, promote cartilage-specific extracellular matrix (ECM) deposition, and can be *in situ* formed (Elisseff et al., 1999; Roberts and Bryant, 2013; Sontjens et al., 2006; Sridhar et al., 2014). PEG hydrogels, like many synthetic hydrogels, offer highly reproducible and tunable properties such as stiffness and degradation rates by controlling for the initial hydrogel crosslink density and incorporating degradable bonds into the crosslinks, respectively (Nicodemus and Bryant, 2008). Hydrogels with low crosslink density show improved ECM deposition and elaboration due to the larger polymer mesh size that permits diffusion of smaller ECM precursor molecules through the hydrogel ((Nicodemus et al., 2011; Park et al., 2009; Sontjens et al., 2006). With the incorporation of degradable linkers, PEG hydrogels have been shown to support the formation of a macroscopic cartilage-like ECM (Bryant and Anseth, 2003; Neumann et al., 2016; Skaalure et al., 2015; Sridhar et al., 2015). In addition, the presence of dynamic compressive loading, which is present in the native environment, is an important parameter in cartilage tissue engineering (Butler et al., 2000). Recent studies have identified physiologically relevant loading regimes that support the chondrogenic phenotype and cartilage-specific ECM deposition in PEG hydrogels (Aisenbrey and Bryant, 2016; Farnsworth et al., 2013). Collectively these and other studies support the use of PEG hydrogels for applications in cartilage tissue engineering.

While characterizing the type of ECM that is formed is important to identifying promising hydrogels for cartilage tissue engineering, characterizing the types of proteins that are secreted by the encapsulated cells may also be important to the overall success. Notably, molecules released by encapsulated cells can lead to paracrine signaling within the hydrogel and for *in vivo* applications with cells in the adjacent native tissue and the synovium. Moreover, studies have reported loss of ECM molecules from hydrogels and even greater loss under dynamic loading (Hunter et al., 2004; Kisiday et al., 2004; Kisiday et al., 2009; Lee et al., 2003), which can arise due to increased transport. Thus, characterizing the

proteins secreted by cells, *i.e.* the secretome (Aebersold, 2003; Aebersold and Mann, 2003), can provide new insights into the different types of proteins that are released from the hydrogel. These proteins may impact the formation of neo-tissue as well as the surrounding cells within the joint capsule. The secretome of chondrocyte-laden PEG hydrogels has not yet been characterized.

Secretomics, and proteomics in general, has been used to identify the types of proteins that are secreted by chondrocytes within cartilage explants (Cillero-Pastor et al., 2013; Stenberg et al., 2013; Yu et al., 2014) and that are present in the synovial fluid (Bennike et al., 2014; Peffers et al., 2015; Chiaradia et al., 2012). For example, in an *ex vivo* culture of cartilage explants treated with or without an inflammatory cytokine, many of the proteins identified were found to be involved in ECM functions, such as repair and stress responses (Clutterbuck et al., 2011). Another study, however, reported that cartilage explants subjected to injurious compression released higher levels of intracellular and extracellular matrix proteins (Stevens et al., 2008). The types of protein that were identified within healthy synovial fluid originated from transudates (of blood plasma), synovial membrane, and cartilage (Bennike et al., 2014). However, a comparison between healthy and osteoarthritic synovial fluid revealed that about one percent of the proteins identified were differentially expressed in osteoarthritis (Peffers et al., 2015). Secretomics has also been used to investigate the effects of culture conditions on isolated chondrocytes *ex vivo*. For example, in two-dimensional culture of chondrocytes, the secretome revealed that the types of protein secreted by chondrocytes were similar to chondrocytes within cartilage explants and included proteins associated with cartilage matrix components and regulators of matrix remodeling (Polacek et al., 2010). However, monolayer cultures secreted proteins associated with dedifferentiation, which were not detected in the secretome in cartilage explants. Collectively, these studies using secretomics/proteomics indicate that chondrocytes secrete a range of different types of proteins, but which depends on their environment.

The objectives for this study were two-fold. First, we aimed to characterize the secretome of chondrocytes encapsulated in PEG hydrogels due to their promise in cartilage tissue engineering. Second, we aimed to determine whether the secretome varied with different culture conditions, specifically hydrogel crosslink density, which alters the stiffness of the hydrogel, and dynamic compressive loading. In the latter, hydrogel crosslink densities and the regime of dynamic compressive loading were chosen based on prior studies, which have shown promise in promoting cartilage-specific ECM deposition (Aisenbrey and Bryant, 2016; Farnsworth et al., 2013; Mauck et al., 2000; Nicodemus et al., 2011; Park et al., 2011). Photoclickable PEG hydrogels formed from a thiol-norbornene step-growth polymerization were employed due to their rapid polymerizations and ease of use (Fairbanks et al., 2009a; Hoyle and Bowman, 2010; Roberts and Bryant, 2013). Experiments were performed using a stable version of the hydrogel to ensure the hydrogel structure did not vary over the course of the experiment and confound the secretome analysis. Juvenile bovine chondrocytes were used as a model cell type given their wide use in cartilage tissue engineering studies, *e.g.*, (Bryant and Anseth, 2002; Mizuno et al., 2001; Skaalure et al., 2015; Tran-Khanh et al., 2005; Xu et al., 2006). Cell-laden hydrogels were cultured under free swelling conditions or dynamic compressive loading and first assessed for cartilage-specific ECM synthesis to confirm maintenance of the chondrocyte phenotype and then their conditioned medium was

analyzed by LC-MS to identify the secreted proteins. Overall results from this study identified more than sixty proteins in the secretome, which were associated with biological regulation, response to stress, and collagen fibril organization, and which were largely similar across the different experimental conditions. Collectively these findings provide new insights into the types of proteins in the chondrocyte secretome for chondrocytes encapsulated in PEG hydrogels.

