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Research Article

Physical and Biological Characterizations of Injectable Thermosensitive Poly(N-Isopropylacrylamide)-Collagen I Hydrogels Intended for Meniscus Repair

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There has been an increase in the incidence of meniscus injuries worldwide, as well as growing needs for injectable hydrogels with the potentials to promote in situ meniscal tissue repair. This work developed poly(N-isopropylacrylamide)-collagen I composite hydrogels, with a series of different collagen I contents. The hydrogels were injectable under room temperature and started to solidify around 32°C due to their thermosensitivities, meeting the requirements of the intended applications. The addition of collagen I to the poly(N-isopropylacrylamide) provided physical reinforcement to the hydrogels. Rheological characterizations showed that, under room temperature, the complex viscosity, the storage modulus, and the loss modulus, respectively, increased from 0.71 ± 0.35 Pa•s to 110.95 ± 23.22 Pa•s, from 3.28 ± 2.02 Pa to 658.06 ± 128.07 Pa, and from 3.30 ± 1.01 Pa to 308.78 ± 56.67 Pa, as the collagen I content in the hydrogel increased from 0% to 1%. Cell proliferation tests and high-density culture also revealed that collagen I promoted bioactivity of the hydrogels and induced fibrochondrocyte phenotype of the chondrocytes. The expressions of collagen I protein and collagen I a1 gene in the PNIPAm-0.5Coll group were 3.50-fold and 3.64-fold of those in the PNIPAm group. In contrast, the expressions of collagen II protein and collagen II a1 gene were less prominent (respectively, 2.36-fold and 3.34-fold in the PNIPAm-0.5Coll group compared with the PNIPAm group). This phenotyping was believed to be conducive to the regeneration of meniscal tissues. The findings of this study have provided an important basis for future in vivo studies and clinical applications of this type of composite hydrogels.

1. Introduction

The menisci are C-shaped pads of fibrocartilage located on the medial and lateral aspects of the knee, between the tibia and the femur. Their main functions include load transition, shock absorption, lubrication, and maintaining stability of the knee [1–3]. Meniscus injuries are common worldwide and are on the rise due to people's increasing involvement in sports activities and the ageing of the society [1, 4, 5]. A significant percentage of the patients with meniscus lesions require surgical treatments, in which partial meniscectomy is the most performed procedure [1]. However, the partial removal of the meniscal tissue will result in increased contact pressure on the tibial plateau and contribute to the development of osteoarthritis [3, 6–8]. Research has shown that the incidence of osteoarthritis in patients with partial medial meniscectomy and partial lateral meniscectomy will increase by 38% and 24%, respectively [9]. Therefore, less destructive treatments, which do not cause secondary conditions, are very much needed.

Injectable hydrogels have the potentials to promote in situ regeneration of the meniscal tissue [10] and are compatible with the minimally invasive arthroscopic surgeries [11].

Hence, they have attracted extensive research interests. Wu et al. and Zhong et al. developed hydrogels from decellularized porcine menisci and applied them (with and without cells, respectively) in vivo. The results suggested that the hydrogels were promising scaffolds and cell carriers for long-term meniscus repair [12, 13]. An et al. and Kim et al. fabricated an injectable pluronic/PMMA-reinforced fibrin hydrogel with improved mechanical properties and an injectable fibrin/polyethylene hydrogel that accelerated tissue repair in the in vivo segmental meniscal defects [14, 15]. Moreover, a self-crosslinking alginate dialdehyde-gelatin hydrogel [16] and a glycol chitosan/multialdehyde functionalized 4-arm-polyethylene glycol hydrogel [10] were also reported to exhibit effective tissue-regenerative abilities.

The authors paid specific attention to a series of studies conducted by Chen and Cheng [17–19], in which the thermosensitive properties of poly(N-isopropylacrylamide) (PNIPAm) were utilized, and the PNIPAm-based hydrogels, incorporated with natural polymers (such as chitosan and hyaluronic acid), possessed desirable injectability, tunable mechanical property, and good biocompatibility for meniscus regeneration. Unlike articular cartilage, the meniscus is abundant in collagen I, with most of the resident cells being fibroblast-like chondrocytes (fibrochondrocytes) [20, 21]. It is, therefore, logical to apply collagen I in the injectable hydrogel, so that a microenvironment similar to native meniscus may be created with biological and chemical cues for tissue regeneration [12, 22].

Although not previously reported in the application of meniscus repair, the combination of PNIPAm and collagen I has already exhibited therapeutic potentials in various other researches. Shimmura et al. made a membrane from PNIPAm-collagen I blend to serve as a cornea-stroma equivalent. It was found that the membrane supported prompt epithelialization and regeneration of corneal nerves [23]. In another ophthalmological application, Fitzpatrick et al. developed an injectable PNIPAm-grafted-collagen I gel and proved its excellent compatibility with and successful entrapment of the retinal pigment epithelial cells, which could be delivered together with the gel to the subretinal space in order to treat retinal degenerative diseases [24]. In the cardiovascular field, a family of PNIPAm-based hydrogels added with collagen I were developed by Li et al., and they demonstrated that those hydrogels not only possessed physical properties suitable for myocardial injection but also promoted cardiosphere-derived cell proliferation and cardiac differentiation [25]. In the present work, the authors have adopted a similar strategy and have assessed the physical and biological properties of a series of PNIPAm-collagen I composite hydrogels. The intention is to, for the first time to the best of our knowledge, develop an injectable PNIPAm-collagen I hydrogel as an effective therapeutic option for future treatment of meniscus lesions.

2. Materials and Methods

2.1. Reagents. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), penicillin, streptomycin, type II collagenase, and TRIzol Reagent were purchased from Thermo Fisher Scientific, Inc. N-Isopropylacrylamide (NIPAm), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and paraformaldehyde were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Water-soluble collagen I was purchased from Guangdong Victory Biotech Co., Ltd. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories Co., Ltd. Safranin-O was purchased from Beijing Solarbio Science & Technology Co., Ltd. Radioimmunoprecipitation assay (RIPA) lysis buffer and bicinchoninic acid (BCA) protein assay kit were purchased from Shanghai Beyotime Biotechnology Co., Ltd. All antibodies were purchased from Cell Signaling Technology, Inc.

