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RESEARCH ARTICLE

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Controlling collagen gelation pH to enhance biochemical, structural, and biomechanical properties of tissue-engineered menisci

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Abstract

Collagen-based hydrogels have been widely used in biomedical applications due to their biocompatibility. Enhancing mechanical properties of collagen gels remains challenging while maintaining biocompatibility. Here, we demonstrate that gelation pH has profound effects on cellular activity, collagen fibril structure, and mechanical properties of the fibrochondrocyte-seeded collagen gels in both short- and long-terms. Acidic and basic gelation pH, below pH 7.0 and above 8.5, resulted in dramatic cell death. Gelation pH ranging from 7.0 to 8.5 showed a relatively high cell viability. Furthermore, physiologic gelation (pH 7.5) showed the greatest collagen deposition while glycosaminoglycan deposition appeared independent of gelation pH. Scanning electron microscopy showed that neutral and physiologic gelation pH, 7.0 and 7.5, exhibited well-aligned collagen fibril structure on day 0 and enhanced collagen fibril structure with laterally joined fibrils on day 30. However, basic pH, 8.0 and 8.5, displayed a densely packed collagen fibril structure on day 0, which was also persistent on day 30. Initial equilibrium modulus increased with increasing gelation pH. Notably, after 30 days of culture, gelation pH of 7.5 and 8.0 showed the highest equilibrium modulus, reaching 150 −160 kPa. While controlling gelation pH is simply achieved compared with other strategies to improve mechanical properties, its influences on biochemical and biomechanical properties of the collagen gel are long-lasting. As such, gelation pH is a useful means to modulate both biochemical and biomechanical properties of the collagen-based hydrogels and can be utilized for diverse types of tissue engineering due to its simple application.

KEYWORDS
collagen, gelation pH, hydrogels, meniscus, tissue engineering

1 | INTRODUCTION

Collagen is the major structural component of connective tissues in vertebrates, such as bones, tendons, ligaments, cartilage, skin, blood vessels, and menisci.[1] Thus, collagen has been widely used as a scaffold material for diverse tissue engineering applications.[2] Although collagen-based biomaterials have shown promising outcomes due to their excellent biocompatibility, several drawbacks including inferior mechanical properties compared with native tissue pose limitations in their clinical application.[3]

There have been significant efforts to enhance the mechanical properties of collagen-based biomaterials. A variety of strategies using chemical or photochemical crosslinking agents or incorporating other stiff materials have been investigated.[4–7] In addition, gelation factors, such as collagen concentration,[8,9] temperature,[8,10,11] ionic strength,[8,12,13] and pH,[9–11,14] have been reported to affect collagen fibrillogenesis, which is

directly related to the mechanical properties of collagen gels. Among these factors, gelation pH influences hydrophobic and electrostatic interactions,[15] major driving forces of collagen self-assembly, and kinetics of fibrillogenesis.[11,16] These influences subsequently affect fibril size and morphology of the collagen gels.[11,16,17] Notably, optimal pH for mechanical properties has been observed to be in the range of basic pH,[11,18,19] which is higher than is typically used for formulating collagen-based hydrogels.[7,20,21] Given that changes in collagen fibril architecture result in changes in mechanical properties, gelation pH can be a useful tool to tune mechanical properties of collagen-based biomaterials.

Although the aforementioned studies have demonstrated the effects of gelation pH on collagen fibrillogenesis and resultant mechanical properties, these studies mainly focused on initial collagen fibril structure and mechanical properties. Little is known about whether such changes persist in the process of remodeling that occurs during tissue repair or regeneration. Furthermore, all of these previous studies employed low concentrations of collagen, which are a factor of 100 lower than that of native tissue[22] and thus far less amenable to engineering load-bearing tissues, and focused on mechanical properties upon gelation. Furthermore, it is unclear whether gelation pH has long-lasting biological effects on cells seeded in a collagen gel. Thus, the influences of gelation pH on structural, biochemical, and biomechanical properties of high-density collagen constructs in long-term culture have not yet been elucidated.

Menisci play an important role in biomechanical functions in the knee joint and are mainly composed of collagen. In multiple previous studies, we have developed tissue-engineered menisci using a fibrochondrocyte-seeded high-density collagen gel and cultured them up to 8 weeks.[23–26] We have also showed that collagen fiber structure can be remodeled by seeded fibrochondrocytes, leading to changes in mechanical properties. Therefore, tissue-engineered menisci represent a useful platform to investigate the short- and long-term effects of gelation pH on cellular activities of fibrochondrocytes, cell-mediated remodeling, and mechanical properties of collagen gels.