Materials and Methods

Materials

8-arm PEG-amine (MW 20000) was from JenKem USA (Plano, TX). Dimethylformamide (DMF), trypan blue, 5-norbornene-2-carboxylic acid, PEG dithiol (MW 1000), L-ascorbic acid, sodium pyruvate, L-proline, dimethylmethylene blue (DMMB), hyaluronidase, chondroitinase-ABC, and keratanase I were from Sigma Aldrich (St. Louis, MO). O-(7-Azabenzotriazol-1-yl)-N,N',N'-tetramethyluronium hexafluorophosphate (HATU) and N,N'-Diisopropylethylamine (DIPEA) were from ChemImpex, Intl. (Wood Dale, IL). Ethylenediaminetetraacetic acid (EDTA) and diethyl ether were from Fisher Scientific (Fair Lawn, NJ). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Irgacure 2959 was from BASF (Tarrytown, NY). Retrievagen A was from BD Biosciences (San Diego, CA). ITS+ Premix, HEPES buffer, penicillin/streptomycin (P/S), Dulbecco's Modified Eagle Medium (DMEM), GlutaGro, and phosphate-buffered saline (PBS) was from Corning (Corning, NY). Fungizone, gentamicin, DMEM/F12, LIVE/DEAD® assay, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), AlexFluor 488 goat anti-rabbit, and AlexFluor 546 goat anti-mouse were from Life Technologies (Grand Island, NY). Bovine knees were from Research 87 (Boylston, MA). Hoechst 33258 dye was from Polysciences, Inc. (Warrington, PA). TissueTek OCT was from Sakura Finetek (Torrance, CA). Collagenase type II and papain were from Worthington Biochemical (Lakewood, NJ). Rabbit anti-collagen type II antibody (C7510-21C) was from US Biologicals (Swamscott, MA). Rabbit anti-collagen VI (ab6588) and mouse anti-aggrecan (ab3778) antibodies were from Abcam (Cambridge, MA). Transforming growth factor- β 3 (TGF- β 3) was from R&D Systems (Minneapolis, MN).

Macromer Synthesis

Eight-arm PEG-norbornene was synthesized by reacting 8-arm PEG-amine (MW 20,000 g/mol) with 5-norbornene-2-carboxylic acid (Fairbanks et al., 2009b; Roberts and Bryant, 2013). Four molar excess (per amine-terminated PEG arm) of 5-norbornene-2-carboxylic acid in DMF was reacted for 5 minutes under argon with 3 M excess HATU and 6 M excess DIPEA at room temperature to activate the norbornene. The activated norbornene solution was added to 8-arm PEG-amine in DMF and reacted overnight under argon at room temperature. The product 8-arm-PEG-NB was precipitated in diethyl ether, passed through 11 μ m filter paper, dialyzed against diH₂O for 4 days and lyophilized. The percentage of PEG arms with conjugated norbornenes was determined to be 87% using ¹H NMR spectroscopy. The peak area of the norbornene vinyl protons (δ = 5.9–6.25 ppm) was compared to the peak area of the methylene protons in the PEG molecule (δ = 3.3–3.9 ppm).

Chondrocyte Isolation

Bovine articular chondrocytes were isolated from the femoral-patellar groove and femoral condyles of a 1–3 week old skeletally immature calf. Cartilage slices were washed in PBS with antibiotics (1% Pen/Strep, 0.5 µg/mL fungizone, and 4 µg/mL gentamicin) and digested at 37 °C in 0.2% collagenase II in DMEM with 5% FBS. After 15–17 hours, undigested tissue was removed with a 100 µm cell strainer. Isolated cells were washed in PBS with antibiotics and 0.02% EDTA and pelleted at 1200 rpm for 10 minutes. Pellets were resuspended in PBS with antibiotics and washed two more times. Cell viability was determined to be 86% using the trypan blue exclusion assay.

Hydrogel Formation

A precursor solution of 10% w/w solution of 8-arm PEG-NB was prepared in PBS with PEG dithiol (MW 1000 g/mol) at a thiol:norbornene ratio of either 0.45:1 and 0.9:1 and 0.05% w/w Irgacure 2959. Chondrocytes were mixed with the precursor solution at 50 million cells/mL. The cell solution (40 µL) was photopolymerized into cylindrical constructs (~ 5 mm diameter × ~2 mm height) under 352 nm light at ~5 mW/cm² for 7 minutes.

Culture of Cell-Laden Hydrogel Construct

Chondrocyte-laden hydrogels were washed 3 times for 1 hour in defined chondrocyte medium (DMEM/F12 with 1% ITS+ Premix, 1% Pen/Strep, 0.5 µg/mL fungizone, 4 µg/mL gentamicin, 10 µM HEPES, 50 µg/mL L-ascorbic acid, and supplemented with an additional 45 µg/mL sodium pyruvate, 0.25 mM L-proline, and 1.5 mM GlutaGro) to remove any unreacted monomers. Gels were allowed to equilibrate in 1.5 mL defined medium with 5 ng/mL TGF-β3 for 24 hours (day 1) at 37 °C in 5% CO₂. Gels were placed in free swelling conditions or in custom bioreactors that were set to apply a compressive strain in a sinusoidal waveform from 0 to 5% at 1 Hz for 1 hour per day. This loading regime was chosen based on previous studies that have shown in PEG hydrogels, the chondrocyte phenotype is maintained and the ECM synthesized is similar or enhanced when compared to free swelling across different donors and cell types (i.e., primary and stem cells) (Aisenbrey and Bryant, 2016; Farnsworth et al., 2013). Medium was replaced every two days for 9 days. Conditioned medium was collected, snap frozen in liquid nitrogen, and stored at –80 °C.