2.2. Preparation of Hydrogels. The hydrogels were prepared according to reported protocols [26, 27], with necessary modifications. Four groups of poly(N-isopropylacrylamide)-collagen I composite hydrogels (namely, PNIPAm-0.1Coll, PNIPAm-0.2Coll, PNIPAm-0.5Coll, and PNIPAm-1Coll) were synthesized by NIPAm polymerization in the presence of selected concentrations of collagen I (0.1%wt/v, 0.2%wt/v, 0.5%wt/v, and 1%wt/v). Briefly, collagen I was first dissolved in deionized water (magnetically stirred for 120 min). Then, the NIPAm monomers were added to the solution and stirred for another 60 min until it was completely dissolved. Subsequently, the initiator (APS) and the accelerator (TEMED) were added, followed by 24 hours of magnetic stirring to complete the reactions. The above process was all carried out in a nitrogen atmosphere at 25°C. Additionally, plain PNIPAm hydrogels were also prepared with the same polymerization approach in the absence of collagen I, to serve as a control group. Detailed formulation of each sample group is listed in Table 1.

Lastly, all the hydrogels were subjected to a dialysis process for 5 days, using a Cellu-sep membrane (Interchim, France), to remove residual NIPAm monomers and any other unreacted chemicals, so that potential biotoxicity was eliminated.

2.3. Differential Scanning Calorimetry (DSC). The lower critical solution temperatures (LCSTs) of the hydrogels were measured using a differential scanning calorimeter (DSC 214 Polyma, NETZSCH, Germany). The analyses were conducted under nitrogen flow (40 mL/min), within a temperature range of 20°C to 45°C. The heating rate was 2°C/min. The LCSTs of the samples were determined from the endothermic peaks of the DSC curves. Three replicates were tested for each hydrogel sample group.

2.4. Rheological Measurements. Rheological measurements were performed on an AR-G2 rheometer (TA Instruments, DE, USA) equipped with a parallel plate geometry (25 mm diameter, 0.5 mm gap). A temperature sweep from 20°C to 40°C (at a heating rate of 0.5°C/s) was performed on each sample with a frequency of 1 Hz and a strain of 0.1%, to monitor the changes in the complex viscosity (η^*), the storage modulus (G'), and the loss modulus (G''). Three replicates were tested for each hydrogel sample group.

2.5. Cell Harvest and Isolation. Experiments involving animals were all conducted in accordance with the "Guiding

Table 1: Detailed formulation of each group.

	PNIPAm	PNIPAm-0.1Coll	PNIPAm-0.2Coll	PNIPAm-0.5Coll	PNIPAm-1Coll
Deionized water (mL)	50	50	50	50	50
Collagen (mg)	0	50	100	250	500
NIPAm (g)	10	10	10	10	10
APS (mg)	200	200	200	200	200
TEMED (μL)	500	500	500	500	500

Principles in the Care and Use of Animals (China)" at the Shaoxing People's Hospital, Zhejiang University School of Medicine, and were approved by its Laboratory Animal Ethics Committee (approval code: 2021-K-Y-170-01).

Murine cartilage tissues were extracted from the knee joints of 2-day-old C57BL/6 mice (Shanghai SLAC Laboratory Animal, China) and cultured in complete DMEM/F-12 medium with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.1% type II collagenase, for 8 h at 37°C with 5% CO_2. After collagenase digestion, primary murine chondrocytes were collected by 800 rpm centrifugation for 5 min and were replated in the same medium without type II collagenase for subsequent experiments. Cells were used upon reaching 90% confluency at passages 1 to 3.

2.6. Cell Proliferation. Murine chondrocytes, at a density of 8×10^3 cells/well, were dispersed in 100 μL/well of respective groups of hydrogels (i.e., PNIPAm, PNIPAm-0.1Coll, PNIPAm-0.2Coll, and PNIPAm-0.5Coll) supplemented with 100 μL/well of complete medium and were cultured in 96-well plates for 96 hours, during which process, additional 100 μL/well of complete medium was topped up at the time point of 48 h. At 24 h, 48 h, 72 h, and 96 h, respectively, assays were performed in selected wells ($n = 6$) with CCK-8 solution (10 μL for each well, incubated for 2 h at 37°C). The optical absorbance of each sample group at 450 nm was then measured by a spectrophotometer microplate reader (Thermo Scientific Multiskan GO, MA, USA).

2.7. High-Density Culture of Chondrocytes. Murine chondrocytes were seeded in 12-well plates at a density of 3×10^5 cells/well with complete medium. After overnight incubation, the culture medium was changed to a mixture of hydrogel (1 mL/well) and complete medium (1 mL/well) and was replenished daily afterwards. At day 7 of culture, the cells were fixed in 4% paraformaldehyde for 30 min and subsequently stained with safranin-O to visualize cartilaginous extracellular matrices (ECM).

2.8. Western Blotting. Chondrocytes were collected after 7 days of high-density culture and subjected to RIPA lysis buffer to extract proteins. Total proteins were quantified according to the instructions of BCA protein assay kit. 20 μg of proteins was then separated on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, CA, USA). After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with anticollagen I antibody (#81375), anticollagen II antibody (ab34712), and anti-β-actin antibody (#3700) overnight at 4°C. Subsequently, after five PBS washes, the membranes were incubated with respective secondary antibodies for 45 min at room temperature. Electrogenerated chemiluminescence (ECL) reagents were added to the membranes, and signals were then detected by an automatic chemiluminescence imaging system (Tanon 4600, Tanon, China). The relative levels of detected proteins were expressed by normalized integrated pixel intensities of respective bands. Three measurements were taken on each band.