In this study, we investigated the effects of gelation pH on cell viability, fibrochondrocyte proliferation, glycosaminoglycan (GAG) and collagen deposition, collagen microstructure, and mechanical properties of tissue-engineered menisci. We hypothesize that gelation pH regulates biochemical, structural, and mechanical properties of tissue-engineered menisci, and that there exists an optimal gelation pH that results in both increased extracellular matrix production and improved mechanical properties of tissue-engineered menisci after extended culture. These results are useful for developing optimal polymerization conditions for enhancing functionality of tissue-engineered menisci and facilitating the transition of tissue-engineered menisci to clinical use.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

Unless otherwise stated, all reagents and materials were purchased from Sigma-Aldrich. Bovine joints were purchased from Gold Medal Packing. Collagenase type II was purchased from Worthington Biochemical Corporation. Dulbecco's Modified Eagle Medium (DMEM) and phosphate-buffered saline (PBS) were purchased from VWR International. Sprague–Dawley rat tails were purchased from Pel-Freez Biologicals. Fetal bovine serum (FBS) was purchased from GemBio.

2.2 | Cell isolation

Fibrochondrocytes were harvested from the menisci of 1–3 day old bovids (Gold Medal Packing, Rome, NY) using a 0.3% (wt/vol) collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in DMEM with 100 μg/mL penicillin and 100 μg/mL streptomycin for 18 h as previously described.[27] After digestion, the cells were filtered through a 100 μm cell strainer, washed, counted, and suspended to a concentration of 150×10^6 cells/mL. Harvested fibrochondrocytes were pooled from all eight menisci to minimize zone-dependent phenotypic variation.

2.3 | Construct fabrication

Collagen was extracted from Sprague–Dawley rat tails (Pel-Freez Biologicals, Rogers, AZ), solubilized, lyophilized, and reconstituted in 0.1% (vol/vol) acetic acid at a 30 mg/mL concentration as previously described.[4,28,29] The stock collagen solution was mixed with a working solution composed of 1 N NaOH, 10X PBS, and 1X PBS using a syringe stopcock. To achieve desired gelation pH of 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, the amounts of 1 M NaOH and 1X PBS were adjusted accordingly. Subsequently, harvested fibrochondrocytes were mixed with the collagen solution to a final concentration of 25×10^6 cells/mL and 20 mg/mL collagen gel and injected into meniscal molds which are 3.5 to 1 mm in height from the outer to inner diameter at a 65° slope across 5.4 mm width.[25] The injected mixtures of collagen and fibrochondrocytes were allowed to gel for 30 min at 37°C. Constructs were cultured in DMEM at a 500 mg/L glucose concentration, which was found to be an optimal glucose concentration for collagen organization,[25] with 10% (vol/vol) FBS (Gembio, West Sacramento, CA), 100 μg/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, 50 μg/mL ascorbic acid, and 0.4 mM L-proline at 37°C and 5% CO_2 for up to 30 days. Culture media were collected and replenished every third day. Photographs of each 30-day cultured construct were obtained on days 0 and 30 to calculate contraction using ImageJ software (NIH, Bethesda, MD).

2.4 | Cell viability

Cell viability was assessed immediately after construct fabrication. Cross sections with 1 mm thickness of each construct were harvested from the middle portion of tissue-engineered menisci using a razor blade and stained for 30 min using 4 μM of ethidium homodimer-1 and 8 μM of calcein AM according to the manufacturer's instruction. After staining, each portion of the constructs was rinsed with PBS for

5 min three times and subsequently imaged on a Zeiss LSM880 confocal/multiphoton inverted microscope. Images were then imported into ImageJ to assess cell viability.

2.5 | Biochemical analysis

Biochemical analysis was performed to measure contents of DNA via a Hoechst DNA assay,[30] GAG via a modified 1,9-dimethylmethylene blue assay,[31] and collagen via a hydroxyproline (Hypro) assay,[32] as previously documented. Prior to the assays, portions of each construct were measured for wet weight (w.w.), lyophilized, and then digested in 1.25 mg/mL papain digestion solution.

2.6 | Histological analysis

Circumferentially cross-sectioned samples were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin blocks, sectioned at 4 μm thickness, and stained with picrosirius red. Images were taken under brightfield and polarized light with a Nikon Eclipse TE2000-S microscope (Nikon Instruments, Melville, NY) with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) to investigate collagen fiber structure.

2.7 | Scanning electron microscopy

Circumferentially cross-sectioned samples were fixed, stored in 100% ethanol, and then critical point dried. The dried samples were sputter-coated with gold at a target current of 20 mA. After coating, samples were scanned using a Zeiss Gemini 500 scanning electron microscope at an accelerating voltage of 1 kV.

2.8 | Mechanical analysis

Confined compression stress relaxation test was performed using an Enduratec ElectroForce 5500 System (Bose, Eden Prairie, MN), as previously reported.[4] Samples were prepared using 4 mm diameter biopsy punches. Six steps of 5% compression were applied to constructs such that circumferentially aligned fibers were axially compressed. Each compression step had a 10-min wait period to ensure full stress relaxation were imposed on the construct samples. The resulting loads were fitted to a poroelastic model using a custom MATLAB program to calculate the equilibrium modulus, hydraulic permeability, and instantaneous modulus.[33–35]

2.9 | Statistical analysis

Normality testing was performed by Shapiro–Wilk's test using GraphPad Prism (GraphPad Prism Software Inc., San Diego, CA). To compare multiple groups, we performed one-way or two-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference post hoc tests. All data are reported as mean ± standard deviation with at least $n = 3$ replicates. Significance was determined with $p < .05$.