Characterization of Cell-Laden Hydrogels

On days 1, 5, and 9, hydrogels (n=3) were collected, assessed for wet and dry weights and compressive modulus. Using a MTS Synergie 100 (10N), hydrogels were compressed to 15% strain at 0.5 mm/min. The modulus was estimated as the slope of the linear region of the stress-strain curve between 10% and 15% strain. Hydrogels were measured for their wet weight and then lyophilized to determine dry weight. The mass swelling ratio is defined as the wet weight divided by the dry weight. The volumetric swelling ratio was determined from the mass swelling ratio using a solvent density of 1 g/mL and a polymer density of 1.07 g/mL. The mesh size was estimated following previously described methods (Canal and Peppas, 1989) using the crosslinking density and polymer-solvent interaction parameter. The latter two were determined from a self-learning model (Akalp et al., 2015), which uses experimentally determined equilibrium volumetric swelling ratio and the secant modulus.

Biochemical Analysis

On days 1, 5, and 9, hydrogel constructs were collected (n=3), snap frozen in liquid nitrogen, and stored at -80°C . Constructs were lyophilized for 48 hours. Dry constructs were homogenized for 10 min at 30 Hz with a Qiagen TissueLyser II. Samples were digested in 0.125 mg/mL papain for 18–24 hours at 60°C . Constructs were assayed immediately after digesting for DNA content using Hoechst 33258 dye, assuming 7.7 pg DNA per chondrocyte (Kim et al., 1988). Constructs were assayed for sulfated glycosaminoglycan (sGAG) content using dimethylmethylene blue (Templeton, 1988) and collagen content using hydroxyproline. Total collagen was estimated by assuming collagen was 10% hydroxyproline (Woessner, 1961). Collagen and sGAG contents in the hydrogel were normalized to DNA content.

Immunohistochemical Analysis

On days 1, 5, and 9, hydrogels (n=2–3) were fixed in 4% paraformaldehyde for 1 day and transferred to 30% sucrose for 3 days. Constructs were then allowed to swell in OCT, frozen, and sectioned to $20\text{ }\mu\text{m}$ with a Leica CM1850 cryostat. Sections were treated with a primary antibody against collagen type II (1:50), collagen type VI (1:100), and aggrecan (1:100). Prior to primary antibody treatment, sections were treated with Retrieval A for antigen retrieval and pretreated with 200 U hyaluronidase, 10 mU chondroitinase-ABC (collagen VI and aggrecan), and 3.4 mU keratanase I (aggrecan). Sections were treated with AlexaFluor 488 or 546-conjugated secondary antibodies and counterstained with DAPI. Images were acquired by laser scanning confocal microscopy at $400\times$ magnification where the microscopy settings were consistent across all samples. Sections that received no primary antibody were used as negative controls. Semi-quantitative analysis was performed with NIH ImageJ software with 2–3 hydrogels per condition and 3–4 images per hydrogel (i.e., n=2–3). The mean fluorescence intensity in arbitrary units was normalized to the number of nuclei per image.

Proteomic Analysis

Media samples were collected on days 1 (24 h post-encapsulation), 3, 5, 7, and 9 and flash frozen under liquid nitrogen and stored at -80°C . At the end of the study, the samples were lyophilized, solubilized and for each hydrogel sample pooled together in a total of 2 mL of 8M urea lysis buffer (ULB) in 100 mM ammonium bicarbonate. Protein was quantified by the BCA protein assay. For each sample, 400 μg of protein was digested in trypsin (1:100) overnight. Samples were acidified to 1% formic acid (FA) and desalted with reverse-phase C18 extraction (Waters Sep-Pak WAT054955). Peptides were eluted with 60% acetonitrile in HPLC-grade water with 0.1% FA and dried in a thermal vacuum concentrator. Samples were resuspended in 2% acetonitrile in HPLC-grade water with 0.1% FA, sonicated in a water bath sonicator, centrifuged at $10,000\times g$, and the supernatant after centrifugation was loaded into LC-MS vials. Samples were analyzed on a Thermo ScientificTM Orbitrap EliteTM mass spectrometer equipped with an EASY-nLC 1000 liquid chromatography system. Peptides were separated with a 90 minute linear LC gradient of 5–35% acetonitrile. The mass spectrometer was run in data-dependent acquisition (DDA) mode with a top 15 method

where the 15 most intense peaks from the precursor mass scan were selected for fragmentation. The method only fragmented peaks with a charge state of +2 and above.

Data Processing and Analysis

MS raw files were processed with MaxQuant and searched with the Andromeda search engine against the uniprot *Bos taurus* database that includes the SwissProt reviewed entries (downloaded September 2015). Reported results have a false discovery rate (FDR) of 1%. MaxQuant was run in the label-free quantification (LFQ) mode with the match-between runs setting turned on. The results were filtered for contaminants using Perseus and LFQ intensities were generated and used to plot intensities in Figure 3. Each biological replicate was injected in technical replicates and protein identifications were only counted if they were identified in at least 2/3 biological replicates. The list of genes observed across the entire experiment above the filtering threshold were uploaded to the STRING database and gene ontology (GO) enrichments were calculated from the online tool (<http://string-db.org>). GO Biological Process (BP) terms are reported along with their calculated false discovery rate (FDR) which represents multiple testing corrected p-values. Additionally, all the proteins found across all conditions were mapped for protein-protein interactions (PPI) using the STRING tool. Edges were the standard set that includes “Textmining”, “Experiments”, “Databases”, “Co-expression”, “Neighborhood”, “Gene Fusion”, and “Co-occurrence” and were mapped with the “medium confidence” setting.

Statistics

Data are reported as the mean with standard deviation shown parenthetically within the text or as error bars in the plots. A one-way ANOVA was performed for each culture condition with time as a factor for sGAG/DNA and collagen/DNA. Tukey’s HSD post hoc analysis ($\alpha=0.05$) was used to determine differences between culture conditions within a specific time point. A one-way ANOVA was performed for the MS intensity plots with culture condition as the factor. P-values are reported to indicate the level of significance.