2.9. Quantitative Real-Time Polymerase Chain Reaction Assay (qPCR). Chondrocytes were collected after 7 days of high-density culture, and total RNA was extracted using the TRIzol Reagent ($n = 3$). 100 ng of total RNA from each sample was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT Master Mix (Takara Bio, Japan). PCR was performed in triplicate with 1 μL of cDNA as template using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), on the StepOnePlus System (Applied Biosystems, CA, USA).

The following primers were used:

Collagen I A1, Forward Primer GCTCCTCTTAGGGG CCACT, Reverse Primer CCACGTCTCACCATTGGGG;

Collagen II A1, Forward Primer CAGGATGCCCGAAA ATTAGGG, Reverse Primer ACCACGATCACCTCTG GGT;

and β-actin, Forward Primer GGCAAATTCAACGGCA CAGTC, Reverse Primer GCTGACAATCTTGAGTGAGTT.

The reaction conditions were as follows: predenaturation at 95°C for 10 min; 40 cycles of 15 s at 95°C, 45 s at 59°C, and 30s at 72°C; and a final extension at 60°C for 5 min. β-Actin was used as the reference gene. Relative quantification of target gene expression was calculated using the comparative CT method.

2.10. Statistical Analysis. All statistical analyses were performed using SPSS 20.0 (SPSS, IL, USA). Quantitative data were presented as means ± standard deviations. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by least square difference (LSD) post hoc test. $P < 0.05$ was considered to denote statistical significance.

3. Results and Discussion

Figure 1 illustrates the thermosensitive behaviours of the PNIPAm-collagen I composite hydrogels, which appear to meet the requirements of the intended application scenarios.

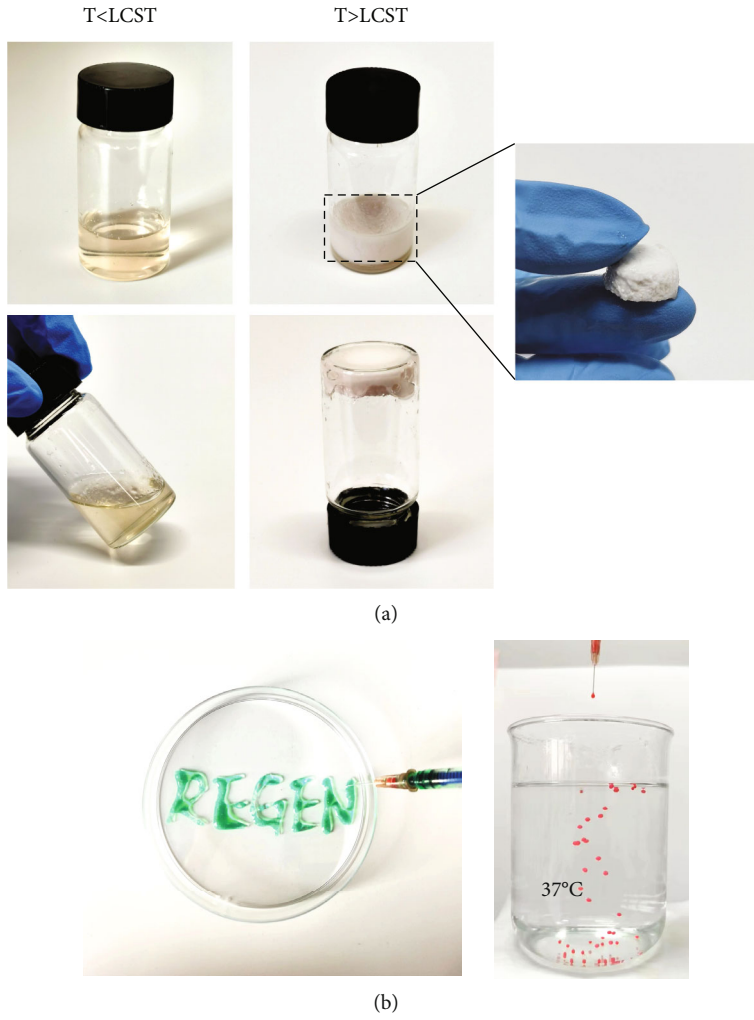


Figure 1: Thermosensitive behaviours of PNIPAm-collagen I composite hydrogels: (a) PNIPAm-collagen I hydrogel (PNIPAm-0.1Coll group) liquefies below and solidifies above the LCST; (b) PNIPAm-collagen I hydrogel (PNIPAm-0.5Coll group) is readily injectable under room temperature and solidifies in 37°C water (colored for better demonstration).

DSC analysis (Figure 2) shows that the LCST of plain PNIPAm hydrogel is around 32°C, agreeing with data from other researches [28, 29], whereas the introduction of collagen I seems not to have caused marked impacts on the LCST. The thermosensitive properties of PNIPAm are governed by the interactions of the hydrophilic amide groups and hydrophobic isopropyl groups with water [30]. Researches have shown that, when the PNIPAm is copolymerized with other monomers, hydrophilic monomers can increase the polymer's hydrophilicity and result in a higher LCST, whereas hydrophobic monomers reduces the LCST [31]. In the present hydrogels, however, the collagen fibrils merely form a physical interpenetrating network with the PNIPAm molecules. The amide groups and hydroxyl groups of collagen I are all participating in the collagen's own stabilization of triple helices and self-assembly of fibrils, leaving very few functional groups to interact with the PNIPAm's amide groups and isopropyl groups [32]. With the hydrophilic and hydrophobic interactions virtually unaffected, the composite hydrogels hence have a constant LCST.

To our delight, the supporting collagen I network has also been reported to reduce the syneresis of PNIPAm during solidification [33]. It is therefore possible to fine-tune the biomechanical and biochemical properties of the PNIPAm-collagen I composite hydrogels by adjusting the collagen contents, without jeopardizing their desirable thermal behaviours.

Figure 3 illustrates the viscoelastic properties, including complex viscosity (η^*), storage modulus (G'), and loss modulus (G''), of the hydrogels with respective collagen I contents. Sharp increases in all characteristics are observed near the LCST due to the thermal phase transition of PNIPAm, which indeed lays the foundation of the applicability of these hydrogels in future clinical practice, i.e., injectability under room temperature and sufficient strength under body temperature.

