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Physically crosslinked polyvinyl alcohol and gelatin interpenetrating polymer network theta-gels for cartilage regeneration

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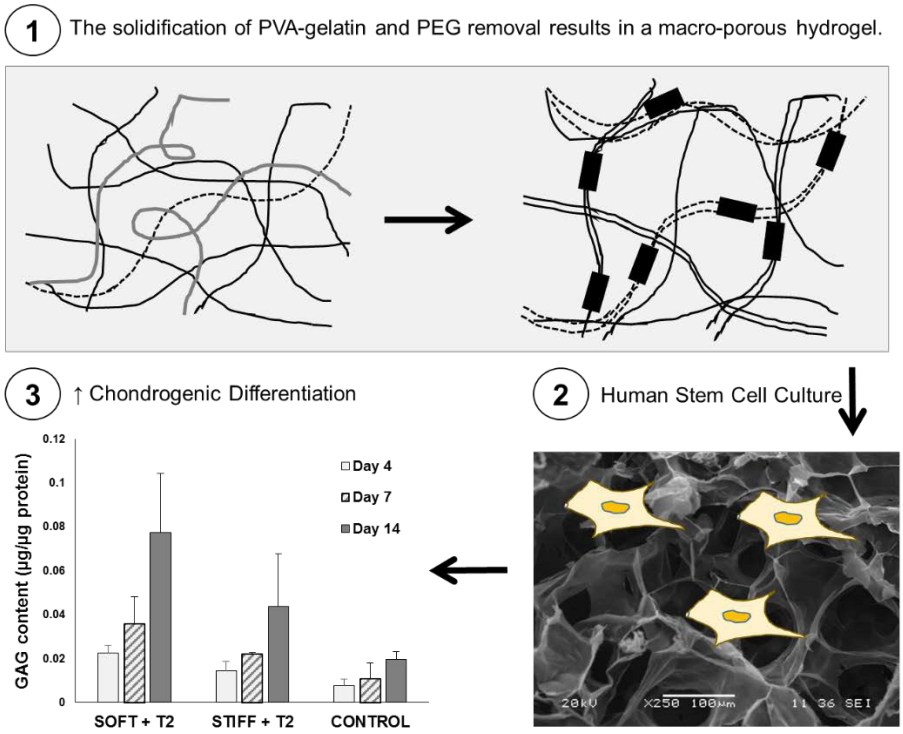
Abstract

Theta-gels are hydrogels that form during the solidification and phase separation of two dislike polymers, in which a low molecular weight polymer behaves as a porogen and is removed through dialysis. For this study, interpenetrating polymer network (IPN) hydrogels were formed between polyvinyl alcohol (PVA) and gelatin using theta-gel fabrication techniques, i.e., in the presence of a porogen. The addition of gelatin to a PVA theta-gel, formed with a porogen, polyethylene glycol (PEG), created macro-porous hydrogels, and increased shear storage moduli and elastic moduli, compared to PVA-gelatin scaffold controls. A reduction in PVA crystallinity was verified by Fourier transform infrared (FTIR) spectroscopy in hydrogels fabricated using a porogen, i.e., PVA-PEG-gelatin, compared to PVA, PVA-PEG, or PVA-gelatin hydrogels alone. Van Geison staining confirmed the retention of gelatin after dialysis. A range of hydrogel moduli was achieved by optimizing PVA concentration, molecular weight, and gelatin concentration. PVA-gelatin hydrogels maintained primary human mesenchymal stem cell (MSC) viability. Soft (~10 kPa) and stiff (~ 100 kPa) PVA-gelatin hydrogels containing type II collagen significantly increased glycosaminoglycan (GAG) production compared to controls. PVA-gelatin hydrogels, formed using

theta-gel techniques, warrant further investigation as articular cartilage tissue engineering scaffolds.



Introduction

Osteoarthritis is a degenerative inflammatory disease that results in irreversible degradation of osteochondral tissue, which consists of surface articular cartilage and underlying subchondral bone.[1] Recent tissue engineering approaches show promise; however, innovative strategies are needed to recapitulate the mechanotransduction pathways found in osteochondral tissue for tissue regeneration, and to maintain tissue homeostasis.[2] The mechanisms by which cells receive signals from their environment can be examined through the manipulation of the structure (e.g., topography and porosity), level of bioactivity (e.g., cell adhesion, stem cell differentiation), and the dynamic mechanical properties (e.g., stiffness, elasticity, toughness, etc.) of the material

systems.[3-5] Therefore, it is critical to define each parameter – structure, bioactivity, and mechanical properties – independently and collectively to define the critical parameters required to regenerate tissue, or treat a disease.[6-9]

Tissue engineering relies on synthetic or natural-based material systems to interact with cells in order to enhance tissue regeneration. Material stiffness,[10] porosity,[11] and bioactivity parameters influence the cellular response, in particular, cell transcription and stem cell differentiation.[5] Thus, various mechanotransduction pathways can be activated to direct stem cell differentiation for specific applications.[12-18] Indeed, mesenchymal stem cells (MSCs) cultured on stiff substrates favor an osteogenic differentiation pathway,[19, 20] while MSCs cultured on soft substrates favor a chondrogenic differentiation pathway.[4] In addition to mechanical cues, MSCs are responsive to biological signals from their physical environment. Collagen is an amino acid based protein that is found in the fibrous tissues of the human body, including bone and cartilage.[21] Collagen increases cell adhesion, migration, and proliferation.[22] Specifically, type II collagen, a component of articular cartilage extracellular matrix (ECM), is known to significantly influence chondrogenic differentiation and the maintenance of chondrocyte phenotype *in vitro* and *in vivo*.[23]

Polyvinyl alcohol (PVA) is a synthetic, biocompatible polymer utilized for soft tissue replacements and tissue regeneration.[24-26] PVA hydrogels are formed by the crystallization of polymer chains through non-covalent linkages, i.e., physical crosslinks.[27,28, 29] Specifically, the formation of theta-gels incorporates the use of a small molecular weight hydrophilic polymer, such as polyethylene glycol (PEG) as a porogen during cooling, or solidification of a PVA solution.[30, 31] During a thermal transition, PEG porogens phase separate from PVA, increasing the density of PVA-rich regions, thus inducing crystallization. The PVA hydrogel network forms during a large thermal transition and the creation of intermolecular hydrogen bonds.[32, 33] PEG is removed through dialysis after the thermal transition, which results in large pores in a rigid hydrogel network.