Results and Discussion

Two nondegradable PEG hydrogels with formulations described in Table 1 were investigated to study the secretome from encapsulated juvenile bovine chondrocytes when cultured under free swelling conditions or dynamic compressive loading. Two formulations were chosen to produce a low crosslinked hydrogel (*i.e.*, 0.45:1 thiol:ene ratio) and a more tightly crosslinked hydrogel (*i.e.*, 0.9:1 thiol:ene ratio), but which span crosslink densities that support cartilage-specific ECM deposition (Bryant et al., 2004; Mauck et al., 2000; Nicodemus et al., 2011; Roberts and Bryant, 2013; Sridhar et al., 2015). The resulting macroscopic properties are described in Table 1. The mechanical properties, specifically the tangent compressive moduli, were determined to be 7.7(0.8) and 46.1(11.5) kPa and are referred to herein as the soft (*i.e.*, low crosslinked) hydrogel and the stiff (*i.e.*, higher crosslinked) hydrogel, respectively (Table 1). While it is possible to match the modulus of cartilage (e.g., ~0.6 MPa (Nieminen et al., 2004)) with increasing crosslink density, the tight mesh size restricts ECM deposition (Nicodemus et al., 2011) and therefore was not chosen for this study. The equilibrium volumetric swelling ratio was determined to be 20 and 13,

respectively. The mesh size of the hydrogel network for the soft and stiff hydrogels was estimated from the volumetric swelling ratio to be 155 nm and 65 nm, respectively, indicating that limitations due to protein diffusion should be minimal in both conditions.

The cell-laden hydrogels were first characterized by their macroscopic properties (Fig. 1A,B), cellular content (DNA) (Fig. 1C), biochemical composition for sGAGs and total collagen contents (Fig. 1D–E), and for cartilage-specific matrix molecules of aggrecan, collagen II, and collagen VI (Fig. 1F–K). The macroscopic properties for the soft and correspondingly for the stiff cell-laden hydrogels remained constant throughout the nine-day culture period as measured by the mass swelling ratio (Fig. 1A), an indicator of water content, and the tangent compressive modulus (Fig. 1B). Total DNA content per construct, and hence cell number, was also consistent among all conditions and remained constant throughout the culture period (Fig. 1C). Chondrocytes synthesized and deposited sGAGs and collagen molecules within the PEG hydrogels across all culture conditions (Fig. 1D–E). In addition, the ECM that was deposited within the PEG hydrogels was comprised of aggrecan, the primary proteoglycan in cartilage that is made up of sGAGs, collagen II, the main collagen type found in cartilage, and collagen VI the primary collagen found in the pericellular matrix of cartilage. These results were confirmed by immunohistochemistry (IHC) as shown in representative confocal microscopy images (Fig. 1F–H) and by semi-quantitative analysis (Fig. 1I–K).

The sGAG content within the hydrogels increased ($p < 0.001$) for both hydrogel formulations and culture conditions with culture time (Fig. 1D), which was also evident in the staining for aggrecan deposition (Fig. 1F, I). The free swelling culture conditions led to higher sGAG content in the hydrogels compared to dynamic loading culture conditions for both hydrogel stiffness. Previous studies have shown similar or improved sGAG synthesis under the same loading regime when accounting for sGAGs that were retained and released from the hydrogel (Farnsworth et al., 2013). Thus, it is possible that the reduced sGAG content within the loaded hydrogels is due to increased transport. Total collagen content generally increased over time with the highest amount found in the softest hydrogel under free swelling culture conditions (Fig. 1E), which is consistent with the staining for deposited collagen II and collagen VI. When collagen fibrils assemble, the large collagen macromolecules will become even more restricted in hydrogels with increased crosslink density due to a smaller mesh size and stiffer hydrogel. Thus, the stiff hydrogel may physically limit collagen deposition. Although, increased catabolic activity could also lead to degradation and/or remodeling of the newly synthesized and deposited ECM (Farnsworth et al., 2013; Hunter et al., 2004; Kisiday et al., 2004; Nicodemus and Bryant, 2010; Roberts et al., 2011) and therefore contribute to the observed reduced ECM within the hydrogel. Nonetheless, these results collectively confirm that chondrocytes when encapsulated in the PEG hydrogels and cultured with or without dynamic loading maintain their chondrogenic phenotype evident by increased sGAG and total collagen with culture time and positive staining for the main cartilage ECM macromolecules, aggrecan, collagen II and collagen VI.

The secretome was assessed as a function of gel structure and dynamic loading. Here, the secretome consists of proteins that have been secreted by the encapsulated chondrocytes and which have diffused through the hydrogel and into the culture medium. Medium was

collected throughout the nine-day culture period, pooled for each sample, and analyzed by LC-MS. Sixty-four proteins were positively identified (Supp. Table 1). Several proteins were identified as contaminants (*i.e.*, human albumin, human keratin, etc.) and were removed from further analysis. The majority of identified proteins was identified in all four culture conditions. Venn diagram plots are shown in Fig. 2A–F illustrating the high number of identified proteins that overlapped between the different culture conditions. The small subset (10) of proteins uniquely identified to a particular condition are listed; however, it should be noted that these proteins may be present, but below the detection limit, in the comparative condition.

Table 2 lists the percentage of genes found within each condition for all gene ontology (GO) Biological Process (BP) terms enriched in each condition, but with a false discovery rate (FDR) of less than 0.05 for all four culture conditions. For several GO BP terms, the FDR varied between conditions, in some conditions being less than 0.05 and in other conditions being greater than 0.05. The complete list for each condition and the corresponding FDR are provided in Supplementary Tables 2–5. Overall, the GO annotations were similar among all four conditions. GO annotations included, for example, cellular functions, such as immune response, cell aging, energy metabolism, and biological assembly. Annotations relevant to tissue production and remodeling include collagen fibril organization and proteoglycan metabolic process.

