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Directing chondrogenic differentiation of mesenchymal stem cells with a solid-supported chitosan thermogel for cartilage tissue engineering

Hongjie Huang¹, Xin Zhang¹, Xiaqing Hu¹, Linghui Dai¹,², Jingxian Zhu¹,², Zhentao Man¹, Haifeng Chen³, Chunyan Zhou²,⁴ and Yingfang Ao¹,⁴

¹ Institute of Sports Medicine, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing 100191, People’s Republic of China
² Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University, 38 Xueyuan Road, Haidian District, Beijing 100191, People’s Republic of China
³ Department of Biomedical Engineering, College of Engineering, Peking University, 5 Yiheyuan Road, Haidian District, Beijing 100871, People’s Republic of China
E-mail: chunyanzhou@bjmu.edu.cn and yingfang.ao@gmail.com

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Abstract

Hydrogels are attractive for cartilage tissue engineering because of their high plasticity and similarity with the native cartilage matrix. However, one critical drawback of hydrogels for osteochondral repair is their inadequate mechanical strength. To address this limitation, we constructed a solid-supported thermogel comprising a chitosan hydrogel system and demineralized bone matrix. Scanning electron microscopy, the equilibrium swelling ratio, the biodegradation rate, biomechanical tests, biochemical assays, metabolic activity tests, immunostaining and cartilage-specific gene expression analysis were used to evaluate the solid-supported thermogel. Compared with pure hydrogel or demineralized matrix, the hybrid biomaterial showed superior porosity, equilibrium swelling and degradation rate. The hybrid scaffolds exhibited an increased mechanical strength: 75% and 30% higher compared with pure hydrogels and demineralized matrix, respectively. After three days culture, bone-derived mesenchymal stem cells (BMSCs) maintained viability above 90% in all three materials; however, the cell retention of the hybrid scaffolds was more efficient and uniform than the other materials. Matrix production and chondrogenic differentiation of BMSCs in the hybrid scaffolds were superior to its precursors, based on glycosaminoglycan quantification and hyaline cartilage marker expression after three weeks in culture. Its easy preparation, favourable biophysical properties and chondrogenic capacity indicated that this solid-supported thermogel could be an attractive biomaterial framework for cartilage tissue engineering.

Keywords: mesenchymal stem cell, thermogel, demineralized bone matrix, chondrogenesis, cartilage tissue engineering

(Some figures may appear in colour only in the online journal)

1. Introduction

The consistently successful repair of articular cartilage defects is still a challenging issue in clinical practice and research. The
paucity of efficient clinical options for treatment has prompted research into tissue engineering approaches involving the combination of chondrogenic cells, biodegradable materials (scaffolds) and/or biologically active factors [1]. With many functions similar to native cartilage matrix, hydrogels are attractive scaffolds for the repair of cartilage defects [2–4]. Hydrogels can adsorb a large amount of water, dictate cell distribution and infiltration in three dimensions, provide integrity to the surrounding tissues following implantation and allow efficient nutrient transportation for the embedded cells [4–6]. The implantation patterns (injectable form or solid gel) and chemico-physical properties of the hydrogel will depend mainly on the time course and mechanism of gelation, and the polymer preparation in the process of tissue engineering [6, 7].

Chitosan is a promising polymer for biomedical applications because of its favourable biocompatibility, biodegradability, nontoxicity and antimicrobial activity [8–13]. A neutral chitosan solution displays a thermosensitive gelation property [14, 15]. This hydrogel system can easily mix with cells and/or growth factors in sol state and retain them in a gel at 37 °C; the cationic character of chitosan in a neutral environment allows for adherence of this thermogel to most human tissue sites [8]. Chitosan-based thermogels have received much attention in tissue engineering because of their simplicity of preparation and controllability of the payload of cells or growth factors [15–18]. However, like other hydrogels, one critical drawback of using a chitosan gel is its inadequate mechanical properties, which have impeded its use for some load-bearing tissues, especially in wet conditions (e.g. articular cavities) [2, 6, 7, 19, 20]. Recently, Oha and Hwang reported that the stiffness of chitosan in hydrated conditions is dramatically reduced, by approximately 87%, because of its ability to absorb water; they made a biomimetic chitosan composite with improved mechanical properties in wet conditions using catechol-mediated cross-linking [21]. However, this biomedical material is a chitosan-based film with reduced swelling properties, which rules it out for three-dimensional (3D) culture or application in tissues with high water content, like cartilage. Therefore, efforts to improve the mechanical properties of chitosan-based hydrogel, without compromising its advantages in the repair of cartilage, are essential for cartilage tissue engineering [8, 21].