PVA hydrogels lack cell adhesion functionalities, and investigators have incorporated biomolecules into the hydrogel network to enhance hydrogel mechanical properties and bioactivity.[34] To enhance bioactivity of PVA hydrogels, polysaccharides and proteins have been added during hydrogel crosslinking.[35, 36] Gelatin, a non-specific derivative of the protein collagen, contains cell adhesion ligands, which provide cell adhesion sites to PVA hydrogels when blended together.[37-39] While cryo-gels of PVA and gelatin blends have been studied, the effect of adding gelatin to PVA theta-gels, in the presence of PEG, has not been investigated.

The aim of this study was to develop and characterize PVA-gelatin hydrogels with improved mechanical properties, through the incorporation of PEG porogens. We hypothesized that the addition of a porogen during fabrication, and subsequent removal of the PEG porogen, would increase the density and interactions of PVA and gelation, compared to the solidification of PVA and gelatin alone, to result in macro-porous hydrogels with increased shear and compressive moduli. We evaluated the potential of PVA-gelatin hydrogels to support chondrogenic MSC differentiation, and varied hydrogel stiffness and the addition of type II collagen.

Results and Discussion

PVA-Gelatin Theta-Gel Formation

Bio-synthetic hydrogels were obtained from the physical crosslinking of PVA and gelatin, the diffusion of PEG, and subsequent removal of the PEG porogen for pore formation (**Figure 1**). The thermal gelation of both PVA and gelatin in the presence of PEG created a macro-porous IPN. The short-chain, hydrophilic PEG molecules behaved as porogens, aggregating into large domains. After cooling, the PVA-gelatin hydrogel was dialyzed for 5 days, allowing the soluble, nucleated PEG molecules to escape the hydrogels, creating a large interconnected porous structure.

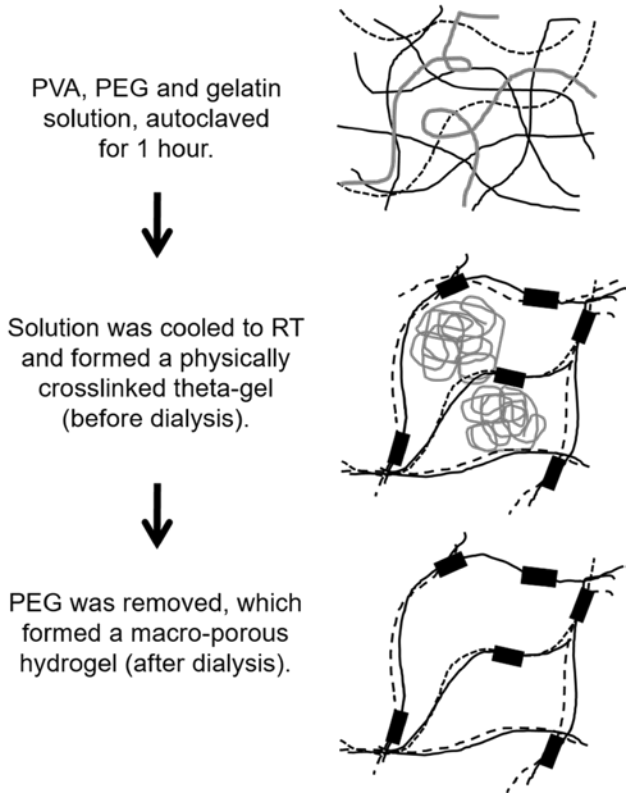


Figure 1. Schematic illustrating the formation of a PVA and gelatin theta-gel, through the physical crosslinking of PVA (solid black lines) and gelatin (dashed black lines), respectively, in the presence and subsequent removal of PEG. Areas of hydrogen bonding (i.e., physical crosslinks) between gelatin and PVA are represented by black rectangles. Nucleation of PEG porogens (gray lines) during solidification and subsequent removal through dialysis resulted in a macro-porous network.

To demonstrate the formation of an IPN, soft hydrogels with 18% PVA (high molecular weight), 1% gelatin were fabricated in the presence of a porogen (20% w/v PEG), and the experimental groups were denoted as PVA-PEG-1Gel; it is important to note however that the samples did not contain PEG after hydrolysis, and the use of PEG in the nomenclature is to signify whether or not a porogen was used. Control samples including 18% PVA (PVA), 1% gelatin (Gelatin), 18% PVA-

1% gelatin (PVA-1Gel) and 18% PVA-20% PEG (PVA-PEG) were also fabricated. These samples were tested under attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy after dialysis in DI water to remove PEG. The physical crosslinking of PVA and PVA-1Gel controls occurred due to numerous inter-chain hydrogen bonds between OH groups, formed during crystallization of the polymer and identified as a peak in the FTIR spectra at 1141 cm^{-1} (**Figure 2E and D, respectively**).[40, 41] The intensity of this peak is related to the C-O stretching vibrations of the intermolecular hydrogen bonds contained within the crystalline regions of PVA. Peaks within 1090 – 1150 cm-1 are also associated with C-O stretching vibrations, and indicate hydrogen bonds formed between two neighboring OH groups. The C-O peak had a lower intensity for PVA and gelatin hydrogels which were fabricated in the presence of PEG porogens – denoted as experimental group PVA-PEG-1Gel (**Figure 2B**). To explain the increased stiffness of the hydrogels and lower signals of the C-O bands, it was hypothesized that crosslinking occurred within the solidifying regions containing PVA and gelatin, and the subsequent generation of van der Waals interactions between hydrocarbon polymer backbones between gelatin and PVA. As a result of using PEG porogens during the solidification process, the PVA and gelatin interacted with each other differently than simply mixing the two polymers together without the use of a, which is supported by the data in **Figure 2**.

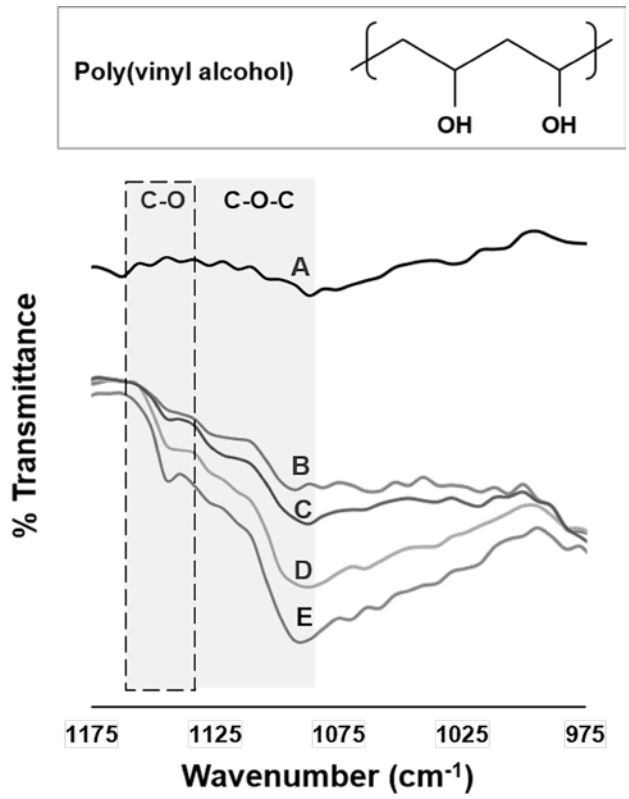


Figure 2. ***Top****:* Chemical structure of PVA; physical crosslinking takes place at OH groups between PVA molecules. *Bottom:* FTIR spectra of (A) gelatin, (B) PVA-PEG-1Gel, (C) PVA-PEG, (D) PVA-1Gel and (E) PVA hydrogel films. C-O stretching vibrational bands, associated with PVA physical crosslinking and crystallization, are shown. Samples were characterized after the removal of PEG porogens; PEG is incorporated into the group names to indicate the fabrication method.