With more detailed analysis, we can see that, under room temperature, η^*, G', and G'' all show positive correlations with the collagen I content (η^* increasing from 0.71 ± 0.35 Pa•s in PNIPAm to 110.95 ± 23.22 Pa•s in PNIPAm-1Coll,

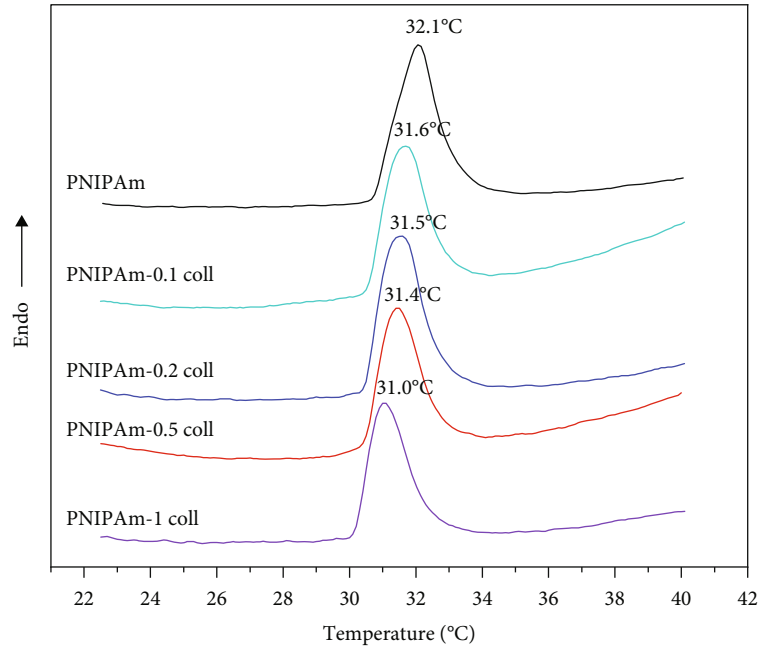


Figure 2: DSC curves of hydrogels with various collagen I contents. Three replicates were tested for each hydrogel sample group. Representative curves of respective groups are shown.

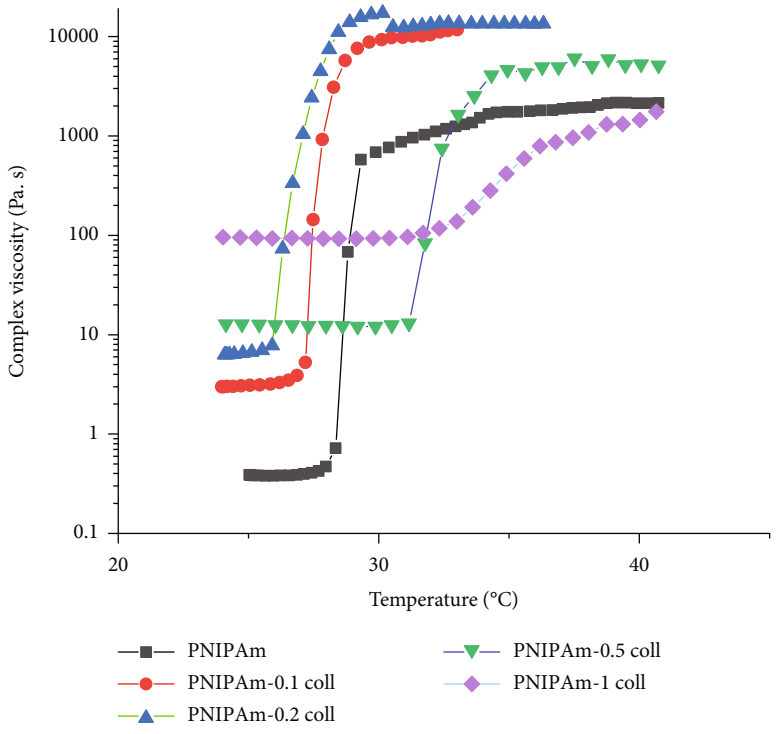
G' increasing from 3.28 ± 2.02 Pa in PNIPAm to 658.06 ± 128.07 Pa in PNIPAm-1Coll, and G'' increasing from 3.30 ± 1.01 Pa in PNIPAm to 308.78 ± 56.67 Pa in PNIPAm-1Coll), offering controllability of the viscoelastic behaviours of the hydrogels. With 1% collagen I, however, the η^* of the hydrogel reaches over 110 Pa•s at 25°C, which exceeds the usual suitable range for injectability [34, 35]. Therefore, the overly viscous PNIPAm-1Coll group is discontinued in further biochemical tests of this study and may be considered for other open surgery scenarios.

Under 37°C, the rheological data of the PNIPAm-0.5Coll group (η^* 4.06 ± 0.77 kPa•s, G' 34.15 ± 4.61 kPa, and G'' 6.07 ± 1.79 kPa) and the PNIPAm-1Coll group (η^* 1.25 ± 0.44 kPa•s, G' 10.36 ± 3.54 kPa, and G'' 1.89 ± 0.68 kPa) have unexpectedly fallen below those of the PNIPAm-0.2Coll group (η^* 14.43 ± 1.94 kPa•s, G' 142.81 ± 22.94 kPa, and G'' 14.26 ± 7.21 kPa). This is due to the fact that high collagen I content has led to much greater extent of solidification and has caused slippage between the sample and the plate in the parallel plate measurement. Nonetheless, the orders of magnitude of the storage moduli (10-100 kPa) and the loss moduli (1 k-10 kPa) of all hydrogels are in line with our design. It has been relatively well-characterized that the native meniscus has a 100-1000 kPa storage modulus and a 10-100 kPa loss modulus [36, 37]. In clinical practice, the cells to be delivered with the hydrogels are believed to be just capable of mechanically bridging this 10-fold gap, forming a repairment close to native tissues [38, 39].

