Effects of cryo-processing on the mechanical and biological properties of poly(vinyl alcohol)-gelatin theta-gels

Cite as: *Biointerphases* **15**, 051004 (2020); doi: [10.1116/6.0000381](https://doi.org/10.1116/6.0000381)

Submitted: 8 June 2020 · Accepted: 31 August 2020 ·

Published Online: 22 September 2020

Patrick N. Charron,¹ Jaime I. Jacobs,² Selina X. Yao,¹ and Rachael A. Oldinski^{1,3}

AFFILIATIONS

¹Department of Mechanical Engineering, University of Vermont, Burlington, Vermont 05405

²Civil and Environmental Engineering Department, University of Vermont, Burlington, Vermont 05405

³Department of Electrical and Biomedical Engineering, University of Vermont, Burlington, Vermont 05405

ABSTRACT

Poly(vinyl alcohol) (PVA), a synthetic, nontoxic polymer, is widely studied for use as a biomedical hydrogel due to its structural and physico-mechanical properties. Depending on the synthesis method, PVA hydrogels can exhibit a range of selected characteristics—strength, creep resistance, energy dissipation, degree of crystallinity, and porosity. While the structural integrity and behavior of the hydrogel can be fine-tuned, common processing techniques result in a brittle, linear elastic material. In addition, PVA lacks functionality to engage and participate in cell adhesion, which can be a limitation for integrating PVA materials with tissue *in situ*. Thus, there is a need to further engineer PVA hydrogels to optimize its physico-mechanical properties while enhancing cell adhesion and bioactivity. While the inclusion of gelatin into PVA hydrogels has been shown to impart cell-adhesive properties, the optimization of the mechanical properties of PVA-gelatin blends has not been studied in the context of traditional PVA hydrogel processing techniques. The incorporation of poly(ethylene glycol) with PVA prior to solidification forms an organized, cell instructive hydrogel with improved stiffness. The effect of cryo-processing, i.e., freeze-thaw (FT) cycling was elucidated by comparing 1 FT and 8 FT theta-cryo-gels and cryo-gels. To confirm the viability of the gels, human mesenchymal stem cell (hMSC) protein and sulfated glycosaminoglycan assays were performed to verify the nontoxicity and influence on hMSC differentiation. We have devised an elastic PVA-gelatin hydrogel utilizing the theta-gel and cryo-gel processing techniques, resulting in a stronger, more elastic material with greater potential as a scaffold for complex tissues.

Published under license by AVS. <https://doi.org/10.1116/6.0000381>

I. INTRODUCTION

Poly(vinyl alcohol) (PVA), a synthetic and nontoxic polymer, is often researched as a soft tissue replacement and cell scaffold owing to its high water content and tunable mechanical properties.^{1–4} Multiple techniques are used to adjust the physico-mechanical properties of PVA, many of which depend on a thermal transition wherein noncovalent intermolecular bonds form during the PVA crystallization process upon cooling to below the freezing temperature of water, resulting in a physically cross-linked polymer network with a porous structure.^{5–10} PVA hydrogels can be further tuned via chemical modification of the PVA backbone or by modification of the hydroxyl groups.^{11–16} One limitation of direct PVA chemical modification is the potential for adverse functional groups to induce toxicity of the hydrogel; therefore, nonchemical modifications can be preferred, despite the decreased thermal stability.^{14,16,17} Crystallinity can be

enhanced through the incorporation of low-weight porogens into the system, which undergo a phase separation from PVA during the thermal transition (i.e., reduction in temperature), resulting in PVA theta-gels with dense PVA regions and increased pore size.^{18–20} Finally, the resulting macroporous structure allows for the removal of the porogen via dialysis.^{19,20} Cryo-processes have also been explored, which form PVA cryo-gels (CGs) through repeated freeze-thaw (FT) cycling of the material, imbuing a semicrystalline structure through the formation of ice crystals within the system. The formation and expansion of ice crystals results in the assembly of PVA molecules into a crystalline structure (inducing secondary bond formation) that is fine-tuned by controlling the number of FT cycles and the freezing temperature.^{21–23}