The pore diameters of PVA-gelatin hydrogels (**Figure 3D**) were approximately ten times larger than the control PVA hydrogels (**Figure 3B**), confirmed through scanning electron micrographs. Van Geison staining qualitatively verified that gelatin was retained in the hydrogels after dialysis (gelatin samples stained red, PVA controls did not; **Figure 3E**). Qualitatively no differences were

seen between groups in the stained images. Indeed, some non-crosslinked gelatin may have also been lost during dialysis, which was physically entangled in the IPN hydrogel, as indicated by the reduction in intermolecular hydrogen bonds with the PVA base material (see **Figure 2**).

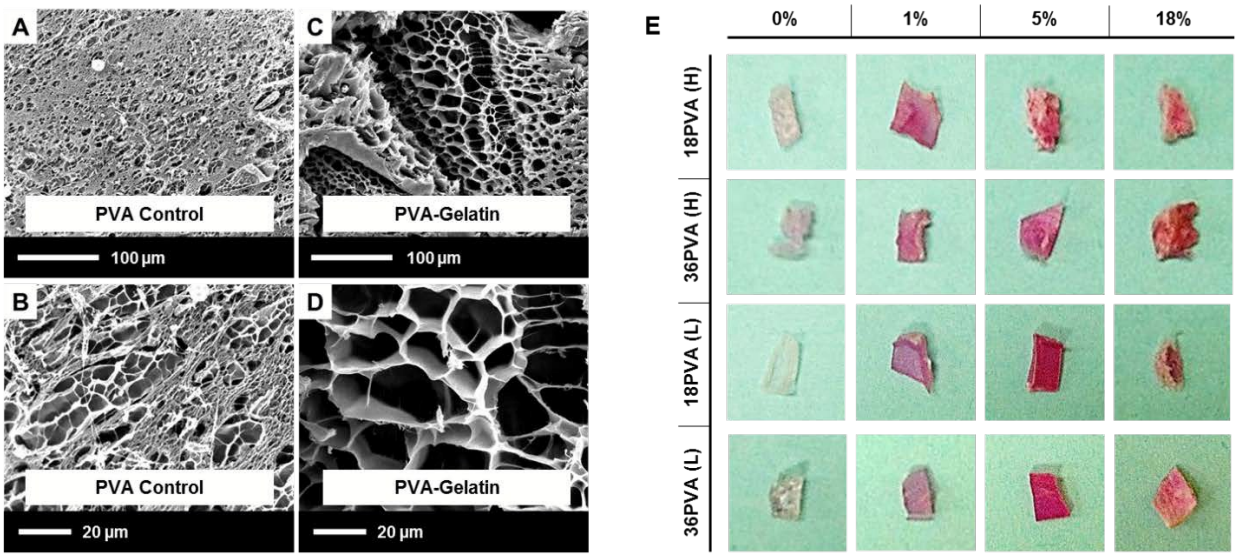


Figure 3. SEM images of lyophilized and cryo-fractured surfaces of 18% PVA hydrogel samples, fabricated in the presence of PEG porogens alone (A, B) or with the addition of 5% gelatin (C, D). Magnifications at 250x, scale bar = 100 µm (A, C) and 800x, scale bar = 20 µm (B, D). (E) Van Geison staining qualitatively verified gelatin retention in the hydrogels, which were fabricated using theta-gel techniques. PVA-gelatin hydrogels consisting of 18 and 36% PVA, using low or high molecular weight PVA, were fabricated. Hydrogels also varied in gelatin content; top numbers represent weight percent of gelatin. Samples containing gelatin displayed a higher intensity of the red stain. Note: the PEG porogen was removed prior to SEM or staining.

Physical Characterization and Mechanical Properties

To demonstrate the formation of an IPN, hydrogels fabricated with and without the use of PEG porogens were characterized via rheology. Experimental groups were fabricated using 18% (w/v) PVA (high molecular weight) aqueous solutions, containing 1% (w/v) gelatin and 20% (w/v) PEG. Experimental groups which were fabricated with PEG porogens are denoted as PVA-PEG-1Gel; groups without PEG porogens are denoted as PVA-1Gel. Control groups included 18% PVA alone (PVA), 1% gelatin alone (Gelatin), and 18% PVA-20% PEG (PVA-PEG) to examine the effect of PEG porogens on the mechanical response of PVA-gelatin hydrogels. Hot polymer hydrogel pre-curser solutions were transferred to an AR2000 stress-controlled rheometer (TA Instruments). A temperature sweep was performed from 60 ºC to 20 ºC to obtain gelation data from each sample (**Figure 4A**). For all samples except 1% gelatin, the storage moduli increased as temperature decreased; the gelatin control remained at a consistent level for the entire test duration. With the addition of gelatin to the PVA to form a hydrogel, storage moduli increased from 24 Pa to 71 Pa at 20˚C. The storage moduli of PVA hydrogels, fabricated in the presence of a PEG porogen, increased to 1863 Pa at 20˚C. However, incorporating all three components into the system during solidification, gelatin, PVA and PEG, the storage modulus increased to 5266 Pa at 20˚C, indicating the positive effect of PEG porogens on the mechanical response of PVA-gelatin hydrogels (**Figure 4A**).

After solidification, all hydrogels were dialyzed against water for one week to remove PEG porogens; sample groups which used PEG during fabrication will be donated as such. Oscillatory time sweeps were then performed to examine the mechanical properties of the hydrogels (**Figure 4B**). PVA and PVA-1Gel hydrogels exhibited similar mechanical properties overtime, each exhibiting storage moduli near 500 Pa. However, hydrogels fabricated in the presence of porogens exhibited much higher storage moduli near 4000 Pa. The stiffest material was the novel theta-gel, PVA-PEG-1Gel, with a storage modulus near 6000 Pa. Therefore, the use of porogens

during the solidification of PVA and gelatin resulted in large porous structures and stiffer scaffolds (**Figure 4B**).