In this study, we constructed a hybrid scaffold comprising a chitosan thermogel and demineralized bone matrix (DBM), which can retain more cells homogeneously because of chitosan’s sol–gel transition and provide sufficient strength for cartilage tissue engineering. To the best of our knowledge, this is the first attempt to integrate a thermosensitive gel and demineralized extracellular matrix (ECM) into one 3D scaffold. The long-term objective of this work was to develop a functional biomaterial based on combining the advantages of a thermogel and solid ECM. In this study, we described the first steps towards this objective by characterizing the material properties, biomechanical strength, in vitro cytocompatibility and chondrogenic capacity of the biomaterial platform.

2. Materials and methods

2.1. Preparation of scaffolds

2.1.1. Chitosan thermogel. Chitosan (93% deacetylation; Aladdin, Shanghai, China) was dissolved in 0.1 M acetic acid to obtain a 2.5 wt% chitosan solution. A pre-cooled β-glycerophosphate solution (GP; Sigma, St. Louis, MO, USA) was then dropped into the chitosan solution resulting in a final chitosan-GP solution with 2.25 wt% chitosan and 6 wt% GP. The pH of the chitosan-GP solution was 7.17 ± 0.3. All procedures for preparing this solution were conducted on ice to prevent premature gelation. Gelation of the final solution was initiated by incubation at 37 °C [14]. This solution was used to re-suspend BMSCs at room temperature to achieve the desired cell density for in vitro 3D culture.

2.1.2. Demineralized bone matrix. DBM was made from the epiphysis of sheep femur according to our previous study [22]. Ethylene diamine tetraacetic acid (EDTA; 0.5 M, pH = 8.3) was used for demineralization. The femur specimens were trimmed and immersed in EDTA solution at 4 °C for one month with a change of solution every day. The replaced EDTA solution was analysed by atomic absorption spectrophotometry to track the demineralization process, and a radiographic check was used to ensure complete demineralization of the specimens. The demineralized matrix were incised into Φ0.5 cm × 0.2 cm cylinders, stored at −80 °C and sterilized with cobalt-60 irradiation for 24 h on the day preceding 3D culture.

2.1.3. Solid-supported chitosan thermogel. The chitosan-GP solution (200 μL) with or without cells was dropped along one side of prefabricated DBM cylinders, allowing the solution to permeate throughout whole DBM cylinder. Gelation was also initiated by incubation at 37 °C. Gel formation and gelation time were determined by the mobility of chitosan-GP solution after inverting the scaffolds, and by monitoring the turbidity of the solution from clear in the liquid state to white/grey in the gel state. There was no difference in gelation time between the pure thermogel and solid-supported thermogel (12 ~ 15 min).

2.2. Characterization of the scaffolds

2.2.1. Scanning electron microscopy. Scanning electron microscopy (SEM) was used to make observations of the surface and internal cross-section of the scaffolds. The scaffolds were fixed for 24 h in 2.5% glutaraldehyde in phosphate buffer solution (PBS; 0.1 M, pH 7.4) and then dehydrated in an ethanol series. The specimens were freeze-dried and then sputter-coated with gold. SEM observations were carried out in a high resolution SEM (Hitachi Ltd, Tokyo, Japan). The images (n = 5) were analysed for pore size and porosity using Image-pro Plus software.

2.2.2. Equilibrium swelling ratio. After 24 h of lyophilization, samples (n = 5) were immersed in PBS at 37 °C. At varying times, the swollen samples were removed and immediately
weighed using a microbalance after the excess of water lying on the surfaces was absorbed with filter papers. The equilibrium swelling ratio (ESR) was calculated as follows:

\[
ESR = \frac{(W_s - W_d)}{W_d}
\]

where \(W_s\) and \(W_d\) are the weights of the scaffolds at the swelling state and at the dry state, respectively \[17\].