3 | RESULTS

3.1 | Cell viability analysis

Cells are known to be sensitive to their surrounding environment, such as stiffness and pH.[36,37] Thus, in order to evaluate the effects of gelation pH on fibrochondrocyte viability, live/dead staining was performed on high-density collagen constructs gelled at pH values ranging from 6.0 to 9.0 (Figure 1). Neutral to slightly basic gelation pH

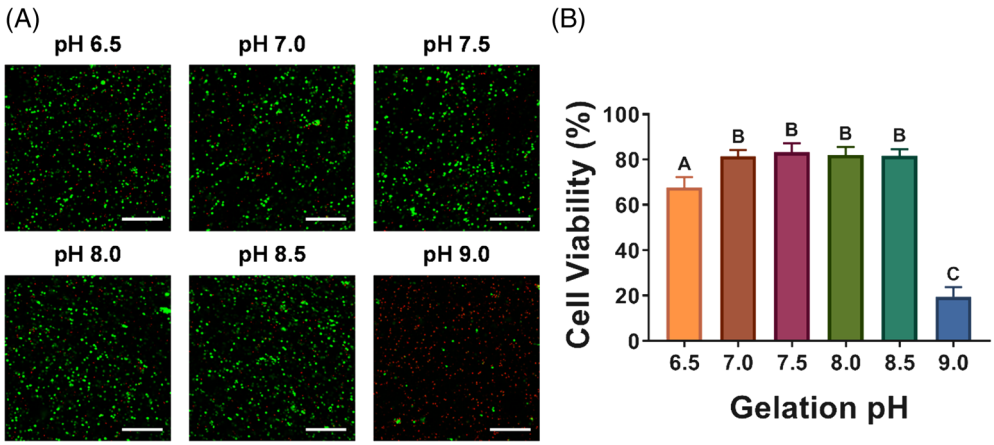

FIGURE 1 Cell viability of fibrochondrocytes seeded in high-density collagen constructs in response to various gelation pH. (A) Representative confocal fluorescent images of live/dead staining at indicated gelation pH. Live cells are stained green, and dead cells are stained red. (B) Viability of fibrochondrocytes at indicated gelation pH ($n = 6–10$). One-way ANOVA with Tukey's Honestly Significant Difference (HSD) tests was performed to determine significant differences between groups. Different letters represent statistical significance between groups ($p < .05$). Scale bars are 200 μm

from 7.0 to 8.5 showed high cell viability, ranging from 81% to 83%. By contrast, acidic pH and basic gelation pH, pH 6.5 and 9.0, resulted in significantly decreased cell viability, 67.7% ± 4.5% and 19.5% ± 4.2%, respectively. Interestingly, gelation pH 9.0 exhibited the lowest cell viability corresponding to one-third of viability at gelation pH 6.5 and one-fourth of that of the rest. As cell viability is strongly associated with success of tissue engineering approaches, gelation pH ranging from 7.0 to 8.5 was chosen for further experiments.

3.2 | Meniscal construct contraction analysis

Considering that anatomic accuracy of tissue-engineered constructs is a key for successful clinical applications, it is critical to prevent dramatic construct contraction. Therefore, we examined construct contraction over the 30-day culture period. Construct contraction appeared to be reduced with increasing gelation pH (Figure 2). Gelation pH 7.0 led to the greatest construct contraction, decreasing 30% from the initial size. Conversely, gelation pH 7.5 to 8.5 displayed a modest contraction to 2%–20% from their initial size. Interestingly, constructs gelled at pH 7.0 dramatically contracted between day 9 and day 18 while the other constructs gradually contracted over time in culture (Figure S1).

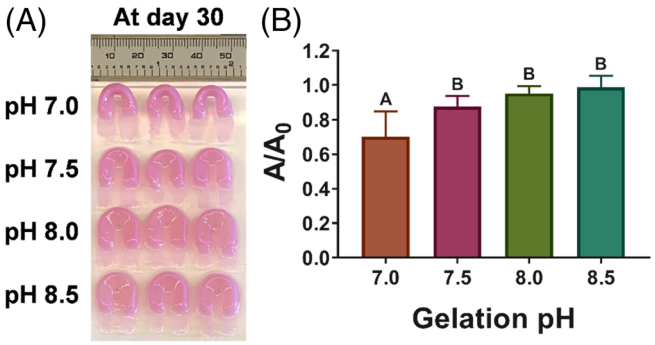

FIGURE 2 Contraction of meniscal constructs after 30 days of culture. (A) Photograph of meniscal constructs on day 30 gelled at indicated pH. (B) Ratio of construct area on day 30 over initial area ($n = 6$). One-way ANOVA with Tukey's Honestly Significant Difference (HSD) tests was performed to determine significant differences between groups. Different letters represent statistical significance between groups ($p < .05$)