The effect of frequency on the shear storage moduli was determined for various different monomers and hydrogels after dialyzing (**Figure 4C**). Oscillatory frequency sweeps were performed at 1% radial strain and 25˚C. The hydrogels exhibited steady-state behavior up to 10 Hz, after which the moduli increased in response to the increasing shear rate. Similar to the previous two experiments, the PVA-PEG-1Gel experimental group revealed the highest storage modulus compared to all of the other groups (**Figure 4C**).

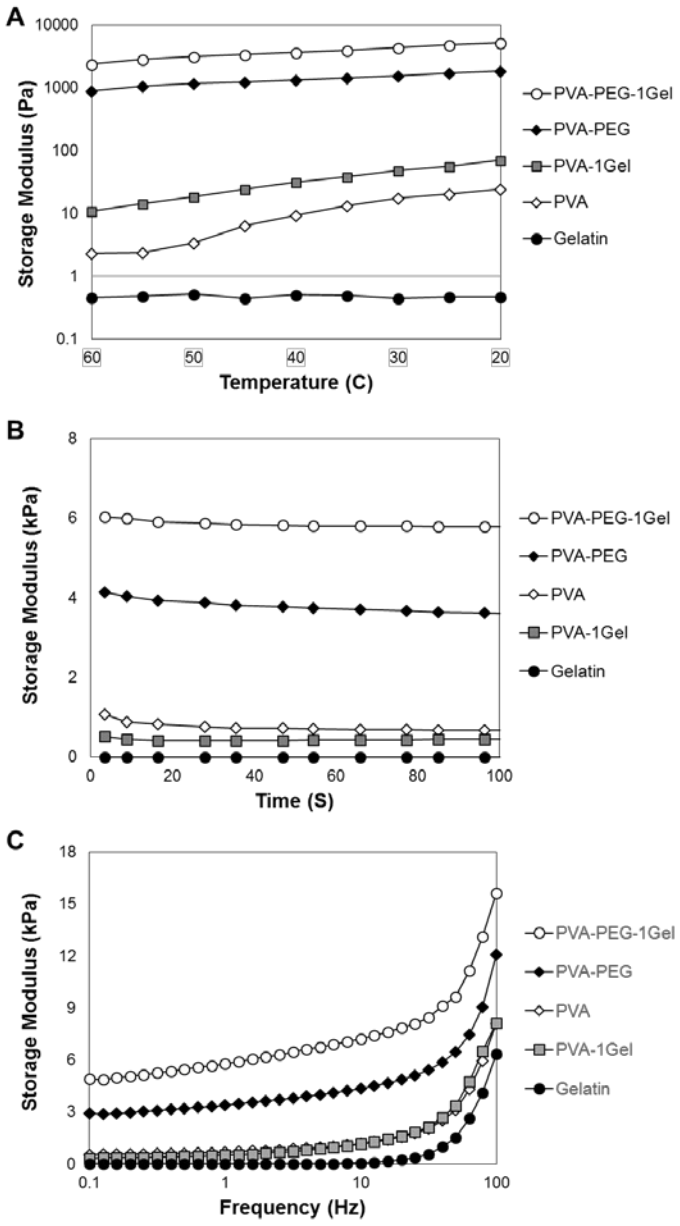


Figure 4. Rheological experiments were performed to examine the effect of PEG porogens during the solicitation of PVA-based hydrogels on the mechanical properties. A) Temperature sweeps of single macromers, control solutions (PVA-1Gel, PVA-PEG), and experimental solutions (PVA-PEG-1Gel) were tested from 60˚C to 20˚C to illustrate the gelation process of each sample. B) Oscillatory time sweep experiments for control hydrogels (gelatin, PVA, PVA-

1Gel, PVA-PEG), and experimental hydrogels (PVA-PEG-1Gel) were conducted after dialysis (i.e., removal of PEG porogens) at 25˚C. C) Oscillatory frequency sweeps were performed on hydrogels at 1% radial strain from 0.1 to 100 Hz at 25˚C.

PVA hydrogels with and without gelatin swelled > 100% after hydration (**Figure 5A**). The PVA molecular weight influenced water content; lower molecular weight PVA-gelatin hydrogels swelled less compared to higher molecular weight PVA-gelatin hydrogels, likely due to an increase in physical crosslinking. A fixed-effect tri-factorial model (e.g., PVA concentration, PVA molecular weight, and gelatin concentration) was generated to study the contribution of each factor to the hydrogel material properties. All three factors contributed significantly to the equilibrium water content of the hydrogel. The addition of gelatin increased the overall water content.

Weight loss was calculated to determine polymer mass lost due to hydrolysis. While some material was lost due to non-crosslinked PVA chains and degraded polymer during physical crosslinking (< 10%), indicated by the white bars in **Figure 5B**, the PVA-gelatin hydrogels, specifically the 18% gelatin samples, black bars in **Figure 5B**, lost more weight (2 – 31%). These results correlate with the FTIR data, which indicated that gelatin interrupted PVA crystallization during cooling, thus allowing more amorphous species to be lost during hydration (**Figure 2**).

Unconfined compression tests were performed on hydrogels directly after dialysis (i.e., removal of PEG porogens). Increasing the gelatin concentration created a stiffer hydrogel up to 5% (w/v); concentrations greater than 5% interfered with the formation of a network, resulting in a more compliant hydrogel (**Figure 5C**); PVA control hydrogels, fabricated with PEG, without gelatin, were too compliant to collect compressive moduli values, thus data is not shown. A fixed effect tri-factorial model (e.g., PVA concentration, PVA molecular weight, and gelatin concentration) was

generated to study the statistical contribution of each factor to the IPN hydrogels with a range of compressive elastic moduli. All three factors had significant contributions to the resulting moduli, among which PVA concentration interacted with PVA molecular weight, and PVA molecular weight interacted with gelation concentration: 18%PVA(H) ranged from 17 to 95 kPa, 36%PVA(H) ranged from 116 to 193 kPa, 18PVA(H) ranged from 26 to 92 kPa, and 36PVA(L) ranged from 189 to 351 kPa. The three factors were also interacting with each other, and contributing of the compressive moduli of the hydrogels. Overall, the formation of a crosslinked network of PVA and gelatin, to form a swollen, stiff hydrogel, was dependent on the PVA concentration and molecular weight, and the concentration of gelatin.