2.2.3. In vitro degradation. Scaffolds \((n = 5)\) were immersed in PBS \((\mathrm{pH} = 7.4)\) and incubated at 37 \(^{\circ}\)C overnight. After equilibrium in PBS, all samples were weighed with a microbalance \((W_0)\) followed by incubation in PBS at 37 \(^{\circ}\)C. At various intervals, samples were removed and immediately weighed with a microbalance after the excess of water was removed \((W_t)\). The degradation ratio of scaffolds was calculated as:

\[
100\% \times (W_0 - W_t)/W_0.
\]

2.2.4. Mechanical testing. For unconfined compression tests, scaffolds \((n = 3)\) were tested with a versatile biomechanical system (MTS Systems Corp., Eden Prairie, MN, USA). Samples were placed on a test plate and a preload of 0.01 N was applied \[23\]. The samples were then compressed at a ramp speed of 0.05\,mm\,s\(^{-1}\) until 50\% strain was reached. Force and deformation data were collected at 2 Hz using TestWorks4 software (MTS Systems Corp.). The elastic modulus was determined from the slope of the linear portion of the stress–strain curve for all samples.

2.3. Characteristics of bone-derived mesenchymal stem cells

Bone-derived mesenchymal stem cells (BMSCs) from multiple Sprague-Dawley rats at passage one were purchased from Cyagen Biosciences Inc., Guangzhou, China. The cells were thawed, cultured and expanded according to the supplier’s instructions. The specific cell surface antigen markers of BMSCs were analysed by flow cytometry analysis using antibodies against CD34 \(\text{(sc-7324 PE; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)}\) and CD45 \(\text{(ab95786; Abcam Inc., Cambridge, MA, USA)}\) for PE; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) cytometry analysis using antibodies against CD34 \(\text{(sc-7324 PE; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)}\) and CD45 \(\text{(ab95786; Abcam Inc., Cambridge, MA, USA)}\) for positive markers of BMSCs \[24–26\].

The multiple lineage differentiation of BMSCs was evaluated by a tri-lineage differentiation experiment towards chondrogenesis, osteogenesis and adipogenesis, according to the supplier’s instruction (Cyagen Biosciences Inc.). Briefly, BMSCs at passage four were used for tri-lineage-induced experiments. For adipogenesis, the BMSCs \((1 \times 10^5 \text{cells/well})\) were cultured in a 6-well plate with rat BMSC adipogenic differentiation medium \((\text{RASMX-90031; Cyagen Biosciences Inc.})\). The cells were evaluated for adipogenesis by oil red O staining after 19 days in culture. The osteogenesis induction of BMSCs was similar to adipogenesis, except that a rat BMSC osteogenic differentiation medium was used for culturing \((\text{RASMX-90021; Cyagen Biosciences Inc.})\). Alizarin red staining was used to check the osteogenic differentiation after 11 days in culture. For chondrogenesis, a standard pellet culture was performed. After three weeks of chondrogenic incubation, the pellet was formalin fixed and paraffin embedded. Toluidine blue staining was then performed to assess the chondrogenic capacity of the BMSCs \[26\].

2.4. Cell encapsulation and culture in scaffolds

The BMSCs from SD rats at passage four were used for \textit{in vitro} 3D culturing. Briefly, BMSCs were collected and re-suspended in chitosan-GP solution to achieve a density of \(3.0 \times 10^5 \text{cells mL}^{-1}\). Then, 200 \(\mu\text{L} of this solution \((6.0 \times 10^5 \text{cells})\) were used for the chitosan gel (CS group) or the solid-supported thermogel (CS-DBM group). After incubation at 37 \(^{\circ}\)C for 15 min, the scaffolds containing hydrogel were transferred into each well of a 24-well plate and washed three times with cell culture medium every 10 min to remove residual \(\beta\)-glycerophosphate. For the pure DBM group, 200 \(\mu\text{L} of culture medium with same cell density were dropped into DBM scaffolds before being transferred into a 24-well plate.

A rat BMSC chondrogenic differentiation medium was used for culturing, which contained 0.1 \(\mu\text{L} \text{mL}^{-1}\) dexamethasone, 3 \(\mu\text{L mL}^{-1}\) ascorbate, 10 \(\mu\text{L mL}^{-1}\) ITS+Supplement, 1 \(\mu\text{L mL}^{-1}\) sodium pyruvate, 1 \(\mu\text{L mL}^{-1}\) proline and 10 \(\mu\text{L mL}^{-1}\) TGF-\(\beta\) \(3\) \((\text{RASMX-90041; Cyagen Biosciences Inc.})\). The culture medium was changed every three days and collected for biochemical analysis.