Several GO terms that were of particular interest were response to stress, response to stimulus and defense response. The proteins in these groups largely overlapped and included complement factor B, macrophage migration inhibitory factor, fibronectin 1, and alpha-2-HS-glycoprotein. Although the response to stress (GO.0006950), response to stimulus (GO.0050896), and defense response (GO.0006952) terms are associated with processes that result in a change in activity or state of a cell produced by damage from some type of stimulus or injury, these proteins may also have other functions within cartilage. For example complement factor B, which is part of the alternative complement pathway of the innate immune response, has been found in the resting zone of cartilage and is thought to be involved in the remodeling of cartilage into bone through endochondral ossification (Andrades et al., 1996; Rutkowski et al., 2010). In other tissues, complement factor B has been shown to be important to bone regeneration after injury (Schoengraf et al., 2013). Thus, complement factor B may play a role in ECM regeneration by freshly isolated cells, in addition to being involved in the more classic stress/defense response.

Macrophage migration inhibitory factor (MIF) has been linked to degenerative cartilage (Xiong et al., 2014), but has also been shown to be expressed by chondrocytes that had formed neo-cartilage *in vivo* in a mouse model (Fujihara et al., 2008). Fibronectin is important during cartilage development (Weiss and Reddi, 1981), and has been shown to be one of the first proteins deposited by freshly isolated chondrocytes (Dessau et al., 1978) and is eventually replaced by collagen II (Zhou et al., 2015). Alpha-2-HS-glycoprotein is present in chondrocytes during cartilage development and has been shown to stimulate alkaline phosphatase activity in chondrocytes, which is an enzyme that is important in mineralization of cartilage (Yang et al., 1991). The exact role that of each of these stress-related proteins play in how chondrocytes respond to the PEG hydrogel culture conditions is not known.

However, the pleiotropic action of these proteins suggest that they may participate in multiple functions. These functions may include a stress response, which could arise as a result of the ‘injury’ as cells are removed from their native matrix, or could arise as the chondrocytes adapt to their new environment and their need to synthesize new matrix.

Several ECM proteins that make-up cartilage along with proteins associated with the GO terms for collagen fibril organization (GO.0030199) and proteoglycan metabolic process (GO.0006029) were identified in the medium. Collectively these proteins are listed in Table 3 and presented in Fig. 3 with their corresponding detection intensity from LC-MS analysis for each of the four conditions. Not all replicates nor all conditions had detectable levels of each ECM protein (*i.e.*, Fig 2). Most notably, the procollagen form of collagen II (*i.e.*, collagen II α 1), the most abundant collagen in cartilage, was identified in all conditions (Fig. 3A). Intracellularly three pro- α 1(II) chains twist together to form the procollagen molecule that then assembles extracellularly to form long, mature collagen II fibers. Although each of the pro- α 1(II) chains are large, with molecular weights of ~140 kDa, the procollagen molecules have a diameter reported to be ~10 nm (Arita et al., 2002) and therefore based on the hydrogel mesh size have the potential to be transported through the hydrogels. Collagen VI is the primary collagen found in the pericellular matrix of cartilage (Hagiwara et al., 1993; Ronzière et al., 1990) and it is comprised of three α chains, all of which were detected in the medium for most conditions (Fig. 3B–D). The secreted procollagen molecule for collagen VI is composed of multiple α chains that form long tetramers that are ~4–14 nm in width (Baldock et al., 2003) and therefore, like procollagen (II) molecules, has the potential to be transported through the hydrogels. Additionally, collagen IX α 1 (Fig. 3E) and collagen type XI α 1 and α 2 (Fig. 3F,G) were also detected. Heterotrimeric fibrils of collagens II, IX, and XI are found throughout cartilage where collagen XI serves to limit the diameter to control fibril growth and collagen IX increases the efficiency of fibril formation (Blaschke et al., 2000). Other less known procollagens were also detected including collagen V α 1 (Fig. 3H) and collagen III α 1 (Fig. 3I). Collagen V α 1 chains have been shown to accumulate as articular cartilage matures (Wu et al., 2009). Collagen III was recently identified in articular cartilage as acting as a covalent modifier to collagen II (Wu et al., 2010). It was hypothesized that collagen III, which is known to be upregulated during wounds, may act as a cohesive molecule that protects structurally damaged collagen II fibers. Among these procollagen molecules, other molecules that are important to collagen II assembly in the cartilage matrix were identified including biglycan (Fig. 3J), lumican (Fig. 3K), and the collagen binding protein, serpin peptidase inhibitor, clade H (Fig. 3L). Overall, these ECM proteins strongly point to the fact that the encapsulated chondrocytes regardless of hydrogel or culture condition are actively secreting molecules that are necessary for the formation of the collagen-rich ECM within cartilage.

A number of the proteins identified in this study overlap with proteins that have been found within articular cartilage vesicles (ACVs) (Rosenthal et al., 2011), which are exosomes secreted by chondrocytes. Exosomes are vesicles secreted by various cell types that may play a role in intercellular communication, transfer of membrane proteins, as well as T-cell stimulation (Théry et al., 2002). These vesicles were originally characterized in chondrocytes for their role in mineralization of cartilage (Ali et al., 1970), which is observed in pathologic conditions such as osteoarthritis (Fuerst et al., 2009). However, recent evidence

has shown that chondrocytes in healthy cartilage also secrete these vesicles (Derfus et al., 1992) and are important to regulating joint homeostasis and aiding in joint development (Malda et al., 2016). It appears that many of the intracellular associated proteins identified in this study are derived from secreted ACVs. Exosomes have been reported to be as small as 30 nm, but can have diameters larger than 100 nm (Raposo and Stoorvogel, 2013) and therefore smaller exosomes may be able to diffuse through the hydrogel. Examples include cytoplasmic proteins and enzymes, such as actin, triosephosphate isomerase, phosphoglycerate mutase, and α -enolase, which are part of the glycolysis cycle and several ECM-related proteins, such as fibronectin, matrix Gla protein, and transforming growth factor-beta-induced protein ig-h3. Matrix Gla protein, a calcium binding ECM protein (Hauschka et al., 1989), functions as a calcification inhibitor in arteries and cartilage (Luo et al., 1997). Several members of the S100 protein family, which have both intracellular and extracellular functions in chondrocytes and cartilage are found in ACVs (Yammani, 2012) and were also found in this study. Finally, ACVs also contain many of the building blocks that make up cartilage ECM including many proteoglycans and collagen alpha chains, as described above.