3.3 | Cellular activity analysis

Next, we measured contents of DNA, GAG, and Hypro (collagen) to determine the biological effects of gelation pH on fibrochondrocytes. Although gelation pH 8.5 appeared to reduce density of fibrochondrocytes over time in culture, no statistical difference was found in the DNA content between the groups (Figure 3A). Since constructs were made of purified type I collagen, all the constructs showed a minimal content of GAG on day 0 without significant difference between the groups (Figure 3B). After 30 days of culture, all meniscal constructs showed significantly increased GAG content compared with the day 0 constructs. However, statistical difference in GAG content was not observed between the different pH conditions on day 30. Similar results were observed in GAG content normalized to dry weight and to DNA content, and total GAG content per construct (Figure S2). Moreover, no statistical difference was observed in the release of GAG to media throughout the culture period (Figure S3). Conversely, while collagen content did not show significant difference between the day 0 constructs, gelation pH 7.5 resulted in the greatest collagen content between the groups and significantly increased collagen content compared with gelation pH 8.0 and 8.5 on day 30 (Figure 3C). However, gelation pH 8.0 resulted in the significantly decreased collagen content normalized to dry weight on day 30 compared with gelation pH 7.0 on day 0 while no statistical difference was found in the other groups (Figure S4A). Furthermore, there was no significant difference in the collagen content normalized to the DNA content and per

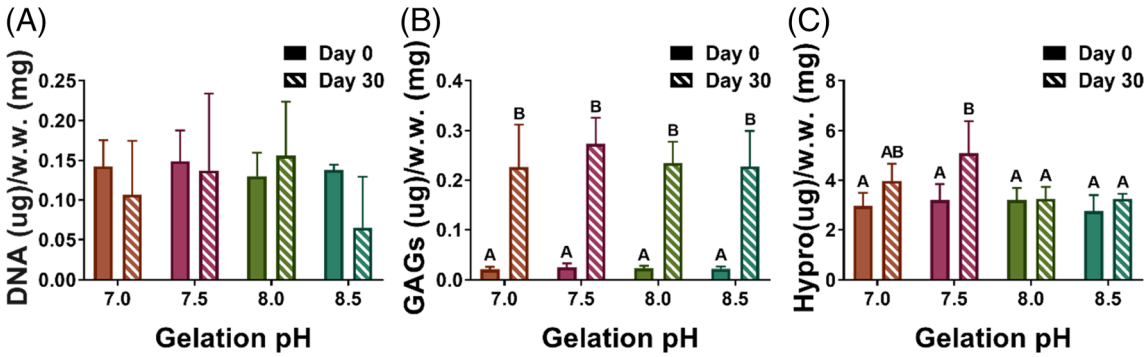

FIGURE 3 Contents of DNA, GAGs, and Hypro (collagen) in high-density collagen constructs on days 0 (filled bar) and 30 (diagonal striped bar). (A) DNA content in meniscal constructs normalized to wet weight ($n = 6$). (B) GAG content in meniscal constructs normalized to wet weight ($n = 6$). (C) Hypro (collagen) content in meniscal constructs normalized to wet weight ($n = 6$). Two-way ANOVA with Tukey's Honestly Significant Difference (HSD) tests was performed to determine significant differences between groups. Different letters represent statistical significance between groups ($p < .05$)

construct (Figure S4B,C) and the release of collagen to media over time in culture between the groups (Figure S5).

3.4 | Collagen microstructure analysis

In order to investigate how gelation pH affects collagen microstructure of meniscal constructs in the short- and long-terms, scanning electron microscope imaging was performed on meniscal constructs on days 0 and 30. Constructs gelled at basic gelation pH exhibited entangled collagen fibrils on day 0 (Figures 4 and S6). This collagen fibril structure was also persistent on day 30. However, constructs gelled at pH of 7.0 and 7.5 showed aligned collagen fibrils on day 0 to some extent and more aligned collagen fibrils on day 30. Interestingly, bundles of collagen fibrils were observed at gelation pH 7.5 on day 30. Intriguingly, fibrochondrocytes were found in lacunae-like spaces surrounded by collagen fibrils at gelation pH 7.0 and 7.5 on day 30. By contrast, fibrochondrocytes within constructs gelled at pH 8.0 and 8.5 were found on the surface of collagen fibrils on day 30, similar to the day 0 groups. These observations might indicate that fibrochondrocytes were able to remodel collagen fibril structure gelled at pH 7.0 and 7.5 but not at pH 8.0 and 8.5. These findings were supported by picrosirius red staining images showing similar collagen fiber organization, enhanced collagen fiber structure at gelation pH 7.5 on day 30 compared with the other groups (Figure 5).