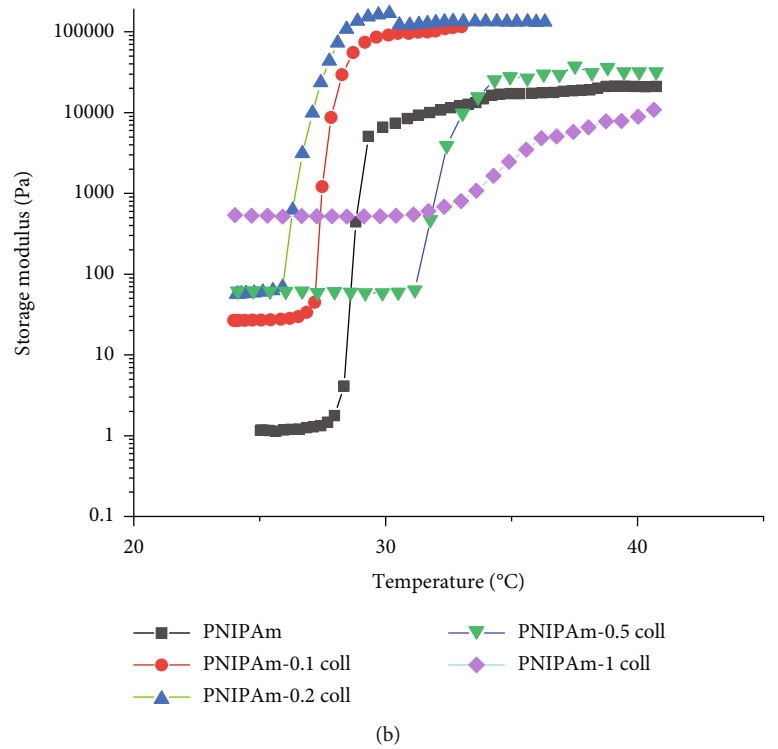
Figure 4(a) shows the morphology of passage 1 murine chondrocytes. While the majority of the chondrocytes exhibit the typical round morphology (believed to be mainly expressing collagen II and aggrecans), some cells with fibroblast-like flattened or fusiform shapes (believed to be mainly expressing collagen I) are also observed [40–42]. This is in agreement with the cartilage composition of newborn mice [43] and also provides a desirable onset for the coculture of chondrocytes and hydrogels, as we can later investigate how the hydrogels could potentially direct the phenotyping of the chondrocytes.

After 96 h of coculture, as can be seen in Figure 4(b), chondrocytes in all groups of hydrogels are well-proliferated, and the increase of collagen I content in the hydrogels has prominently promoted cell proliferation. This is because collagen binds to integrin receptors and activates integrin-based pathways that upregulate proliferation [44, 45]. Many natural polymers have been investigated to form composites with PNIPAm, and collagen seems to have excelled in bioactivity amongst them. Chen and Cheng and Mellati et al. found PNIPAm/chitosan scaffolds to be inhibiting cell growth, due to the positive surface charges (NH_3^+) of chitosan [17, 46]. Tan et al. cultured human adipose-derived stem cells with hyaluronic acid-PNIPAm hydrogels, and cells decreased in viability by different extents depending on the content of hyaluronic acid [47]. In contrast, all of the PNIPAm-collagen I hydrogels in this study are already enjoying at least 200% cell proliferation at 96 h. It should also be noted that, in the cell proliferation test, the chondrocytes were cultured in a 3D environment, and the results could therefore indicate satisfactory bioactivity of the hydrogels when injected to 3D meniscal tissues.

High-density cell culture is widely used for in vitro studies of chondrogenesis [48, 49]. Zhang et al. reported upregulated collagen II and aggrecan and downregulated collagen I and collagen X in high-density-cultured human mesenchymal stem cells (HMSCs) [50]. This is desirable for inducing articular cartilage tissue but does not meet the authors' specific requirements of meniscus regeneration. Therefore, we

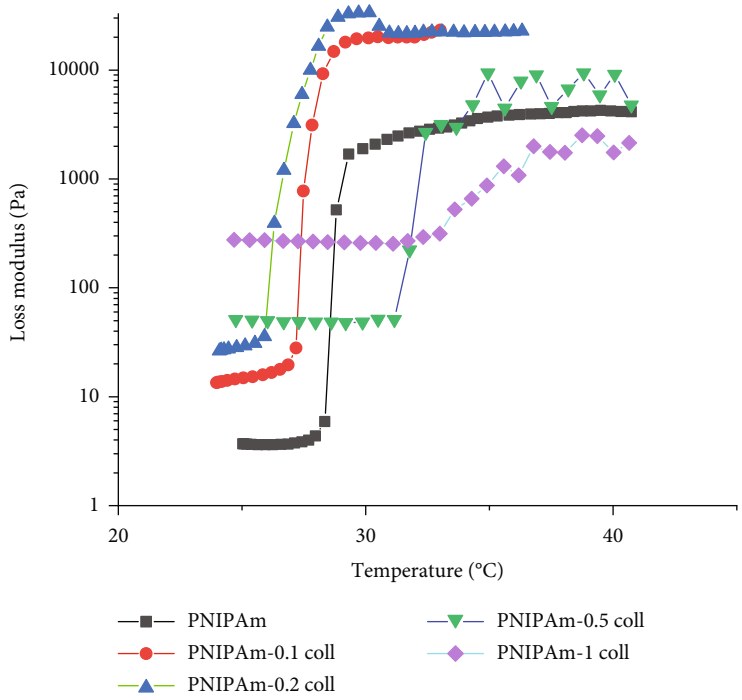


(a)



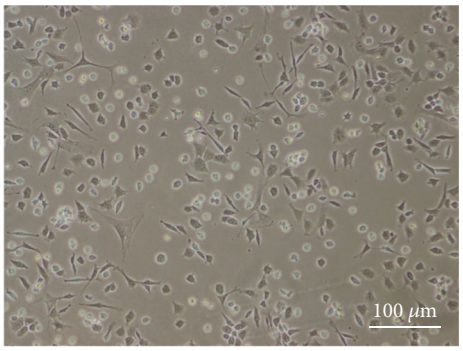
(b)

Figure 3: Continued.