Gelatin is a thermoresponsive polymer derived from collagen capable of forming uniformed complex structures^{24–26} and

promoting cell adhesion and viability.²⁷ The addition of gelatin to the PVA cryo-gel during formation has been previously shown to increase the resilience and elasticity of the cryo-gels due to the bond formation between the PVA and gelatin components during processing.^{10,19,20} PVA-gelatin theta-cryo-gels (TCGs) were fabricated utilizing both the theta-transition imparted by the incorporation of a polyethylene glycol (PEG) porogen into the system, followed by repeated FT cycles to modify the structure-function relationships of the material.

This study expands on a previous study that focused on the fabrication and mechanical characterization of PVA-based theta-cryo-gels.¹⁰ The current study focuses on the tunability of the material properties and the ability of the material to support human mesenchymal stem cell (hMSC) growth and soft tissue matrix component production. Chemical and mechanical characteristics were compared to observe how the material can be altered via cryo-processing methods. Cell studies were performed to ascertain the cytotoxicity and bioactive support of the hydrogel materials. Substrate mechanics, physical properties, and growth factor-supplemented cell culture media were varied to support tissue formation. We hypothesized that the addition of enhanced cryo-processing methods would increase the physical cross-linking and overall stiffness, while also increasing the energy dissipation of the hydrogel system due to viscoelastic properties, allowing for tunable mechanics for soft tissue replacement.

II. MATERIALS AND METHODS

A. PVA-gelatin TCG and CG fabrication

PVA-gelatin TCGs and CGs were prepared using base solutions previously reported.^{10,19,20} High molecular weight PVA (145 kg/mol, 99+% hydrolyzed), PEG (400 g/mol), and gelatin (bovine derived, type B powder) were purchased from Sigma-Aldrich. To make TCGs, a 15% (w/v) PVA solution supplemented with 1% (w/v) gelatin and 15% (w/v) PEG was prepared in de-ionized (DI) water and uniformly heated to 105 °C until homogeneous. While hot, the solution was poured into open-faced 3-mm tall molds and cooled overnight at room temperature, initiating the theta-gel transformation and PEG phase separation during the thermal transition; solutions without PEG did not form theta-gels. Next, the materials were placed in a −20 °C freezer overnight to initiate the cryo-gel transformation, i.e., cryo-processing was conducted specifically at temperatures below the freezing temperature of water to allow the formation of ice crystals. The materials were removed from the freezer and then allowed to thaw at room temperature for ~2 h. Half of the hydrogels were further processed for use and were subsequently identified as the 1 FT group. To explore the effect of additional cryo-processing cycles, the remaining half of the hydrogels were subsequently frozen and thawed for an additional seven cycles in a similar manner and are subsequently identified as the 8 FT group. The resulting TCGs from the 1 FT and 8 FT groups were dialyzed in DI water for three days to remove any remaining PEG porogen and stored at 4–8 °C prior to testing. CGs were processed in an identical fashion to the TCGs, with PEG being excluded from the initial solution to prevent the theta-transition effects.

B. Van Gieson staining

To verify the retention of gelatin in the hydrogels after fabrication, TCG and CG 1 and 8 FT groups were stained with a Van Gieson dye. Hydrogel specimens (8-mm diameter, 3.0 ± 0.5-mm thick) were placed in a Van Gieson solution (Electron Microscopy Sciences) and allowed to equilibrate for 5 min. The specimens were removed from the staining solution and rinsed in DI water until no stain leached from scaffolds. Color images were captured using a digital camera.