2.5. Cell retention and viability assessment in scaffolds

After 72 h of culture, cell viability in the scaffolds \((n = 3)\) was evaluated with a LIVE/DEAD assay (Invitrogen, Carlsbad, CA, USA) under a Leica TCS-SP8 confocal microscopy (Leica, Nussloch, Germany), according to the manufacturer’s protocol. The scaffolds were washed in sterile PBS for 2 min, and immersed in 500 \(\mu\text{L} of working solution containing 2 mM calcein AM and 4 mM ethidium homomider-1 reagents before incubation for 1 h at room temperature. Excitation wavelengths of 488 or 568 nm were used to detect the visualization of calcein AM (green fluorescence: labelling live cells) or ethidium homomider-1 (red fluorescence: labelling dead cells). The morphology of the cells in the scaffolds was also observed under confocal microscopy. Briefly, the scaffolds were washed with PBS, and fixed with 4\% paraformaldehyde. Rhodamine phalloidin \(160 \text{nM; Cytoskeleton Inc., Denver, CO, USA}) was used to stain the cytoskeleton of the cells for 4 h at 37 \(^{\circ}\)C. The nuclei were then counter-stained with Hoechst33258 \((1:800; Fanbo, Beijing, China)\).

2.6. In vitro proliferation and matrix formation

A 1,9-dimethylmethylene blue \((\text{DMMB; Sigma})\) dye-binding assay \[27\] was used to quantify the sulphated-glycosaminoglycan \((\text{sGAG})\) content in the scaffolds. For each group, three specimens were weighed with a microbalance and digested in a pre-prepared papain solution \((\text{Sigma})\) at 60 \(^{\circ}\)C overnight. Samples of the digest \((20 \mu\text{L})\) were mixed with 200 \(\mu\text{L} of DMBB reagent, and the absorbance was measured on a plate reader at 525 nm. A standard curve based on chondroitin 6-sulphate from shark \((\text{Sigma})\) was established to determine the sGAG content.
Table 1. Primer sequences used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers (5′–3′)</th>
<th>Reverse primers (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGC</td>
<td>CATTGCACGAGGACAGCCCA</td>
<td>TGGGTTCCGTGGGGTACACA</td>
</tr>
<tr>
<td>COL1</td>
<td>CTGCCCCCTCGACGGGTTTGC</td>
<td>GCCCTGACATGTGGGCGAG</td>
</tr>
<tr>
<td>COL2</td>
<td>CAGCCTACGCTCCAGATGAC</td>
<td>GGAAGGCGTGGAGTTCTTCTTGT</td>
</tr>
<tr>
<td>COL10</td>
<td>GCCAGCGACATATGACCCACCA</td>
<td>ACAGGCCCTACCCAAACGTCAGTCC</td>
</tr>
<tr>
<td>ALP</td>
<td>GTACCACCCACAAACACGGCGGA</td>
<td>TCCAAAGCTGATGAGTGTCAC</td>
</tr>
<tr>
<td>18s RNA</td>
<td>GTAAACCCGTTGAACCCCATT</td>
<td>CCATCCAATCGTAGTACG</td>
</tr>
</tbody>
</table>

A commercial enzyme-linked immunosorbent assay kit (ELISA; Cloud-Clone Corp., Houston, TX, USA) was used to examine the alkaline phosphatase (ALP) content, according to the standard protocols provided by the manufacturer. Briefly, aliquots of the same sample digestion (n = 3) were dissociated by sonication in 0.1 M Tris (pH 7.4) containing 1% Triton X-100 and 5 mM MgCl₂. ALP production was measured at 460 nm using a plate reader and presented as a ratio of the ALP concentration normalized by the DNA content [28].

A fluorometric assay was used to measure the DNA content. Briefly, aliquots of the same sample digestion were incubated at 37 °C for 1 h with 200 μL Hoechst33258 working solution (2 μg mL⁻¹). The fluorescence intensities were then measured at 360 nm for excitation and 460 nm for emission. The DNA content was determined against a standard curve of calf thymus DNA (Sigma). A Cell Counting Kit-8 assay (CCK-8; Dojindo Laboratories, Kamimashiki Gun, Kumamoto, Japan) was used to quantify metabolic activity. Briefly, the CCK-8 solution (200 μL per scaffold) was added to the medium and incubated at 37 °C for 6 h. The optical density (OD) was then measured at 450 nm using a plate reader [28, 29].