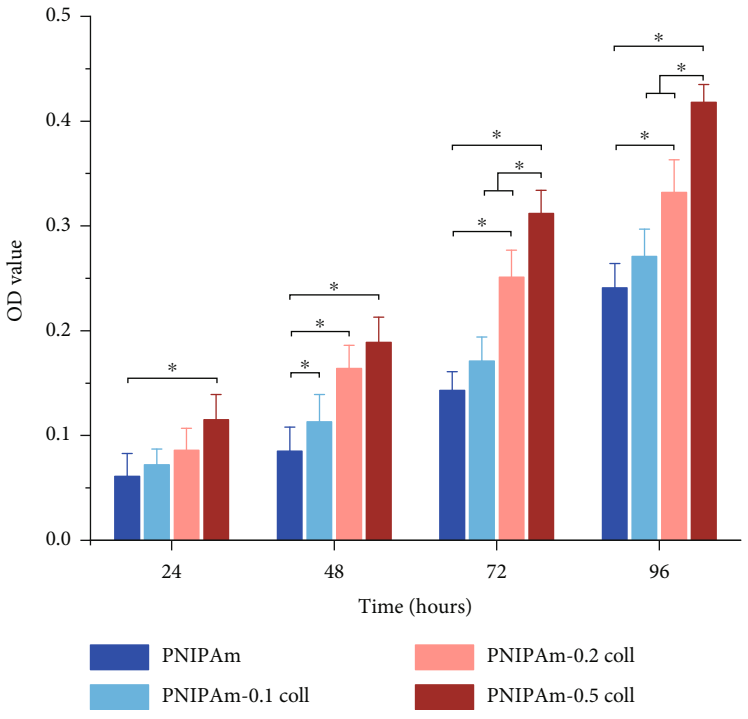


(c)

Figure 3: Thermosensitive viscoelastic properties of hydrogels with various collagen I contents: (a) complex viscosity (η^*), (b) storage modulus (G'), and (c) loss modulus (G''). Three replicates were tested for each hydrogel sample group. Representative data are shown.



(a)



(b)

Figure 4: (a) Optical micrograph of passage 1 murine chondrocytes. Scale bar = 100 μm. (b) Proliferation of murine chondrocytes cocultured with hydrogels with various collagen I contents. Error bar represents one standard deviation from mean. * denotes $P < 0.05$.

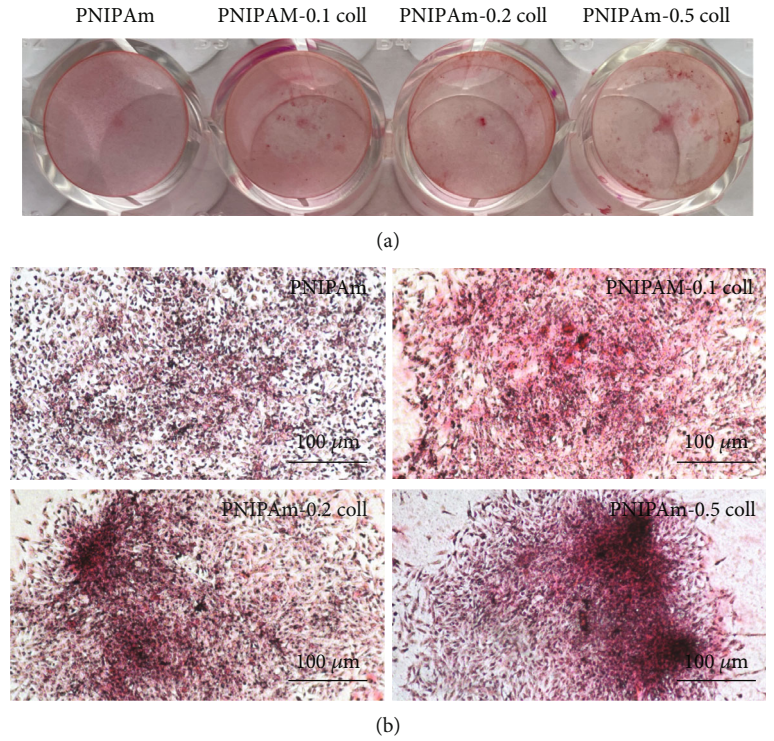


Figure 5: (a) Macrograph and (b) micrographs of murine chondrocytes stained with safranin-O after 7 days of high-density culture with hydrogels. Scale bar = 100 μm.

have designed the meniscus-like (collagen I-rich) microenvironment for high-density cell culture, attempting to guide the phenotyping of the chondrocytes.

Histochemical analysis (Figure 5) shows that, in high-density culture, all groups of cells retain their chondrogenic characteristics and are positively stained by safranin-O [51]. Coculture with higher collagen I content hydrogel seems to have resulted in stronger staining. This is directly due to the different cell density in each group at day 7, to which the collagen I content is known to be a major influencing factor. Collagen I has also been reported to promote formation of cell aggregates [52], which agrees with the findings in Figure 5(a). The greater extent of cell aggregation in the PNIPAm-0.5Coll group suggests that there is a faster process of neotissue formation [53] and cell maturation [52].

Western blot data (Figure 6) reveals more phenotypic information of the cells. Both collagen I and collagen II are visibly expressed, indicating the existence of both articular chondrocytes and fibrochondrocytes. It is also discovered that higher collagen I content in the microenvironment has more remarkable effects on the collagen I expression in the cells. For instance, the PNIPAm-0.5Coll group shows a 3.50-fold relative intensity of collagen I compared with the PNIPAm group (Figure 6(b)), whereas that of collagen II is 2.36-fold (Figure 6(c)).