C. Swell ratio

In preparation for swell ratio measurements, rectangular hydrogel specimens were prepared and characterized following ASTM D2765-11.²⁸ Dehydrated specimens were weighed and a dry weight (W_D) was recorded. The specimens were then submerged in DI water at room temperature for 24 h. The hydrated hydrogels were weighed and a wet weight (W_W) was recorded. Hydrogel swell ratio percentages were calculated as the difference between the wet weight and the initial dry weight, divided by the dry weight and multiplied by 100,

$$\text{Swelling Ratio} = \frac{W_W - W_D}{W_D} \times 100.$$

D. ATR-FTIR spectroscopy

Chemical characterization was performed on the TCG and CG 1 and 8 FT groups to identify the presence of PVA, gelatin, and the formation of physical cross-links (i.e., crystal formation) between PVA itself and between PVA and the blended gelatin. Prior to characterization, specimens were dried via lyophilization (FreeZone, Labconco). Spectra were obtained using an FTIR (IRAffinity-1S, Shimadzu) with an ATR head. Spectra were collected from 800–4000 cm^{-1} at a resolution of 2 cm^{-1} .

E. Uniaxial unconfined compression testing

Uniaxial unconfined compression tests were performed using a DHR-3 rheometer at room temperature, using a Peltier plate and 8-mm-diameter stainless steel plate geometry. TC-gel samples (8-mm diameter, 3.0 ± 0.5-mm thick) were loaded onto the Peltier plate and a pre-load of 0.01–0.03 N was applied. Samples were compressed at 0%–15% unconfined axial; the axial strain rate was 10 $\mu\text{m/s}$. Data were analyzed using analytical software (TA DATA ANALYSIS). The compressive elastic modulus (E) was calculated using the linear region of the loading curve from the initial cycle of the hysteresis test. The area between the loading and unloading curves from the cyclic hysteresis testing was calculated using a MATLAB code and the trapezoidal numerical integration (TRAPZ) function.

F. Uniaxial tensile testing

Tensile tests were performed on a benchtop testing machine (1000M, TestResources) at room temperature. Rectangular films were cut from cast hydrogels to obtain standard samples (30 × 20 mm) with a uniform thickness of 3 mm. Samples were

clamped in position and axially strained at a rate of 500 $\mu\text{m/s}$ until failure. Engineering axial stress and strain were calculated from force and displacement data. The tensile Young's modulus (E) was calculated using the linear region (20%–40% strain) of the loading curves. Percent elongation was calculated from initial and final clamp positions.

G. hMSC cytotoxicity

Human bone marrow derived MSCs (RoosterBank™-hBM-1M, MSC-004, RoosterBio) were received frozen at passage 1 and stored in liquid nitrogen. Cells were thawed and placed in T75 tissue culture polystyrene flasks with 13 ml of basal medium (alpha minimum essential medium, 10% MSC-screened fetal bovine serum, and 1% antimycotic/antibiotic). Cells were incubated at 37 °C, 5% CO_2 , and the medium was changed every 2–3 days until the cells reached 80% confluency. Trypsin-EDTA was added and cells were incubated at 37 °C, 5% CO_2 , for 8 min. Cell solution was diluted with 8 ml of basal medium and centrifuged at 600g for 5 min. Cell pellets were resuspended in the basal medium, diluted in trypan blue, and viable cells were counted using a hemocytometer. Cells were then seeded in T75 flasks as described above. hMSCs at passages 2–3 were used in this study.

The cytotoxicity of the PVA-based hydrogel samples was evaluated using a lactose dehydrogenase (LDH) cytotoxicity assay kit according to the manufacturer's instructions (Thermo Scientific™ Pierce 88954, Fisher Scientific). PVA hydrogel specimens were placed in trans-well plates after equilibrating in the basal medium at 37 °C. hMSCs were seeded into 24-well tissue culture treated plates at 6000 cells/cm². After 24 h of culture, 50 μl of each sample medium and 50 μl of the reaction mixture was added to a well in a 96-well plate. An LDH positive control was created by adding a 10 $\times$ lysis buffer (Cell Lytic™, Sigma) to blank cells and incubating for 45 min. An LDH positive control, nontreated cells, and spontaneous LDH activity controls (DI water-treated cells) were analyzed in the same fashion as the samples. The plate was incubated for 30 min and then 50 μl of stop solution was added. The absorbance was measured at 490 and 680 nm using a plate reading spectrophotometer. The corrected LDH activity for each cell control and hydrogel sample was determined as the difference between absorbance values measured at 490 and 680 nm, and the average was calculated ($n = 6$). The cytotoxicity (%) was calculated by subtracting the spontaneous LDH activity control from the samples and LDH positive control, and then dividing the samples by the LDH positive control and multiplying by 100. The assay was performed in triplicate.