The protein-protein interaction (PPI) network plotted as the result of the STRING database analysis depicts all of the proteins found in the study across all conditions (Figure 4). As the inter sample variability in protein identifications across the entire experiment was low, the mapping of all of the proteins together provides a summarized picture of the secretome that is similar for all conditions. As such, it can be seen that many interacting known ECM molecules including many collagen members were found throughout as well as numerous signaling factors and some remodeling enzymes.

Overall, this study describes the secretome for freshly isolated bovine chondrocytes that are cultured in PEG hydrogels. When chondrocytes are removed from their native ECM, they begin synthesizing a new ECM, which was evident in our biochemical and histological assessment, as well as in the secretome analysis. Interestingly, the secretome identified that in addition to collagen II ($\alpha 1$), the main collagen in cartilage, and collagen VI ($\alpha 1$, $\alpha 2$, $\alpha 3$) the main collagen in the pericellular matrix of cartilage, procollagen forms for types III, V, IX, and XI as well as biglycan were also present, several of which are important to the assembly and formation of mature collagen II fibers (Mendler et al., 1989). This indicates that the chondrocytes are actively producing many of the ECM proteins that are needed to re-synthesize their matrix. The loss of these molecules to the culture medium may be attributed to either their initial loss before a pericellular matrix develops or as part of degraded collagen fragments that result from the cells actively remodeling their newly deposited matrix. However, due to the relatively tight mesh size of the hydrogel, it is likely that the collagen molecules in the secretome represent the procollagen precursors that have diffused through the hydrogel rather than degraded collagen. This observation is supported by the fact that degraded collagen will be much larger in size than the procollagen molecules and previous studies have identified degraded collagen fragments within the pericellular matrix in non-degradable hydrogels (Farnsworth et al., 2013). Beyond these ECM-related molecules, chondrocytes also appear to be actively secreting exosomes, which may be important in paracrine signaling particularly for *in vivo* applications, and therefore requires further analysis in future work. Despite the different culture conditions, the encapsulated

chondrocytes secrete similar types of proteins. These results suggest that while chondrocytes appear to be highly active within the PEG hydrogels, they may be less sensitive to smaller changes in their environment (*i.e.*, via hydrogel crosslinking and dynamic loading) with respect to the type of proteins synthesized, but may instead influence the magnitude of the proteins synthesized (Clutterbuck et al., 2011).

There are several limitations of this study. Non-degradable hydrogels and short culture times were employed in this study. The former was necessary to minimize confounding factors that could influence the secretome. Since in non-degradable hydrogels ECM deposition is limited to the pericellular region due to the relatively small mesh size of the hydrogel (Dhote et al., 2013; Nicodemus et al., 2011), short culture times were chosen. As a result, robust neo-tissue had not yet developed within the nine-day culture period. In addition, the effects of dynamic loading on ECM synthesis and deposition was limited. Previous studies have reported anabolic effects due to dynamic compressive loading, but only after extended culture times (Mauck et al., 2000; Mauck et al., 2002; Waldman et al., 2004) and thus it was not expected that loading would have a significant effect on ECM deposition in this study at least during the short culture period. The bovine genome has been sequenced, but a comprehensive annotation of all of its genes has not yet been completed (Reese et al., 2010). As a result, it is likely that gaps exist in the GO annotations for many proteins. While many ECM proteins were detected in this analysis, aggrecan, which is the primary proteoglycan found in cartilage was not detected. We have previously reported the presence of sGAGs, which are derived from aggrecan, in the culture medium (Roberts et al., 2011; Skaalure et al., 2015). In this study, the secretome proteins were not subjected to deglycosylation protocols that would allow for the detection and identification of heavily glycosylated proteins, like aggrecan. Similarly, collagen XI $\alpha 1$ and $\alpha 2$ were detected, but not $\alpha 3$, despite all three being important to forming collagen XI. Collagen XI $\alpha 3$ is heavily glycosylated (Mendler et al., 1989) and therefore may not have been detected. Thus, future studies may require protocols more amenable to detecting glycosylated peptides that are derived from proteins such as aggrecan that are expected to be present. Additionally, it is important to note that there may have been low abundance proteins present in the culture medium, but which were not detected due to the presence of several high abundance proteins in the defined medium, which can reduce the sensitivity of the LC-MS. Finally, defined media was used to avoid the presence of serum proteins in the secretome and instead defined media with ITS was supplemented with TGF- $\beta 3$ to maintain the chondrocyte phenotype (Chua et al., 2005). It is possible that the secretome may be partially influenced by the presence of TGF- $\beta 3$; however the concentrations employed herein were similar to its concentration in synovial fluid (Albro et al., 2012; Brennan et al., 1990).

Conclusion

PEG hydrogels are highly tunable platforms that are promising cell delivery vehicles for chondrocytes and cartilage tissue engineering. Chondrocytes encapsulated in these synthetic hydrogels deposited cartilage-specific ECM molecules that increased over time. The chondrocyte secretome was comprised of proteins that have been identified in chondrocyte exosomes as well as proteins that are important to the formation and organization of the cartilage ECM. The identified proteins were largely similar across the culture conditions

investigated in this study, suggesting that although there were small, but significant differences in the amount of ECM deposited, the different culture conditions did not affect the overall secretome. However, the concentrations of the secreted proteins may be affected by loading and/or hydrogel structure and require further analysis. In summary, chondrocytes are highly active within PEG hydrogels secreting many different types of proteins that diffuse through and out of the hydrogel, which were similar across different hydrogel culture environments. These findings suggest that it may be possible to design PEG hydrogels for cartilage tissue engineering with a range of initial properties and loading environments without drastically affecting the chondrocyte secretome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number 1R01AR065441. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors acknowledge the National Institute of Health (NIH) Institutional Pharmaceutical Training fellowship to MCS.