3.5 | Mechanical property analysis

It has been shown that collagen organization is critical for mechanical properties of native tissue and collagen-based biomaterials. Thus, we performed confined compression tests to obtain the equilibrium modulus, instantaneous modulus, and hydraulic permeability of meniscal constructs. For day 0 groups, the equilibrium modulus increased with increasing gelation pH with the greatest equilibrium modulus observed at gelation pH 8.5 showing a twofold increase relative to that of gelation pH 7.0, 111 kPa versus 55 kPa, respectively (Figure 6A). Interestingly, over time in culture, constructs gelled at pH 7.0 and 7.5 showed significantly enhanced equilibrium modulus compared with their day 0 groups while constructs gelled at pH 8.0 and 8.5 also showed an increase in equilibrium modulus but was not significant. The highest equilibrium modulus, ~162 kPa, was observed in gelation pH 7.5 on day 30. Instantaneous modulus also peaked at gelation pH 7.5 on day 30 while there was no difference between the other groups (Figure S7). Gelation pH 7.0 and 7.5 showed higher hydraulic permeability than that of gelation pH 8.0 and 8.5 on day 0 (significantly higher at gelation pH 7.0) but substantially decreased

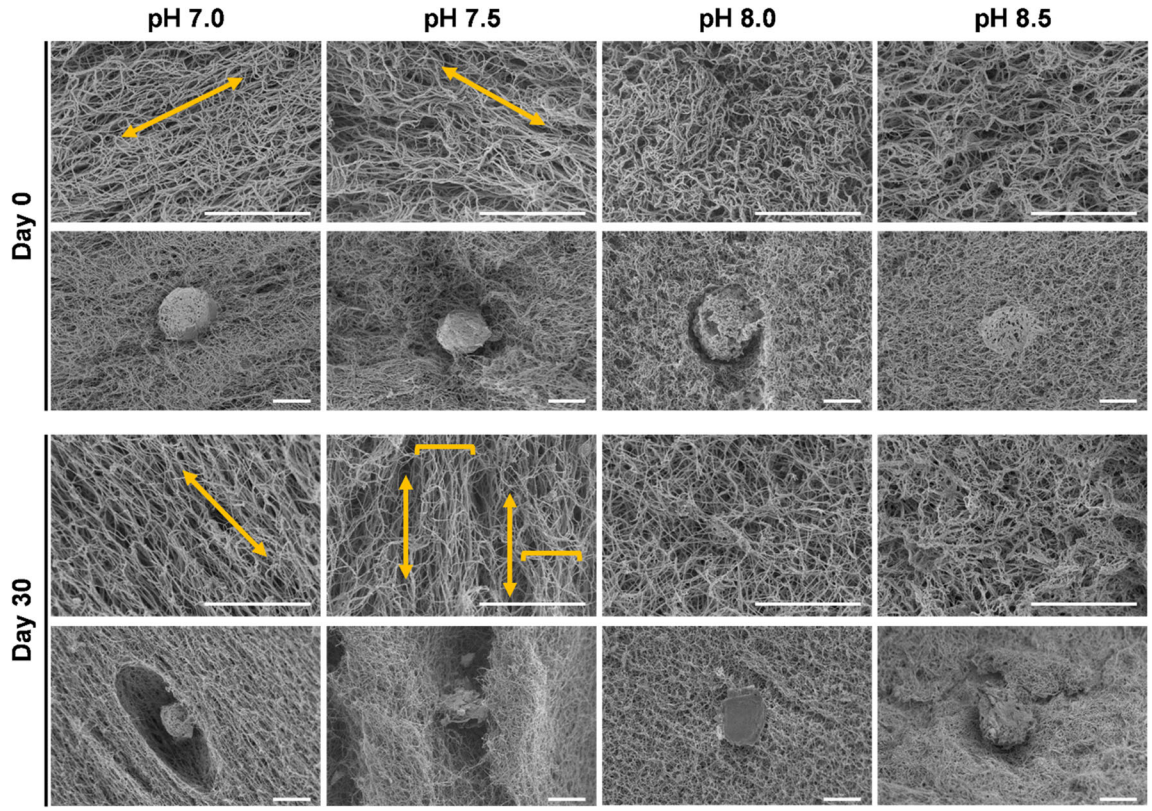

FIGURE 4 Representative scanning electron microscope images of fibril organization of meniscal constructs after 0 and 30 days of culture (8–10 images were taken per condition). Double-headed arrows indicate a direction of collagen fibril alignment. Brackets indicate a bundle of collagen fibrils. Scale bars are 4 μm