H. hMSC short-term bioactivity study

PVA-based hydrogel material slabs were punched into 3-mm-diameter disks, disinfected with 70% ethanol, washed with sterile phosphate buffered saline (PBS), and soaked in the basal medium for 30 min. Meanwhile, a chondrogenic differentiation medium was prepared as previously reported.^{19,29} Briefly, recombinant human transforming growth factor beta 1 (TGF- β 1, HEK293 derived, 100-21, PeproTech) was reconstituted to a concentration of 10 ng/ μl in 4 mM hydrochloric acid (HCl) supplemented with

1 mg/ml bovine serum albumin, and sterile filtered. Prepared hydrogel specimens were placed into individual wells of 48-well tissue culture treated polystyrene plates. Next, each hydrogel specimen was seeded with 300 000 hMSCs in either 900 μl of basal media or TGF- β 1 (10 ng/ml) supplemented media. Cells were maintained in a cell culture incubator at 37 °C, 5% CO_2 , for a total time of 14 days; basal and chondrogenic media, respectfully, were replaced every 2–3 days.

1. sGAG binding assay

The production of sulfated glycosaminoglycan (sGAG) by hMSCs cultured in either basal or chondrogenic differentiation medium was assessed to evaluate the potential for soft tissue matrix production.^{19,29,30} After 1, 7, and 14 days of cell culture, the medium was aspirated off each well and hydrogel specimens ($n = 4$) were rinsed with sterile PBS. Specimens were transferred to new wells with 200 μl of 10 $\times$ lysing solution; the specimens were incubated for 20 min at room temperature and then frozen at –20 °C until use. A standard curve was created using chondroitin sulfate solutions with known concentrations ranging from 0 to 30 $\mu\text{g/ml}$. Using a 96-well plate, cell lysate or standard solution (50 μl) was added to 150 μl of 1,9-dimethylmethylene blue (DMMB) solution, consisting of 16 mg DMMB, 3.04 g glycine, 2.37 g sodium chloride, 95 ml 0.1M HCl, and 1L distilled water. The absorbance was measured at 525 nm using a spectrophotometer (Synergy H1, BioTek). The assay was performed in triplicate.

2. Protein assay

To normalize the sGAG production to cell protein content, a protein assay was performed, and the sGAG mass was divided by the protein mass. Cellular protein concentration was measured using the cell lysate (see above) collected after 1, 7, and 14 days of cell culture. The total protein content of the lysate was measured using a commercially available kit (Pierce® BCA Protein Assay Kit, Thermo Scientific™23227) following the manufacturers'

FIG. 1. Swell ratio (%) depicted as average $\pm$ standard deviation ($n = 3$); significant difference ($p < 0.05$) was observed between all groups except TCG 1 FT-TCG 8 FT. (Top) Van Gieson stained hydrogel samples confirmed the retention of gelatin after the theta-gel and cryo-processing techniques.

FIG. 2. ATR-FTIR spectra between 975–1175 and 2750–3625 cm^{-1} indicating increased bonding and spectra shift due to material processing techniques (i.e., physical cross-linking and hydrogen bond formation between PVA and gelatin).

instructions. The final reaction volume of $210\mu\text{l}$ in each sample well was composed of $200\mu\text{l}$ of working solution and $10\mu\text{l}$ of the cell lysate or standard solution. Absorbance of the solution was measured using a spectrophotometer (Synergy H1, BioTek) at a wavelength of 562 nm and then converted to protein content using the standard curve to determine the amount of intracellular protein. The assay was performed in triplicate.

I. Statistics

Swell ratio and results from the compression and tensile testing are presented as mean $\pm$ standard deviation. The LDH and sGAG assay results from the cell study are presented as mean $\pm$ standard error of the mean. Mechanical and cell analyses were performed using a t-test with an unpaired two-tailed distribution and two-sample unequal variance (type 2), where $p \leq 0.05$ was statistically significant, and verified with single factor one-way analysis of variance.