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Figure 1.

Analysis of cell-laden photoclickable PEG hydrogels for soft and stiff hydrogels cultured under free swelling or dynamic loading conditions as a function of culture time. A) Mass swelling ratio of cell-laden hydrogels defined as swollen mass / dry mass as an indicator of water content. B) Tangent compressive modulus of cell-laden hydrogels. C) Total DNA content per hydrogel. D) Sulfated glycosaminoglycan (sGAG) content per DNA. E) Total collagen content per DNA. Immunohistochemical staining for F) aggrecan, G) collagen II, and H) collagen VI at day 9. Scale bars are 20 μm. Semi-quantitative image analysis for I) aggrecan, J) collagen II, K) and collagen VI. Data is taken from 3–4 images of 2–3 constructs per condition. Data are presented as mean with standard deviation (n=2–3) as error bars. P-values are presented for a one-way ANOVA or after post-hoc analysis.

Figure 2.

Venn diagrams showing the overlap of proteins identified with mass spectrometry for different comparisons of A) soft hydrogel versus stiff hydrogel regardless of culture condition, B) free swelling (FS) versus dynamic loading (DL) culture conditions regardless of hydrogel, C) soft hydrogel under FS versus DL culture conditions, D) stiff hydrogel under FS versus DL culture conditions, E) FS for soft hydrogel versus stiff hydrogel, F) DL soft hydrogel versus stiff hydrogel. Uniquely identified proteins are listed next to the Venn diagram in each panel.

Figure 3.

Intensity plots (arbitrary units) of ECM and ECM related proteins for A) COL2A1, B) COL6A1, C) COL6A2, D) COL6A3, E) COL9A1, F) COL11A1, G) COL11A2, H) COL5A1, I) COL3A1, J) biglycan, K) lumican, and L) SERPINH1. Data are shown for soft, free swelling hydrogels (white bar), soft, dynamic loading hydrogels (gray bar), stiff, free swelling hydrogels (black bar), and stiff, dynamic loading hydrogels (black stripe bar). Data are presented as mean with standard deviation as error bars. P-values are presented for a one-way ANOVA.

Figure 4.
The protein-protein interaction (PPI) network shown for both hydrogels (soft and stiff) and culture conditions (free swelling and dynamic loading) combined.

Table 1

Hydrogel formulation and properties

	Soft	Stiff
PEG-NB (wt%)	10	10
Norbornene Conjugation	87%	87%
Thiol:ene Molar Ratio	0.45	0.9
Compressive Modulus (kPa)	7.7(0.8)	46.1(11.5)
Volumetric Swelling Ratio	19.7(0.6)	12.8(1.8)
Mesh Size (nm)	155	65

Table 2

GO Terms-Enriched in all conditions

GO Term	Description	Soft, DL		Soft, FS		Stiff, DL		Stiff, FS	
		%	%	%	%	%	%	%	%
GO.0065007	biological regulation	46.6	44.4	51.0	44.6				
GO.0044699	single-organism process	44.8	41.3	47.1	42.9				
GO.0008150	biological_process	43.1	41.3	47.1	41.1				
GO.0009987	cellular process	43.1	41.3	47.1	41.1				
GO.0044763	single-organism cellular process	43.1	39.7	45.1	41.1				
GO.0050789	regulation of biological process	43.1	41.3	47.1	41.1				
GO.0050794	regulation of cellular process	36.2	34.9	41.2	35.7				
GO.0008152	metabolic process	29.3	28.6	33.3	28.6				
GO.0044238	primary metabolic process	29.3	28.6	33.3	28.6				
GO.0044707	single-multicellular organism process	29.3	27.0	31.4	28.6				
GO.0048518	positive regulation of biological process	29.3	27.0	29.4	26.8				
GO.0050896	response to stimulus	29.3	27.0	29.4	26.8				
GO.0071704	organic substance metabolic process	29.3	28.6	33.3	28.6				
GO.0048519	negative regulation of biological process	27.6	27.0	29.4	25.0				
GO.0065008	regulation of biological quality	27.6	25.4	29.4	26.8				
GO.0048522	positive regulation of cellular process	25.9	23.8	25.5	23.2				
GO.0048523	negative regulation of cellular process	25.9	25.4	29.4	25.0				
GO.0044767	single-organism developmental process	24.1	22.2	25.5	23.2				
GO.0006950	response to stress	22.4	20.6	23.5	21.4				
GO.0051239	regulation of multicellular organismal process	22.4	20.6	21.6	19.6				
GO.0060255	regulation of macromolecule metabolic process	22.4	22.2	25.5	21.4				
GO.0048856	anatomical structure development	20.7	19.0	21.6	19.6				
GO.0007275	multicellular organismal development	19.0	17.5	19.6	17.9				
GO.0044710	single-organism metabolic process	19.0	17.5	21.6	19.6				
GO.0048583	regulation of response to stimulus	19.0	17.5	17.6	16.1				
GO.0048731	system development	19.0	17.5	17.6	16.1				
GO.0050793	regulation of developmental process	19.0	17.5	17.6	16.1				