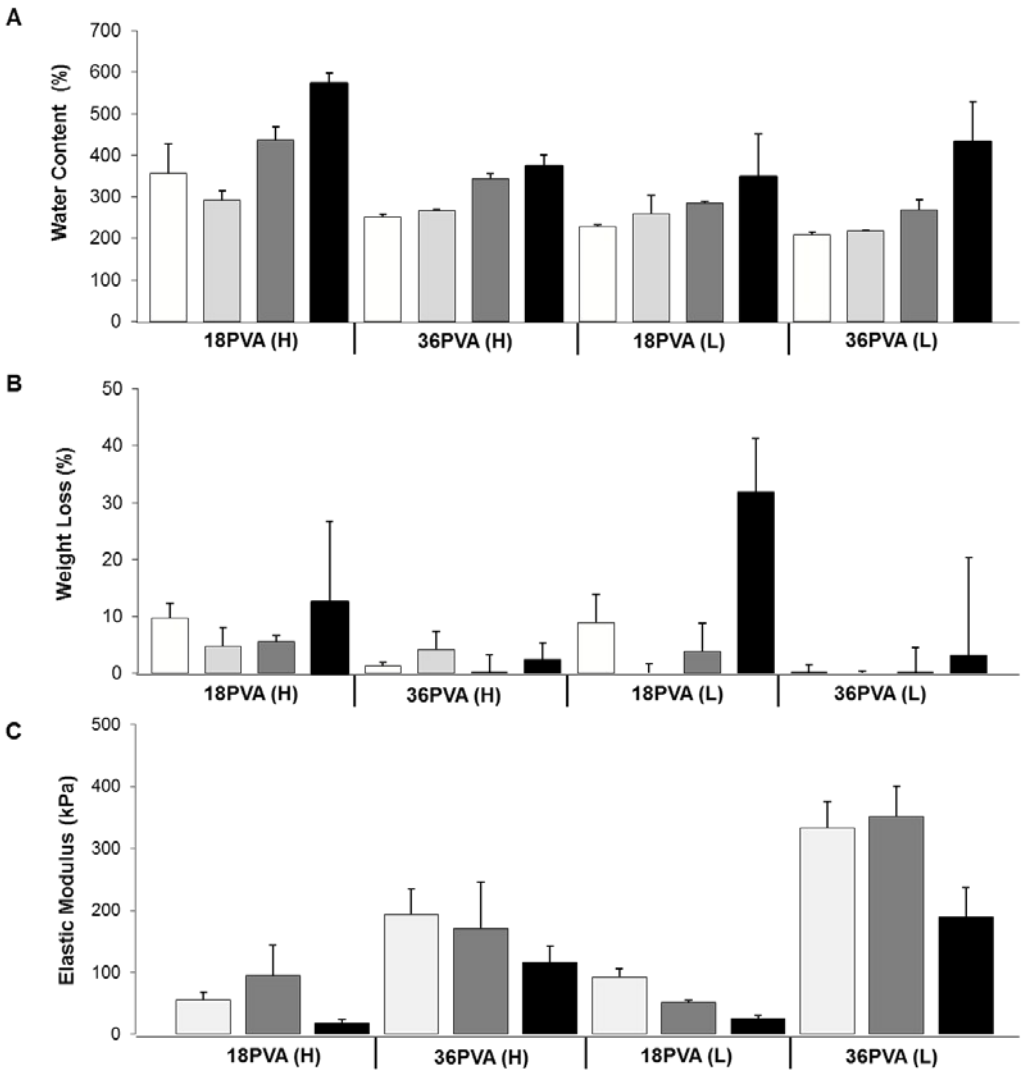


Figure 5. The physical and mechanical properties of PVA-gelatin hydrogels, developed using theta-gel techniques, are reported. PVA-gelatin hydrogels consisting of 18 and 36% (w/v) PVA, using low (L, 95 kDa) or high (H, 145 kDa) molecular weight PVA, were fabricated. Hydrogels also varied in gelatin content; white = no gelatin, light gray = 1% gelatin, dark gray = 5% gelatin, black = 18% gelatin. (A) To evaluate hydration, the equilibrium water content of PVA-gelatin hydrogels and PVA controls was calculated; lyophilized hydrogel samples were hydrated in phosphate buffered saline (PBS), pH 7.4, for 24 hours at room temperature. (B) Weight loss values were calculated after removal of the hydrogel samples from solution. Measurements were used to determine the loss of soluble low molecular species and hydrolytic degradation. (C) Unconfined compression tests were performed and elastic moduli of PVA-gelatin hydrogels were calculated using a linear regression of the stress-strain curve between 5 – 15% axial compressive strain; average ± standard deviation are reported (n = 4). PVA samples were left out of the study due to high compliance.

In Vitro Chondrogenic Differentiation

The bioactive properties of the PVA-gelatin hydrogels were investigated for cartilage tissue engineering applications. Two different PVA-gelatin hydrogel formulations were selected for *in vitro* cell culture based on the physical characterization and mechanical analysis (see **Figure 5**). Hydrogel formulations were chosen to reflect differences in elastic moduli, and with or without the addition of a cell-signaling molecule, type II collagen. Prior to chondrogenic differentiation, an *in vitro* MTT-based cytotoxicity assay was used to measure the mitochondrial activity of human MSCs after 24 hours of culture in the presence of PVA-based hydrogels in standard MSC culture media. All of the PVA-gelatin groups displayed non-cytotoxic effects and maintained mitochondrial activity levels > 75% (**Figure 6A**). There were no significant results among the groups; however, the cytotoxicity assay results indicate a dependence on gelatin content.