III. RESULTS AND DISCUSSION

A. PVA-gelatin TCG and CG characterization

Herein, the physicochemical characteristics of PVA-gelatin TCGs and CGs for biomedical applications were investigated. Traditional cryo-processing techniques, which depend on a thermal transition wherein noncovalent intermolecular bonds form during the PVA crystallization process upon cooling below the freezing temperature of water, were incorporated into the hydrogel fabrication process resulting in a physically cross-linked polymer network with a porous structure.^{5–10} Swell ratio data show slightly higher swell ratios in the TCG groups compared to the CG groups, suggesting that the TCG groups are both hydrophilic and exhibit a cross-linked network with structural integrity (Fig. 1). However, the 1 FT groups have slightly increased values compared to the 8 FT groups, though this trend is less pronounced in the TCG groups. While these mean differences exist, the swell ratios may also depend on the amount of gelatin held within the hydrogel during

FIG. 3. (a) Compressive modulus (kilopascal) was calculated using the linear elastic region of a one-cycle hysteresis curve, generated from unconfined compression testing. Results reported as average $\pm$ standard deviation ($n = 3$); significant difference ($p < 0.05$) was observed between all groups except CG 8 FT–TCG 8 FT. (b) Energy dissipated (i.e., area between) loading and unloading curves of one-cycle hysteresis testing reported as average $\pm$ standard deviation ($n = 3$); significant difference ($p < 0.05$) was observed between all groups except CG 8 FT–TCG 8 FT. (c) Percent energy dissipated in one-cycle hysteresis loop, reported as average $\pm$ standard deviation ($n = 3$); significant difference ($p < 0.05$) was observed between CG 1 FT–CG 8 FT and TCG 1 FT–CG 8 FT.

FIG. 4. (a) Young's modulus (kilopascal) was calculated from the linear elastic regime of a stress-strain curve, generated from tensile loading to failure, and reported as average $\pm$ standard deviation ($n = 3$); significant difference ($p < 0.05$) was observed between all groups except CG 1 FT-TCG 1 FT and CG 8 FT-TCG 8 FT. (b) The elongation percent to material failure under uniaxial tensile loading to failure, reported as average $\pm$ standard deviation ($n = 3$); no significant difference ($p < 0.05$) was observed.

the fabrication process; a limitation of the study is that the gelatin content was not quantitatively evaluated. However, the retention of gelatin was qualitatively assessed via Van Gieson staining. All the samples retained dye after washing (Fig. 1, top), indicating the presence of gelatin. The intensity between the images is similar, suggesting that neither the incorporation of porogens nor increased FT cycles had a deleterious effect on the gelatin content.

ATR-FTIR spectra for the experimental groups are shown in Fig. 2. Physical cross-linking between PVA and gelatin was verified by the increase in interchain hydrogen bonds between the hydroxyl groups formed during crystallization, associated with the peak at 1141 cm^{-1} and peak formations within the $1090\text{--}1150\text{ cm}^{-1}$ region.^{10,19,21} Peak intensity was influenced by C–O stretching vibrations within the crystalline regions. These peaks are exaggerated in the TCG groups compared to the CG groups, suggesting enhanced intermolecular hydrogen bonding between the PVA and gelatin components. These observations are supported by a slight shift in the spectra data in groups containing a porogen (i.e., TCGs), evidenced by the peaks near 2915 and 3315 cm^{-1} . This is further supported by the increase in mechanical properties within these groups, shown in Sec. III B.