GO Term	Description	Soft, DL		Soft, FS		Stiff, DL		Stiff, FS	
		%	%	%	%	%	%	%	%
GO.0051716	cellular response to stimulus	19.0	17.5	17.5	17.6	16.1			
GO.0080090	regulation of primary metabolic process	19.0	19.0	19.0	21.6	17.9			
GO.0007154	cell communication	15.5	14.3	14.3	15.7	14.3			
GO.0009966	regulation of signal transduction	15.5	14.3	14.3	13.7	12.5			
GO.0032268	regulation of cellular protein metabolic process	15.5	14.3	14.3	15.7	14.3			
GO.0032879	regulation of localization	15.5	14.3	14.3	13.7	12.5			
GO.0050790	regulation of catalytic activity	15.5	14.3	14.3	15.7	14.3			
GO.0006952	defense response	13.8	12.7	12.7	13.7	12.5			
GO.0009892	negative regulation of metabolic process	13.8	14.3	14.3	15.7	12.5			
GO.0010604	positive regulation of macromolecule metabolic process	13.8	12.7	12.7	13.7	12.5			
GO.0042981	regulation of apoptotic process	13.8	12.7	12.7	11.8	10.7			
GO.0044281	small molecule metabolic process	13.8	12.7	12.7	15.7	14.3			
GO.0048513	organ development	13.8	12.7	12.7	13.7	12.5			
GO.0048584	positive regulation of response to stimulus	13.8	12.7	12.7	11.8	10.7			
GO.0051240	positive regulation of multicellular organismal process	13.8	12.7	12.7	11.8	10.7			
GO.2000026	regulation of multicellular organismal development	13.8	12.7	12.7	11.8	10.7			
GO.0009653	anatomical structure morphogenesis	12.1	11.1	11.1	11.8	10.7			
GO.0009967	positive regulation of signal transduction	12.1	11.1	11.1	11.8	10.7			
GO.0043436	oxoacid metabolic process	12.1	11.1	11.1	13.7	12.5			
GO.0051093	negative regulation of developmental process	12.1	11.1	11.1	11.8	10.7			
GO.0071822	protein complex subunit organization	12.1	11.1	11.1	13.7	12.5			
GO.0030162	regulation of proteolysis	10.3	9.5	9.5	11.8	10.7			
GO.0031324	negative regulation of cellular metabolic process	10.3	11.1	11.1	13.7	10.7			
GO.0032101	regulation of response to external stimulus	10.3	9.5	9.5	9.8	8.9			
GO.0032787	monocarboxylic acid metabolic process	10.3	9.5	9.5	11.8	10.7			
GO.0042060	wound healing	10.3	9.5	9.5	11.8	10.7			
GO.0043066	negative regulation of apoptotic process	10.3	9.5	9.5	9.8	8.9			
GO.0043086	negative regulation of catalytic activity	10.3	9.5	9.5	9.8	8.9			
GO.0051241	negative regulation of multicellular organismal process	10.3	9.5	9.5	9.8	8.9			
GO.0052547	regulation of peptidase activity	10.3	9.5	9.5	9.8	8.9			

GO Term	Description	Soft, DL		Soft, FS		Stiff, DL		Stiff, FS	
		%	%	%	%	%	%	%	%
GO.1901135	carbohydrate derivative metabolic process	10.3	9.5	11.8	10.7				
GO.0001775	cell activation	8.6	7.9	9.8	8.9				
GO.0005975	carbohydrate metabolic process	8.6	7.9	9.8	8.9				
GO.0006954	inflammatory response	8.6	7.9	9.8	8.9				
GO.0010951	negative regulation of endopeptidase activity	8.6	7.9	7.8	7.1				
GO.0032269	negative regulation of cellular protein metabolic process	8.6	7.9	9.8	8.9				
GO.1902533	positive regulation of intracellular signal transduction	8.6	7.9	9.8	8.9				
GO.0001501	skeletal system development	6.9	6.3	7.8	7.1				
GO.0007417	central nervous system development	6.9	6.3	7.8	7.1				
GO.0030168	platelet activation	6.9	6.3	7.8	7.1				
GO.0044723	single-organism carbohydrate metabolic process	6.9	6.3	7.8	7.1				
GO.0060348	bone development	6.9	6.3	7.8	7.1				
GO.0006096	glycolytic process	5.2	4.8	5.9	5.4				
GO.0006953	acute-phase response	5.2	4.8	5.9	5.4				
GO.0030199	collagen fibril organization	5.2	4.8	5.9	5.4				
GO.0002062	chondrocyte differentiation	3.4	3.2	3.9	3.6				
GO.0006029	proteoglycan metabolic process	3.4	3.2	3.9	3.6				
GO.0007569	cell aging	3.4	3.2	9.8	8.9				
GO.0014002	astrocyte development	3.4	3.2	3.9	3.6				
GO.0035025	positive regulation of Rho protein signal transduction	3.4	3.2	3.9	3.6				
GO.0048708	astrocyte differentiation	3.4	3.2	3.9	3.6				
GO.0060350	endochondral bone morphogenesis	3.4	3.2	3.9	3.6				
GO.0060351	cartilage development involved in endochondral bone morphogenesis	3.4	3.2	3.9	3.6				
GO.2000257	regulation of protein activation cascade	3.4	3.2	3.9	3.6				

Table 3

ECM and ECM related proteins

UniProt Accession	Protein	Gene
P02459	collagen, type II, alpha 1 ^{1,2}	COL2A1
Q08E14	collagen, type III, alpha 1	COL3A1
G3MZI7	uncharacterized protein (probably collagen 5)	COL5A1
E1BI98	uncharacterized protein (probably collagen 6)	COL6A1
F1MKG2	uncharacterized protein (probably collagen 6)	COL6A2
E1BB91	uncharacterized protein (probably collagen 6)	COL6A3
F1MND9	uncharacterized protein (probably collagen 9)	COL9A1
F1N0K0	collagen alpha-1(XI) chain	COL11A1
Q32S24	collagen, type XI, alpha 2	COL11A2
P21809	biglycan ²	BGN
Q05443	lumican ¹	LUM
Q2KJH6	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1) ¹	SERPINH1

¹Collagen fibril organization

²Proteoglycan metabolic process