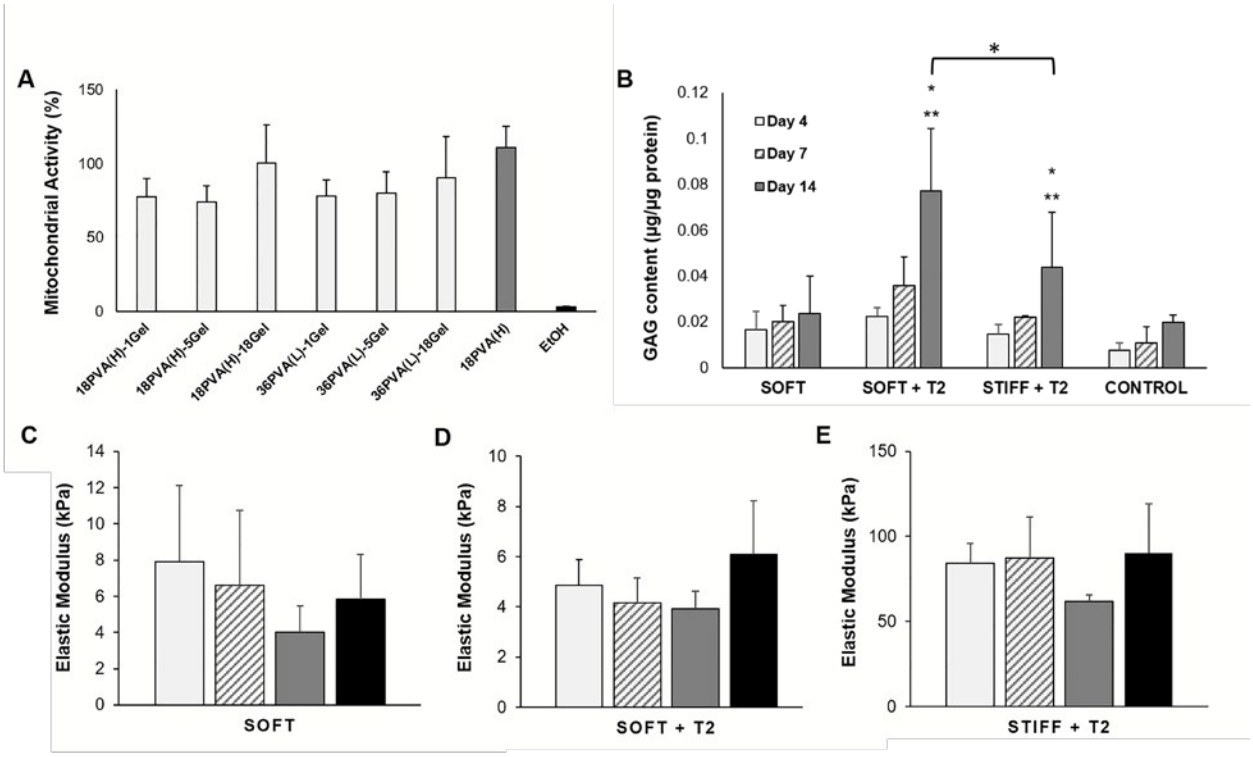


Figure 6. (A) Mitochondrial activity was determined using an MTT-based cytotoxicity assay. The absorbance values for PVA-gelatin and PVA hydrogels were normalized to non-modified cell controls cultured on tissue culture polystyrene; ethanol (EtOH) was used as a negative control. Confluent human MSCs were cultured with PVA-based hydrogels for 24 hours in standard MSC culture medium at 37 ˚C and 5% $C0_2$ (n = 4). (B) Sulfated GAG production was quantified for MSCs cultured in PVA-gelatin scaffolds and in non-modified treated polystyrene culture plates in chondrogenic media. Hydrogel experimental groups consisted of: 18PVA(H)-1Gel (soft), 18PVA(H)-1Gel + type II collagen (soft + T2), and 36PVA(L)-5Gel + type II collagen (stiff +T2); results are reported as average ± standard deviation (n = 4). (* = $p < 0.005$ compared to control samples at day 14; ** = $p < 0.03$ compared to day 4 results within the same sample group; † = $p< 0.004$ comparing day 14 SOFT+T2 and STIFF+T2 results.) Unconfined compression tests were performed and the elastic moduli of human MSC-seeded hydrogels and non-seeded hydrogel

controls were calculated using a linear regression of the stress-strain curve from 5 – 15% axial compressive strain. Hydrogel experimental groups consisted of: 18PVA(H)-1Gel (soft), 18PVA(H)-1Gel + type II collagen (soft + T2), and 36PVA(L)-5Gel + type II collagen (stiff +T2); results are reported as average ± standard deviation (n = 4).

The efficacy of using PVA-gelatin hydrogels, formed using theta-gels techniques, to support chondrogenic differentiation was evaluated by measuring initial matrix content production. The chondrogenic differentiation of the MSCs was measured using a dimethylmethylene blue (DMMB) assay 4, 7, and 14 days post-seeding onto PVA-gelatin hydrogels. PVA-gelatin hydrogel groups were selected according to elastic moduli values and two groups were chosen to reflect high and low moduli (soft ~ 10 kPa, stiff ~ 100 kPa). The presence of a cartilage matrix protein, type II collagen, was investigated; hydrogel groups varied in bioactivity (with or without type II collagen). Reports from the literature suggest that compliant hydrogels promote and/or enhance chondrogenic differentiation.[42-44] Therefore, the effect of the bioactive molecule was investigated in the soft hydrogel groups.

The intracellular sulfated glycosaminoglycan (GAG) content was measured in primary human MSCs cultured on PVA-gelatin hydrogel scaffolds in chondrogenic media over a period of 14 days. Soft PVA-gelatin hydrogels, without the addition of type II collagen, did not show any significant differences compared to non-modified cell controls at all three time points (**Figure 6B**). However, both soft and stiff hydrogels containing type II collagen significantly increased intracellular GAG content compared to the controls after 14 days of culture ($p < 0.005$). In addition, both the soft+ T2 and stiff+ T2 hydrogel groups significantly increased intracellular GAG content after 14 days compared to 4 days of culture ($p < 0.03$). The results also suggest that the soft + T2 hydrogel samples had a significantly greater influence on intracellular GAG production compared to the stiff + T2 hydrogels (p value = 0.0037). Thus, the combination of both a selective moduli value

and the presence of a bioactive molecule will significantly enhance the ability for PVA-gelatin hydrogels to support chondrogenic differentiation, and potentially tissue regeneration. While the two week duration of the *in vitro* is a limitation of the study, the efficacy of using PVA-gelatin hydrogels, which incorporate type II collagen, for cartilage tissue engineering applications is supported.

Unconfined compression tests were conducted on cell-seeded hydrogels to determine what effect the cells may have on the mechanical properties of their physical environment, i.e., the hydrogel scaffold. PVA-gelatin hydrogel controls were incubated in standard MSC culture media until testing. At day 4, 7, and 14 the elastic moduli of the MSC-seeded experimental groups were not significantly different compared to the controls (**Figure 6**, day 4 = light gray bars, day 7 = striped gray bars, day 14 = dark gray bars, hydrogel controls = black bars). However, a trend showing the decrease in elastic moduli of cell-seeded hydrogels with time may reflect the cells remodeling the hydrogel scaffold during differentiation. Longer time points may reveal significant data, and future work will examine degradation and the dynamic mechanical properties of cell-seeded hydrogels.