B. Mechanical analysis

After chemical characterization, unconfined compressive and tensile tests were performed to elucidate the effects of various processing techniques on the materials' mechanical response. Hydrogels fabricated with more FT cycles had significantly increased compressive moduli for both cryo- and theta-cryo-gels [Fig. 3(a)]. The increase in modulus can be attributed to increased crystallinity, a function of the porogen and FT techniques. The addition of the porogen significantly increased the modulus in the 1 FT group, due to the increased crystallinity imbued by the porogen during the theta-transition. The difference in moduli diminished in higher FT groups, as the FT mechanics dominate the increase due to the porogen. Similar trends appeared after comparing the amount of energy the materials dissipated during a one-cycle hysteresis test [Fig. 3(b)]. Energy dissipation is heavily influenced by the compressive moduli, as

stiffer materials require more energy to displace during testing. No significant differences were observed between the 8 FT groups, further indicating that the FT process dominates the theta characteristics at higher FT cycles. Interestingly, these trends do not appear when comparing the overall percentage of energy dissipated in the material during testing [Fig. 3(c)]. Compressive data suggest the ability to greatly alter material properties by varying theta- and cryo-processes independently. In addition, the effects of the theta-process are overpowered by the cryo-process at higher FT cycles.

A significant difference in Young's moduli was observed between the low and high FT hydrogel groups after tensile testing [Fig. 4(a)]. The addition of PEG, i.e., use of a porogen, effected the material structure by promoting densification and subsequent bonding between PVA and gelatin molecules, thereby increasing tensile modulus. Similarly, multiple FT cycles induce secondary bond formation and crystallinity, increasing the hydrogel

FIG. 5. LDH assay was performed on PVA-gelatin CG and TCG groups after cryo-processing for one or eight FT cycles. Cytotoxicity (%) was calculated reported as average $\pm$ standard deviation ($n = 4$); no significant difference ($p < 0.05$) was observed.

modulus as well. The effects of the porogen are dominated by FT cycling at higher FT cycles, with no significant difference between the 8 FT groups. No significant differences between groups were observed in percent elongation values calculated during tensile testing [Fig. 4(b)]. All groups exceeded 100% elongation, with average values ranging from 160% to 230% elongation. These results suggest that the incorporation of theta-processing techniques has less impact on the tensile mechanics of the hydrogels than the compressive mechanics. Furthermore, the effect of processing technique appears to be less influential on the tensile properties, suggesting anisotropic behavior. While compressive properties of the hydrogels were

able to be tuned significantly, the same level of control was lacking in the tensile mechanics.

C. hMSC cytotoxicity

The cytotoxicity of the PVA-based hydrogel samples was evaluated using an LDH assay. The cytotoxicity (%) of the samples was quantitatively compared to a blank cell control. After 24 h of culture of the PVA-gelatin hydrogels, hMSCs indicated a slight decrease in cell viability, although the cytotoxicity of the hydrogels was <20% (Fig. 5). The percent cytotoxicity was 12% for the CG hydrogel groups and ranged from 13% to 19% for the TCG

FIG. 6. Production of sGAGs normalized to the cell protein content was used to determine the efficacy of PVA-gelatin hydrogels for supporting matrix production. hMSCs were seeded onto 3 mm-diameter CG and TCG materials processed at varying FT cycles; nontreated cells were used as a control. Cells were cultured on materials in either basal or TGF-β1 supplemented media, and analyzed after 1, 7, and 14 days of culture. All results reported as average $\pm$ standard deviation ($n = 3$).

hydrogel groups. No significant differences at $p < 0.05$ were observed between groups. Thus, while some percentage of cells were affected, the hydrogel materials overall did not indicate the cause for concern and the materials were deemed appropriate for analyzing cell activity for time points up to 14 days.

D. hMSC short-term bioactivity study

While the material fabrication techniques provide some control over mechanical properties, herein the efficacy of using PVA-based TCGs and CGs as soft tissue replacements or scaffolds was investigated by examining the ability of the PVA-gelatin hydrogels to support matrix production and maintain cell viability for at least two weeks.