The same trend is confirmed in qPCR analysis. There is a more drastic upregulation of collagen I a1 than collagen II a1 when the cells are cocultured with higher collagen I content hydrogels. The PNIPAm-0.5Coll group shows a 3.64-fold expression of collagen I a1 compared with the PNIPAm group (Figure 7(a)), whereas that of collagen II a1 is 3.34-fold (Figure 7(b)). The PNIPAm-0.5Coll group also exhibits significantly higher collagen I a1 expression than the PNIPAm-0.1Coll and PNIPAm-0.2Coll groups, while the increase of collagen II a1 expression has not reached statistical significance. Gigante et al. reported increased conversion to fibrochondrocyte phenotype on collagen I membranes when culturing human chondrocytes [54]. In contrast, Ibusuki et al. found that rabbit chondrocytes remained predominantly in chondrogenic phenotype when cultured in PNIPAm-gelatin hydrogels [29]. It is therefore speculated that the collagen I in the hydrogels of this study has also been capable of inducing a greater proportion of fibrochondrocytes in the high-density cultured murine chondrocytes, possibly through a focal adhesion kinase (FAK) signaling pathway activated by collagen-integrin interaction [55, 56].

The regeneration of meniscal tissue indeed needs a spectrum of chondrocytic and fibroblastic characteristics. Hoben et al. [53] delicately cocultured fibrochondrocytes and articular chondrocytes at a 50 : 50 ratio and achieved after 4 weeks promising meniscal tissue constructs. Zhang et al. [57] applied biochemical stimulation (connective tissue growth factor and transforming growth factor-β3) and biomechanical stimulation (dynamic loading) simultaneously to induce fibrogenic phenotype in the "outer region" and chondrogenic phenotype in the "inner region" of the meniscal scaffold. Both of these studies aimed for meniscus implantation, and certain level of sophistication was necessary for desirable cell phenotyping and ECM composition. In an injection-based treatment, however, a simpler method is more welcome. Just by adjusting the collagen I content, the injectable hydrogels developed in the present study

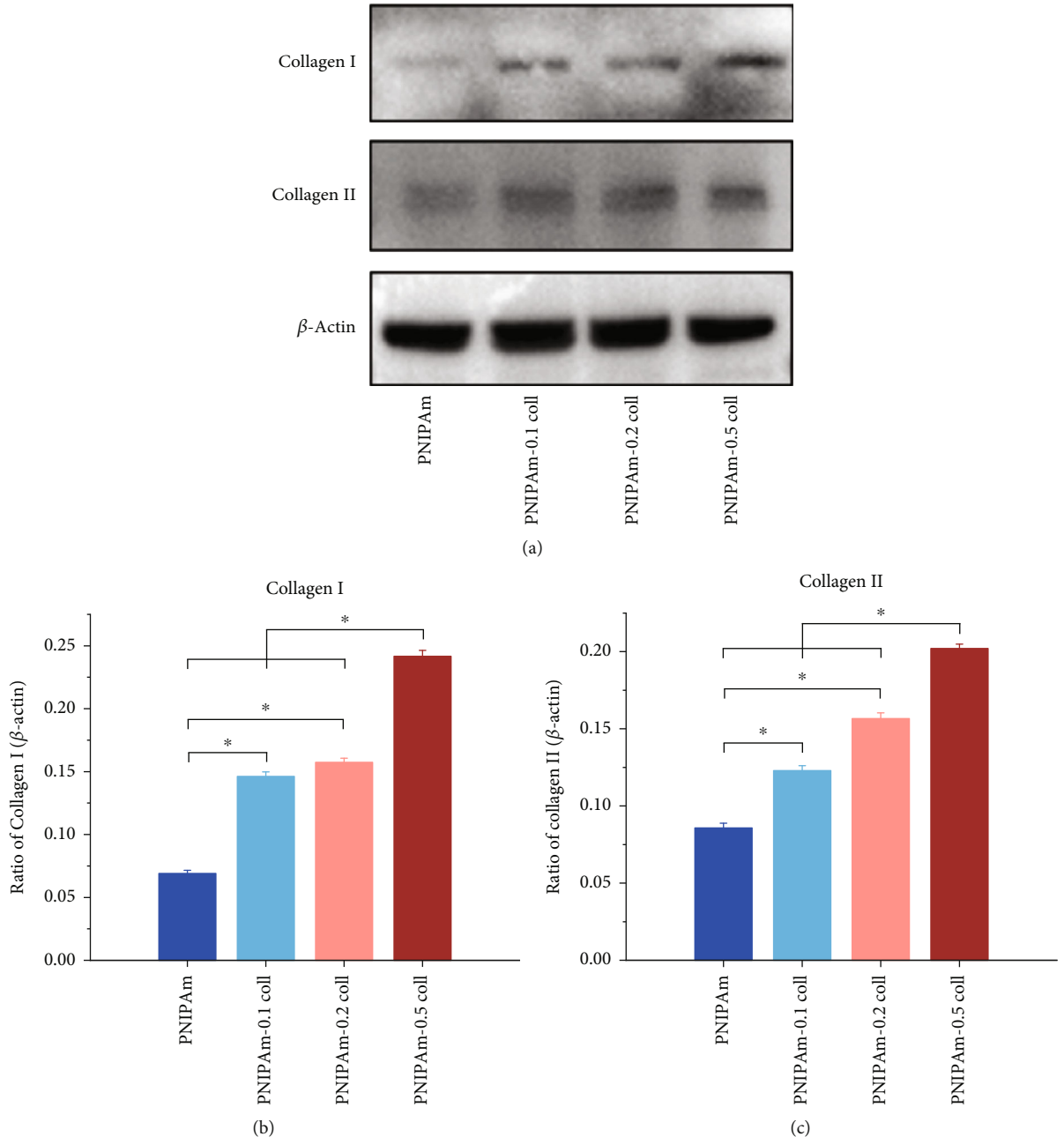


Figure 6: Expression of collagen I and collagen II in murine chondrocytes after 7 days of high-density culture with hydrogels: (a) Western blot bands of collagen I and collagen II, with β-actin as reference; (b) normalized intensities of collagen I bands; (c) normalized intensities of collagen II bands. Error bar represents one standard deviation from mean. * denotes $P < 0.05$.

may be capable of achieving the suitable zonal phenotypic composition from primary chondrocytes, which may provide new insights into strategies of meniscus cell therapy.