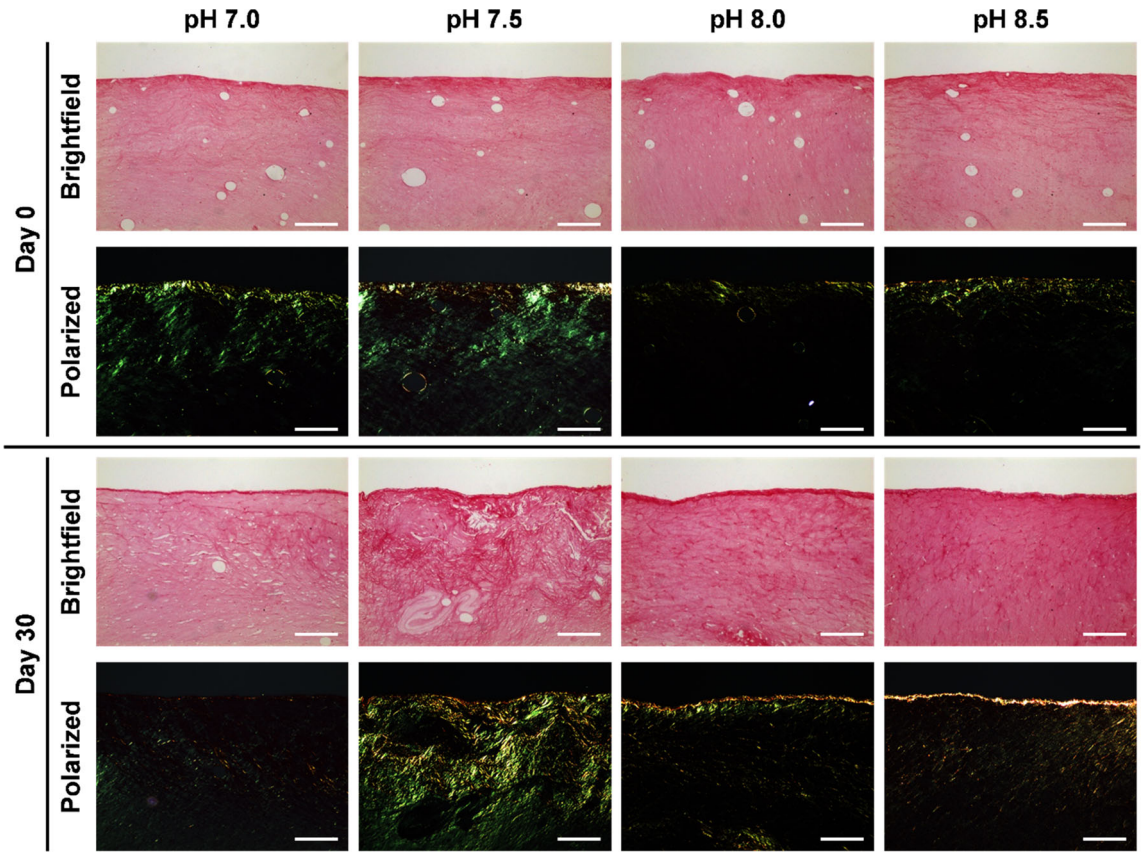

FIGURE 5 Picrosirius red stating images of collagen fiber organization of meniscal constructs under brightfield and polarized light on days 0 and 30 (8–10 images were taken per condition). Scale bars are 200 μm

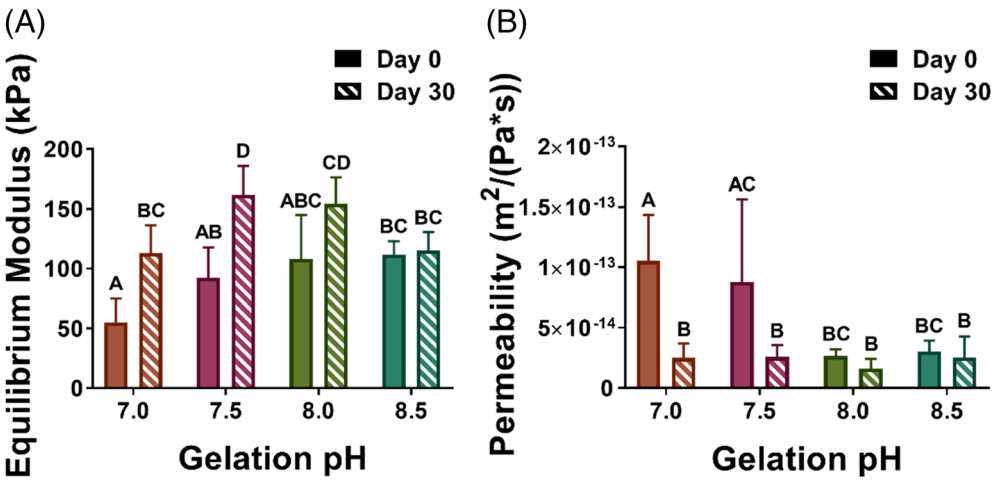

FIGURE 6 Mechanical properties of meniscal constructs on days 0 (filled bar) and 30 (diagonal striped bar). (A) Equilibrium modulus of meniscal constructs ($n = 4–6$). (B) Hydraulic permeability of meniscal constructs ($n = 4–6$). Two-way ANOVA with Tukey's Honestly Significant Difference (HSD) tests was performed to determine significant differences between groups. Different letters represent statistical significance between groups ($p < .05$)

hydraulic permeability on day 30 comparable to that of gelation pH 8.0 and 8.5 (Figure 6B). On the other hand, gelation pH 8.0 and 8.5 retained hydraulic permeability throughout the culture period.