The mass of sGAG per mass of protein was calculated to determine how actively cells were producing a soft tissue matrix component when cultured on PVA-gelatin hydrogels of varying mechanical properties and in either basal or TGF- β 1 supplemented media. At days 1 and 7 (Fig. 6, top and middle), the values of sGAG normalized to the amount of cell protein were small, as expected, and there was little difference between hydrogel groups or between hydrogel groups and the cell control. Also, at days 1 and 7, no consistent trend showing the benefits of TGF- β 1 on sGAG production was evident. At day 14 (Fig. 6, bottom), the CG 1 FT hydrogel groups tripled the sGAG production for cells cultured in TGF- β 1-supplemented media; thus, the added biological benefit (i.e., effect on stem cell differentiation) resulted in significantly higher sGAG production compared to the nonmodified cell control.³¹ However, the TCG 8 FT hydrogel group nearly tripled the sGAG production for cells cultured in both the basal and TGF- β 1-supplemented media, possibly indicating that the physicomaterial properties of that sample group may be enough to support soft tissue matrix production without the need for biological supplements. The TCG 8 FT samples exhibited consistently high compressive (100 kPa) and tensile (121 kPa) moduli compared to the other material groups, whose values more closely approach the mechanical properties of various soft tissues in the body.^{32–34} Indeed, the stiffer material may lead to more sGAG production, even when cultured in basal media. It is also interesting to point out that the materials processed with 8 FT cycles behave in a more viscoelastic manner, which may also influence the cellular response. Based on our previously published data, we indicated that TCGs exhibit larger pore sizes compared to CGs, which may also contribute to the significant biological response.¹⁹

IV. CONCLUSIONS

Herein, we have described an elastic PVA-gelatin hydrogel utilizing theta-gel and cryo-gel processing techniques, resulting in a stronger, more elastic material with greater potential for support soft tissue matrix component production (i.e., sGAGs). Chemical, mechanical, and cell-based tests were performed to verify the viability of the system to host cells and perform under varying loading regimes. Neither the TCG nor CG fabrication techniques had a qualitative negative effect on the gelatin content in the system, as Van Gieson staining confirmed the presence of gelatin after all processing and FTIR spectra indicated increased bonding between

gelatin and PVA—secondary bonds were formed as a result of both the theta-processing and increased FT cycling. At higher FT cycles, the effects of the theta-processing were dominated by cryo-processing, evidenced more in the compression data compared to the tensile results. While the current study focused on maintaining cell viability and inducing sGAG production in hMSCs, future studies will focus on cell migration and viability throughout the scaffold, and a more rigorous differentiation study will be needed to further analyze PVA-gelatin TCGs for soft tissue repair and/or engineering.

ACKNOWLEDGMENTS

This work was funded in part by the National Institutes of Health (NIH) [Grant No. R01 EB020964-01 (A. Oldinski)] and by the University of Vermont College of Engineering and Mathematical Sciences. There are no conflicts to declare.