Compared with other injectable hydrogels intended for the same application, the hydrogel in this study also exhibits merits on ease of use. The ECM hydrogel developed by Wu et al. takes 30 minutes to solidify [12], and the pluronic/PMMA-reinforced fibrin hydrogel developed by An et al. needs to be applied by a dual-syringe system [14], whereas the presented hydrogel can be applied with a single homogeneous injection and solidifies instantly under body temperature.

In the specific topic of PNIPAm-based thermosensitive hydrogels for meniscus repair, Chen and Cheng are believed to have conducted the pioneering works. In their studies, they also evaluated PNIPAm hydrogels incorporated with chitosan and hyaluronic acid and illustrated that both articular chondrocytes and meniscus cells grown in those hydrogels maintained chondrogenic phenotype, judging from the spherical morphology of the cells [19]. This finding was not counter-intuitive, given that hyaluronic acid was abundantly found in articular cartilage. In the present study, the same logic seems to apply, i.e.,

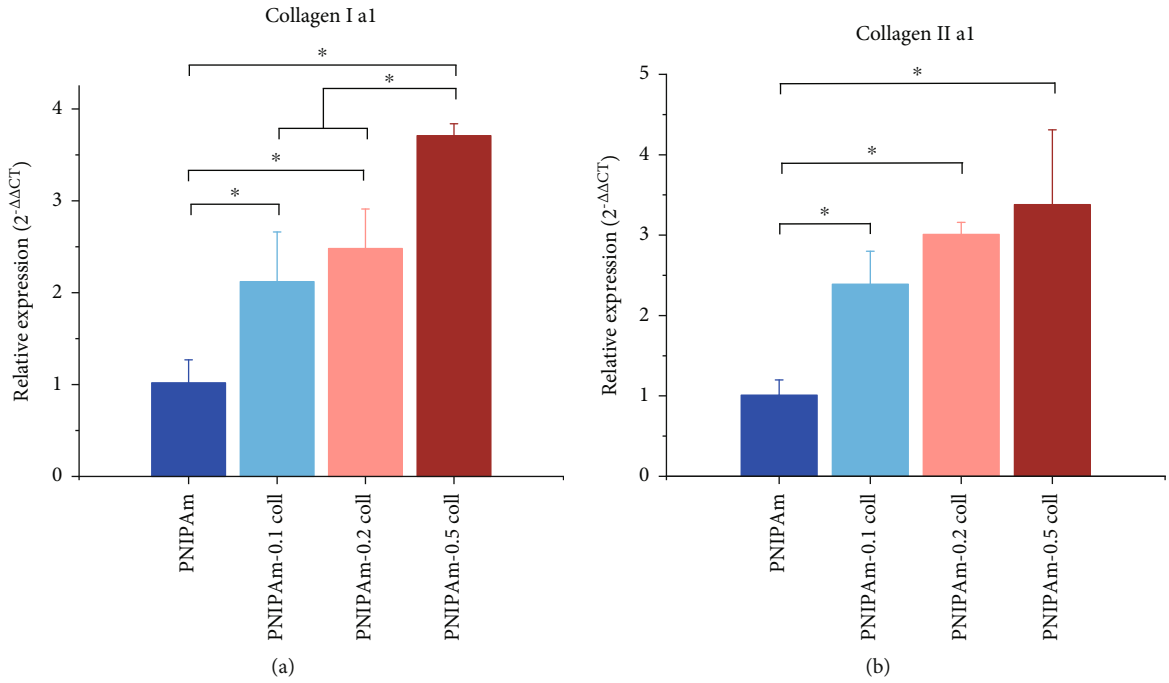


FIGURE 7: qPCR analysis ($2^{-\Delta\Delta CT}$ values) of (a) collagen I a1 gene expression and (b) collagen II a1 gene expression in murine chondrocytes after 7 days of high-density culture with hydrogels. Error bar represents one standard deviation from mean. * denotes $P < 0.05$.

a collagen I-rich microenvironment mimicking native meniscus is able to induce fibrochondrocyte phenotype.

One limitation of this study was that, in the high-density culture, the cells were still grown in a 2D-like manner, because spatially mixing the cells with hydrogels would have made medium change practically infeasible. Therefore, our future work will be focusing on in vivo studies of cell-bearing hydrogels in 3D meniscal environments, to further verify their regenerative and phenotype-inducing properties. Based on the preliminary results of physical and biological characterizations, we expect that the novel PNIPAm-collagen I hydrogels in this study will be able to successfully entrap and retain chondrocytes or stem cells and stimulate in situ phenotyping or differentiation for the regeneration of meniscus. The PNIPAm-0.5Coll hydrogel is likely to be the first promising candidate to be trialed for this application.

4. Conclusion

In this study, injectable thermosensitive poly(N-isopropylacrylamide)-collagen I composite hydrogels were successfully fabricated, and their physical and biological properties were characterized. The incorporation of collagen I did not affect the LCST of PNIPAm but was able to mechanically reinforce the hydrogels, rendering higher complex viscosities, storage moduli, and loss moduli. Preliminary results of coculturing murine chondrocytes with the hydrogels showed satisfactory cell proliferation, as well as desirable fibrochondrocyte phenotype induced by collagen I. To the authors' knowledge, this family of hydrogels has first-of-its-kind compositions specifically designed for meniscus repair. Compared with other hydrogels intended for the same indication, these novel PNIPAm-collagen I hydrogels possess favorable features such as undemanding fabrication procedure, good injectability and thermosensitivity, tunable mechanical properties, and superior bioactivities. The PNIPAm-0.5Coll hydrogel is believed to be the optimal choice in this series. The findings of this study provide an important basis for the next-step in vivo investigation and bode well for the hydrogels' future clinical applications.

Data Availability

All data used to support the findings of this study are available upon requests made to Qi Chen (chenqimike@gmail.com).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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