4 | DISCUSSION

The goal of this study was to investigate the biological, structural, and biomechanical effects of gelation pH on high-density collagen-based constructs. In this current study, we demonstrated that gelation pH affects the biological activity of fibrochondrocytes, initial collagen fibril structure, cell-mediated collagen remodeling, and mechanical properties of the collagen gel in both short- and long-terms. We showed that gelation pH of 7.5, a physiologic condition, is beneficial for both biological and mechanical properties of the meniscal constructs, supported by both enhanced collagen deposition and equilibrium modulus comparable to native tissue[38] after 30 days of culture.

Live/dead staining revealed that fibrochondrocytes retained a relatively high cell viability within the range of physiologic gelation pH (Figure 1). Beyond this range, substantial cytotoxicity was detected, indicating that there might exist a threshold of gelation pH that is suitable for fibrochondrocytes. Previous studies have shown that fibroblasts, keratinocytes, and stromal cells maintain a higher cell viability at physiologic pH compared with acidic and basic pH,[39,40] while chondrocytes retain a high cell viability at acidic pH comparable to physiologic pH.[41] Such data suggest that the effect of pH on cell viability is dependent on cell type and thus that the optimization of gelation pH should be taken into consideration for collagen-based biomaterials using diverse cell types.

Significant construct contraction should be avoided due to the possibility of anatomical mismatching which can lead to failure after implantation.[42] Contraction analysis demonstrated that gelation pH of 7.0 resulted in a significant contraction compared with the other groups although there was no statistical difference in cell viability between these groups (Figures 2 and S1). Contraction of collagen gels is attributed to weak mechanical properties and/or cell-mediated remodeling,[43] which is consistent with our findings in this study that increasing initial equilibrium modulus resulted in decreasing construct contraction (Figures 2B and 6A). These data underscore the importance of initial construct mechanics in achieving sufficient shape fidelity of implants.

It is well known that collagen and GAGs are major components of native meniscus and also give rise to the biomechanical functions of native menisci.[44,45] Cell proliferation and extracellular matrix production by cells have been shown to change in response to the surrounding environment, such as dimensionality (2D vs. 3D) or stiffness.[46,47] Biochemical analysis revealed that maintenance of cell content throughout the culture was independent of gelation pH. GAG content also did not show any significant difference between groups on day 30. However, only gelation pH of 7.5 showed the significantly increased collagen content compared with the day 0 groups and the groups of gelation pH of 8.0 and 8.5 on day 30. This result was consistent with increased picrosirius red staining observed at gelation pH 7.5 (Figure 5). In previous studies, the effects of extracellular pH on biosynthetic activity have widely been investigated using various cell types, including bovine chondrocytes in agarose gel[41] and in suspension culture,[48] bovine chondrocytes and nucleus pulposus cells in alginate beads,[49] and human osteoblasts[50] and human bone marrow stromal cells[51] in monolayer culture. These studies demonstrate that extracellular pH influence both catabolic and anabolic activities of cells, such as GAG and collagen production or matrix metalloproteinase activity, and that these activities have been shown to dramatically change in response to a small change in extracellular pH. Interestingly, collagen production of chondrocytes at extracellular pH of 7.4 was twofold higher than that at pH 7.0, a similar trend to our finding.[48] These findings suggest that physiologic gelation pH is not only beneficial for cell viability but also biosynthetic activity.

Many studies have shown that collagen fibrillogenesis is mainly driven by hydrophobic and electrostatic interactions and influenced by various factors including the presence or absence of telopeptide, ionic strength, temperature, and gelation pH.[11,52] Scanning electron microscope images demonstrated that gelation pH affects collagen fibril organization of high-density collagen constructs. Whereas basic gelation pH, 8.0 and 8.5, appeared to generate compact, entangled collagen fibril structures, gelation pH of 7.0 and 7.5 resulted in focal collagen microstructures (Figures 5 and 6, top panels). This structural difference became more apparent in the constructs cultured for 30 days (Figures 5 and 6, bottom panels). Constructs gelled at pH 7.0 and 7.5 showed a lateral growth of collagen fibrils after 30 days of culture, which was not observed in those gelled at pH 8.0 and 8.5. It has been shown that gelation pH ranging from 8.5 to 10 leads to randomly interconnected collagen fibril structure due to enhanced hydrophobic interaction, while physiologic pH leads to organized collagen fibril formation mediated by thermodynamically preferred interactions.[11,16,19] Interestingly, in 12 m*M* NaCl or in the absence of salts, the isoelectronic point of collagen is reported to be ~9.3,[16,53] which could have a strong effect on association of fibrils. Furthermore, flexibility of collagen molecules has been found to be greater at pH 7.4 than at pH 4.0, and such flexibility might aid in assembly of organized collagen fibril networks.[54] In addition, collagen fiber stiffness and length of collagen gels have been shown to determine cellular responses.[55] Soft and long collagen fibers led to greater cell spreading and collagen recruitment around cells with enhanced focal adhesion formation compared with stiff and short collagen fibers. The exact mechanism of structural differences observed in this study is not clear, but it can be speculated that entangled and interconnected collagen fibril structure with stiff and short collagen fibers is not favorable for cell-mediated remodeling due to sterically hindered binding sites for cells, limited space between collagen fibrils, and eventually prohibited focal adhesion formation. This phenomenon could explain why fibrochondrocytes were found in lacunae-like spaces only at gelation pH 7.0 and 7.5, but not at higher pH.