REFERENCES

- J. C. Iatridis, S. B. Nicoll, A. J. Michalek, B. A. Walter, and M. S. Gupta, *Spine* **13**, 243 (2013).
- S. Bryant, C. Nuttelman, and K. Anseth, *Biomed. Sci. Instrum.* **35**, 309 (1999).
- C. R. Nuttelman, S. M. Henry, and K. S. Anseth, *Biomaterials* **23**, 3617 (2002).
- P. J. Martens, S. J. Bryant, and K. S. Anseth, *Biomacromolecules* **4**, 283 (2003).
- J. A. Stammen, S. Williams, D. N. Ku, and R. E. Guldberg, *Biomaterials* **22**, 799 (2001).
- M. H. Alves, B. E. Jensen, A. A. Smith, and A. N. Zelikin, *Macromol. Biosci.* **11**, 1293 (2011).
- H. Bodugoz-Senturk, J. Choi, E. Oral, J. H. Kung, C. E. Macias, G. Braithwaite, and O. K. Muratoglu, *Biomaterials* **29**, 141 (2008).
- D. A. Bichara, X. Zhao, H. Bodugoz-Senturk, F. P. Ballyns, E. Oral, M. A. Randolph, L. J. Bonassar, T. J. Gill, and O. K. Muratoglu, *Tissue Eng. Part A* **17**, 301 (2010).
- S.-Y. Lee, A.-S. Wee, C.-K. Lim, A. A. Abbas, L. Selvaratnam, A. M. Merican, T. S. Ahmad, and T. Kamarul, *J. Mater. Sci. Mater. Med.* **24**, 1561 (2013).
- P. N. Charron, T. A. Braddish, and R. A. Oldinski, *J. Mech. Behav. Biomed. Mater.* **92**, 90 (2019).
- K. S. Lim, M. H. Alves, L. A. Poole-Warren, and P. J. Martens, *Biomaterials* **34**, 7097 (2013).
- S. M. Shaheen and K. Yamaura, *J. Controlled Release* **81**, 367 (2002).
- S. Kudo, E. Otsuka, and A. Suzuki, *J. Polym. Sci. Part B Polym. Phys.* **48**, 1978 (2010).
- A. V. Reis, A. R. Fajardo, I. T. Schuquel, M. R. Guilherme, G. J. Vidotti, A. F. Rubira, and E. C. Muniz, *J. Org. Chem.* **74**, 3750 (2009).
- P. Martens and K. Anseth, *Polymer* **41**, 7715 (2000).
- E. G. Crispim, J. F. Piai, A. R. Fajardo, E. R. F. Ramos, T. U. Nakamura, C. V. Nakamura, A. F. Rubira, and E. C. Muniz, *Express Polym. Lett.* **6**, 383 (2012).
- E. A. Kamoun, E.-R. S. Kenawy, and X. Chen, *J. Adv. Res.* **8**, 217 (2017).
- M. V. Badiger, M. E. McNeill, and N. B. Graham, *Biomaterials* **14**, 1059 (1993).
- T. Miao, E. J. Miller, C. McKenzie, and R. A. Oldinski, *J. Mater. Chem. B* **3**, 9242 (2015).
- P. N. Charron, S. E. Blatt, C. McKenzie, and R. A. Oldinski, *Biointerphases* **12**, 02C409 (2017).
- N. A. Peppas and S. R. Stauffer, *J. Controlled Release* **16**, 305 (1991).
- V. Lozinsky, L. Damshkaln, I. Kurochkin, and I. Kurochkin, *Colloid J.* **70**, 189 (2008).
- M. E. Koch, "Mechanical optimization of poly(vinyl alcohol) cryogels to activate osteochondral mechanotransduction pathways," M.S. thesis (University of Vermont, 2014).
- N. T. B. Linh and B.-T. Lee, *J. Biomater. Appl.* **27**, 255 (2012).

- ²⁵M. E. Hoque, T. Nuge, T. K. Yeow, N. Nordin, and R. Prasad, *Polym. Res. J.* **9**, 15 (2015).
- ²⁶D. Liu, M. Nikoo, G. Boran, P. Zhou, and J. M. Regenstein, *Annu. Rev. Food Sci. Technol.* **6**, 527 (2015).
- ²⁷N. Davidenko, C. F. Schuster, D. V. Bax, R. W. Farndale, S. Hamaia, S. M. Best, and R. E. Cameron, *J. Mater. Sci. Mater. Med.* **27**, 148 (2016).
- ²⁸*ASTM Volume 08.01 Plastics (I): C1147–D3159* (ASTM International, 2006).
- ²⁹A. Galperin, R. A. Oldinski, S. J. Florczyk, J. D. Bryers, M. Zhang, and B. D. Ratner, *Adv. Healthc. Mater.* **2**, 872 (2013).
- ³⁰G. Muller and M. Hanschke, *Connect. Tissue Res.* **33**, 243 (1996).
- ³¹D. Bosnakovski, M. Mizuno, G. Kim, S. Takagi, M. Okumura, and T. Fujinaga, *Biotechnol. Bioeng.* **93**, 1152 (2006).
- ³²T. Wang, J. H. Lai, L. H. Han, X. Tong, and F. Yang, *Tissue Eng. Part A* **20**, 2131 (2014).
- ³³H. Lv, L. Li, M. Sun, Y. Zhang, L. Chen, Y. Rong, and Y. Li, *Stem Cell Res. Ther.* **6**, 103 (2015).
- ³⁴C. M. Murphy, A. Matsiko, M. G. Haugh, J. P. Gleeson, and F. J. O'Brien, *J. Mech. Behav. Biomed. Mater.* **11**, 53 (2012).