EXPERIMENTAL

Fabrication of PVA-Gelatin Theta-Gels

PVA-gelatin theta-gels, i.e., hydrogels, were prepared using different molecular weights and concentrations of PVA, PEG, and gelatin. PVA (Mw = 145 kg/mol (H) and 95 kg/mol (L), Sigma Aldrich) was combined with 20% (w/v) PEG (Mw = 400 g/mol, Sigma Aldrich) in DI water. PVA concentrations, for both molecular weights, were 18 and 36% (w/v). Experimental groups contained gelatin (Sigma Aldrich) at 1, 5, and 18% (w/v). The control and experimental polymer blend solutions were autoclaved for 1 hour and the warm, homogenous solutions were transferred to curing molds consisting of glass slides and 3.2 mm thick Teflon spacers, preheated to 90 °C.

The molds containing the polymer solutions were cooled to room temperature and cured for 24 hours. The hydrogel sheets were removed from the molds and dialyzed in DI water for 5 days to remove PEG porogens; dialysis water was changed every 12 hours.

Fourier-Transform Infrared (FTIR) Spectroscopy

All sample hydrogels were prepared prior to performing spectroscopy: 18% (w/v) PVA, high molecular weight, and 1% (w/v) gelatin solutions were prepared and used as controls, 20% (w/v) PEG was used for all theta-gels. The hydrogels were dialyzed for 5 days, to ensure removal of the porogen and air-dried after dialysis and cut into small pieces. Then they were tested using Thermo-Nicolet IR200 FTIR spectrometer with an attenuated total reflectance (ATR) head for 32 scans.

Rheological Characterization

All experiments were performed using an AR2000 stress-controlled rheometer (TA Instruments) fitted with a 20 mm diameter steel cone geometry at 25°C, however, the temperature sweep study included a temperature range. All sample solutions were prepared prior to performing the temperature sweep: 18% (w/v) PVA, high molecular weight, and 1% (w/v) gelatin solutions were prepared and used as controls, 20% (w/v) PEG was used for all theta-gels. The hot solutions were transferred to the rheometer directly after heating to collect the gelation data via a temperature sweep, which was performed at 1 Hz and 1% strain, with a cooling rate of -5°C*min^{-1} from 60 to 20°C. After all of the hydrogels were cooled down and crosslinked, they were dialyzed for 7 days to remove the PEG porogens. Additional oscillatory time and frequency sweeps were performed to test the storage moduli under various conditions. Oscillatory time sweeps for experimental (PVA-PEG-1Gel) and control hydrogels (gelatin, PVA, PVA-1Gel) were tested directly after hydrolysis, and were deformed at 1% radial strain and 10 Hz over 100 s. Oscillatory

frequency sweeps were performed at 0.5% radial strain with increasing frequency from 0.1 to 100 Hz.

Scanning Electron Microscopy

Hydrogel samples, containing PVA and PEG, with and without gelatin, were flash frozen in liquid nitrogen after dialysis in DI water, cryo-fractured, lyophilized, and characterized by scanning electronic microscopy (SEM, JEOL 600). Samples were sputter coated with 10 nm of Au-Pd prior to imaging. SEM was used to quantify pore diameters and characterize the inner structure of PVA-PEG-Gel hydrogel cross-sections compared to PVA-PEG controls.

Van Geison Staining

To characterize the retention of gelation, hydrated hydrogels were placed in Van Geison staining solution (ThermoFisher) for 2 minutes and rinsed in DI water several times until the water remained clear. PVA-PEG-Gel hydrogels consisting of 18 and 36% (w/v) PVA, using low (L, 95 kDa) or high (H, 145 kDa) molecular weight PVA, and 18% (w/v) PEG, were fabricated. Hydrogels also varied in gelatin content: 1, 5, and 18% (w/v). Control groups included PVA-PEG hydrogels with no gelatin content. Samples were dried at room temperature, and color images were taken using a digital camera.

Equilibrium Water Content and Weight Loss

Cylindrical hydrogel specimens (6 mm x 3 mm) were lyophilized to ensure all moisture was alleviated in preparation for equilibrium water content measurements and weight loss values. The dehydrated specimens were weighed, then placed in 5 mL PBS, pH 7.4, to rehydrate the scaffolds for 48 hours at 37 °C. Each specimen was weighed, and the wet weight was recorded. Equilibrium water content was calculated as the percentage of wet weight divided by initial dry weight. After each specimen's wet weight was recorded, each specimen was again froze to prepare for

lyophilization. Each specimen was then lyophilized for 24 hours. A second dry weight was then recorded to test for how much weight was lost through the dehydration process. The weight loss was calculated as final mass subtracted from the initial mass, divided by the initial mass. Dry scaffolds were weight to determine the initial amount of polymer. To determine the amount of non-crosslinked polymer, scaffolds were hydrated for 24 hours in order to dissociate non-crosslinked PVA and gelatin. Scaffolds were then lyophilized and massed. To determine the polymer loss due to hydration and diffusion of non-crosslinked molecules, the mass of a dry sample after the crosslinking process was performed was measured.

Unconfined Compression Testing

The unconfined compressive moduli of various hydrogel groups were determined directly after dialysis. Cylindrical hydrogel specimens (6 mm x 3 mm) were tested at 25 ˚C using an AR2000 rheometer (TA Instruments) equipped with a Peltier plate and normal force transducer. Specimens were placed on the rheometer and the geometry was lowered until a force of 0.01 N was measured, and the force was normalized. The gap height was recorded as the original gage length for the modulus calculation. A 20% uni-axial compressive strain was applied at a rate of 10 μm/s. Force (N) and changes in gap height (μm) were obtained using analytical software (TA Universal Analysis) and were subsequently used to calculate elastic strain (ε, %) and stress (σ, kPa). The elastic modulus (E) was calculated as the slope of a linear fit between 5 and 15% compressive strain within the linear-elastic region. A minimum of four replicates from each group were tested.

Cytotoxicity Assay

The cytotoxicity of PVA-gelatin hydrogels to primary bone-marrow derived human MSCs was assessed as a function of mitochondrial activity in living cells. MSCs were seeded in treated 48-well tissue culture polystyrene (TCPS) plates at a density of 20,000 cells/well in 100 μL/well of

standard MSC growth media (alpha minimum essential medium (MEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin) and allowed to adhere for 24 hours. MSCs were incubated in the presence of various PVA-gelatin hydrogels (n = 4) at 37 °C and 5% CO_2. Spherical hydrogel specimens were 4 mm wide and 2 mm tall. After 24 hours of incubation, media was removed and cells were rinsed two times in sterile PBS. Mitochondrial activity was analyzed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) based *In Vitro* Toxicology Assay Kit (Sigma Aldrich) following the manufacturer's protocol with a plate reader (H1 Synergy, BioTek). Absorbance values were recorded at 570 nm with background absorbance at 690 nm deducted. Average absorbance values for the experimental samples were compared to positive cell control values recorded for wells containing media and cells alone.