Confined compression testing revealed that initial equilibrium and instantaneous moduli of meniscal constructs are dependent on gelation pH since higher gelation pH led to an increasing trend of both moduli. However, equilibrium modulus on day 30 appeared to result from the interplay between collagen content and collagen fibril/fiber structure, which is consistent with previous studies showing that increased extracellular matrix molecules and enhanced collagen fiber organization are correlated with mechanical properties of collagen-based constructs.[4,23] On day 0, gelation pH 7.0 and 7.5 showed higher hydraulic permeability than the groups of gelation pH 8.0 and 8.5, suggesting collagen fibril structure determines initial permeability of the constructs. However, on day 30, all constructs presented similar permeability although they showed differing collagen fibril/fiber structure. Notably, these conditions had similar GAG content, which is known to have a strong influence on hydraulic permeability.[34,56] As such, initial mechanical properties are not likely to be a good indicator of eventual mechanical properties of collagen-based scaffolds after time in culture, which are rather accumulative effects of extracellular matrix molecule production and collagen fiber structure.

Ionic strength, as well as the types of electrolytes, has profound effects on collagen fibrillogenesis.[9,11,12] In this context, it should be

noted that gelation pH was regulated by adjusting the amounts of 1 M NaOH and 1X PBS, which also resulted in different ionic strength. However, the difference in ionic strength between the groups is less than 0.1%, and thus the effects of ionic strength might be negligible compared with those of gelation pH in this study.

A number of strategies have been investigated to enhance mechanical properties of collagen-based biomaterials. Chemical or photochemical crosslinking methods have been shown to increase the mechanical properties of collagen constructs.[5,6] However, these methods have several limitations, such as cytotoxicity or pigment production. Although incorporation of synthetic or natural polymers to fabricate collagen-based composites also showed promising outcomes, these methods require additional processing steps, which might limit the clinical applications. Compared with these methods, controlling gelation pH is relatively simple since it does not require any additional steps while achieving significantly enhanced extracellular matrix production and mechanical properties. This strategy can be further extended to various fields of tissue engineering. For examples, 3D bioprinting has gained a significant amount of attention as it enables the fabrication of tissue-engineered constructs with complex structures. As such, 3D bioprinting techniques have been utilized to generate scaffolds for diverse tissues, including skin,[57] cartilage,[58] bone,[59] and trachea.[60] Notably, regardless of types of cells, pH of collagen-based bioinks were adjusted mostly to be neutralized. Therefore, considering the biological and structural effects of gelation pH on cells and collagen gel, the cell-specific validation of gelation pH is likely to enhance advantages of 3D printing technique. Furthermore, we previously showed that mechanical properties and printability of collagen-based bioinks can be modulated by varying gelation pH without hampering cell viability,[14] indicating the versatile applications of gelation pH. Moreover, gelation pH can also be used to enhance functionality of injectable collagen gels to repair tissue defects. Matching mechanical properties of implants to those of tissues at the implantation site is one of the most important criteria.[61] As such, injectable collagen gels can be gelled at appropriate pH to match mechanical properties of corresponding native tissues. Thus, this approach can provide insight in diverse collagen-based scaffold fabrication.

5 | CONCLUSION

Overall, this study demonstrated that gelation pH has profound effects on cellular activity, collagen fibril structure, and mechanical properties of collagen-based hydrogels in both short- and long-terms. Notably, whereas slightly super-physiologic pH, 8.0 and 8.5, produced the highest equilibrium modulus on day 0, physiologic gelation pH showed the highest collagen production and equilibrium modulus of the tissue-engineered constructs along with well-aligned, laterally joined fibrils after 30 days of culture. Collectively, these data highlight the importance of controlling gelation pH for collagen-based hydrogels and the versatility of gelation pH as a means to regulate biological and biomechanical properties of the collagen hydrogels.

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CONFLICT OF INTEREST

Dr. Bonassar is a co-founder of and hold equity in 3DBio Corp.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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