Chondrogenic Differentiation

PVA-gelatin hydrogels selected for cell-seeding were coated with type II collagen using the following protocol. After fabrication and dialysis, PVA-gelatin hydrogels were formed into cylinders, 3 mm x 2 mm, using a 4mm biopsy punch. The hydrogels (soft and stiff scaffold groups) were immersed in type II rat collagen (50 mg/mL, Sigma-Aldrich) in phosphate buffered saline (PBS), which was pre-filtered to obtain cell culture sterile solution, for 1 hour at room temperature.[45] The hydrogel samples were removed from the collagen solution, rinsed in sterile PBS, and prepared for cell culture.

Primary bone-marrow derived human MSCs (Lonza), passage 4, were seeded onto PVA-gelatin hydrogels with or without type II collagen. 38 µL of 700,000 cells suspended in standard MSC culture media were placed onto cylindrical hydrogel scaffolds (3 mm x 2 mm), and the scaffolds were placed in 96-well TCPS plates. Cell-seeded scaffolds were incubated at 37 ˚C, 5% CO_2 for 30 minutes, and then the wells were supplemented with 150 µL of chondrogenic media (standard

MSC growth media supplemented with 10 ng/mL of human transforming growth factor beta one (TGF-β1, Peprotech)).[11]

Dimethylmethylene Blue (DMMB) Assay

Intracellular sulfated GAG content was quantified using a DMMB assay.[11] Cell lysate was collected after 4, 7, and 14 days of culture. Scaffolds were removed from tissue culture plates, trypsin-EDTA was added, and the scaffolds were incubated for 5 minutes. Equal volumes of standard MSC culture media were added to the cell suspensions, cell suspensions were placed in micro-centrifuge tubes, and centrifuged at 4000 rpm for 2 minutes. The supernatant was collected and 150 µL of cell lytic solution was added. Standard chondroitin sulfate solutions with concentrations ranging from 0 to 30 µg/mL were used to form the standard curve. Using a 96-well plate, 25 µL of cell lysate sample or standard was added to each well. Next, 150 µL of DMMB solution was added to each well. The absorbance at 525 nm was measured. Controls were MSCs cultured in chondrogenic media in 96-well tissue culture treated polystyrene plates.

GAG production was normalized to the cell population per sample using intracellular protein and the Pierce™ Protein Assay Kit (ThermoFisher). Briefly, standard bovine serum albumin (BSA) solutions with concentrations ranging from 20 to 2000 µg/mL were used to form the standard curve. Using a 96-well plate, 25 µL of cell lysate sample or standard was added to each well. Next, 175 µL of working solution was added to each well. The absorbance at 562 nm was measured. Controls were MSCs cultured in chondrogenic media in 96-well tissue culture treated polystyrene plates.

***In Vitro* Mechanical Testing**

MSC-seeded scaffolds were tested in unconfined compression using the same methods detailed above. Briefly, cylindrical scaffolds (3 mm x 2 mm) were subjected to uni-axial unconfined compressive strain at 10 μm/s to a final strain of 20%. Samples were tested after 4, 7, and 14 days of culture with MSCs in chondrogenic media. At day 14, non-seeded PVA-gelatin hydrogel controls were mechanically tested as degradation controls.

Statistical Analysis

All experiments were performed in triplicate with results reported as mean ± standard deviation. Statistical analysis was performed with a GLM procedure using Statistical Analysis System software. A fixed effect multi-factorial (concentration, molecular weight, and gelatin concentration, eliminated inside the model if one of the factor is not significant relevant) model was generated to study the contribution of each factor to PVA-gelatin hydrogels with a range of compressive elastic moduli, swell ratio and weight loss based on ANOVA analysis. The multiple comparisons were performed with Tukey adjustment. For analyzing cytotoxicity, similar GLM procedures were preformed to obtain one-way ANOVA results. A $p < 0.05$ was considered significantly different for all analysis. GAG assay results were analyzed using a one-way ANOVA. In addition, multiple comparison of each treatment group of gag content at different day point were performed with Fisher's LSD Method (Protested T-test).

Conclusion

Cell-instructive PVA-gelatin hydrogels were fabricated in the presence of a PEG porogen to enhance network integrity (i.e., physical entanglement of gelatin and PVA during solidification). Theta-gels with macro-porous structures formed through the physical interactions of PVA and gelatin, and supported chondrogenic differentiation of MSCs and cartilage matrix deposition (i.e., GAGs) in the presence of chondrogenic media. Adding gelatin to PVA hydrogels formed in the presence of PEG porogenssignificantly increased the hydrogel stiffness and pore size.[34] Bio-

synthetic IPN hydrogels were formed using theta-gel techniques and are promising candidates for cartilage regeneration scaffolds due to their large pore diameters (10 – 50 μm), moderately high compressive elastic moduli (20 – 400 kPa), and ability to significantly increase chondrogenesis. Future work will investigate the role of the gelatin during cell culture and longer *in vitro* culture time points. In addition, the effect of dynamic culture will be investigated.

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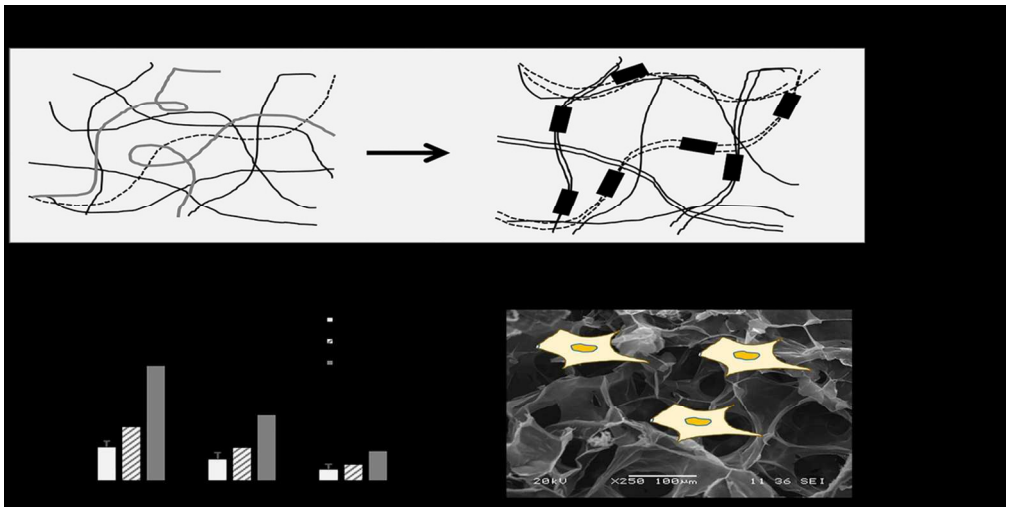

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