2.7. Cartilage-special gene expression analysis

At various intervals, samples (n = 3) were removed from culture and briefly rinsed with PBS. All samples were snap-frozen in liquid nitrogen and pulverized in a mortar pre-cooled in liquid nitrogen. Sample powders were used for RNA extraction using the TRIzol reagent (Invitrogen). Isolated RNA was reverse-transcribed using a commercial kit (Promega, Madison, WI, USA), and real-time PCR analysis was performed using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The conditions for real-time PCR were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A dissociation stage was added at the end of the amplification procedure. The dissociation curve showed non-specific amplification. Aggrecan (AGC) and type II Collagen (COL2) were tested for chondrogenic differentiation; type I Collagen (COL1) and ALP for osteogenesis; type X Collagen (COL10) for hypertrophy. The PCR primers are listed in table 1; 18s RNA was used as the housekeeping gene. The relative expression changes of these target genes was quantified by normalizing their expressions to that of 18s RNA using the ∆∆Ct method [23, 30]. Briefly, ∆Ct values were obtained by the difference between the Ct values of the target genes and the 18s RNA gene at day 1, day 10 or day 21 of culture. The ∆∆Ct values were calculated by subtracting the ∆Ct values at day 1 from their respective ∆Ct values at day 10 or day 21. Each value represents the average of at least three independent experiments.

2.8. Immunostaining evaluation

Immunostaining was performed to observe the presence of type II collagen in the scaffolds. After three weeks in culture, the scaffolds were washed with PBS and fixed with 4% paraformaldehyde. The scaffolds were blocked with 10% FBS for 8 h at room temperature, and then incubated with rabbit polyclonal antibody against collagen II (1:50 dilution, ab53047; Abcam Inc., Cambridge, MA, USA) at 4 °C overnight. The scaffolds were then washed with 10% FBS, followed by incubation with TRITC-conjugated goat anti-rabbit IgG (1:800 dilution, ZF-0316; Zhongshan, Beijing, China) for 4 h at room temperature. Finally, the scaffolds were counter-stained with Hoechst33258. A positive control was performed with native rat cartilage tissue. The images of specimens after immunofluorescent staining were captured using confocal fluorescence microscopy.

2.9. Statistical analysis

All data were expressed as means ± standard deviation and represented at least three independent experiments. Data analysis was performed using PASW Statistics 18 software. After testing for homogeneity of variances, an ANOVA test was used to evaluate the results among the three groups; at the same time Pairwise Post Hoc Tests were performed with an LSD multiple comparisons procedure. A Student’s t-test was used to evaluate results between two groups. P-values <0.05 were considered significant.

3. Results

3.1. Fabrication of scaffolds

The chitosan-GP solution could be infused into the DBM easily and uniformly. There was no difference in gelation time between pure chitosan thermogel and solid-supported hydrogel (12~15 min). Pure chitosan hydrogels exhibited a grey gel structure, while pure DBM scaffolds showed a white fibrous network (figure 1(A)). Mixed CS-DBM materials exhibited an interconnected structure that chitosan gel interspersed within the DBM matrix (figure 1(A)).

SEM observation showed that the CS gel (30~70 μm) had significantly smaller pore sizes than that of DBM...
Figure 1. Gross morphology and scanning electron microscopy of scaffolds. (A) Gross morphology of chitosan thermogel (CS), demineralized bone matrix (DBM) and solid-supported chitosan hydrogel (CS-DBM). (B) Scanning electron microscopy showing CS-DBM effectively intertwined its precursors with different pore sizes.

Figure 2. Equilibrium swelling ratio of chitosan thermogel (CS), demineralized bone matrix (DBM) and solid-supported chitosan hydrogel (CS-DBM). (*) represents significant differences between CS and DBM (n = 5, p < 0.05).

Figure 3. In vitro degradation rate of scaffolds in PBS over time. The total degradation rate of CS and CS-DBM groups was significantly higher than that of pure solid scaffolds (n = 5, #p < 0.05 versus DBM).

In the degradation test, the CS-DBM scaffolds demonstrated unique kinetics of degradation. Unlike the CS or DBM scaffolds, which had an even rate of degradation, CS-DBM scaffolds degraded faster than the CS group during the early period of culture (first week), and then displayed a rate that was as slow as the DBM scaffolds during late period (figure 3). Overall, the degradation rate of the CS-DBM group was somewhere between that of the CS and DBM groups. The total degradation rate of CS and CS-DBM groups was significantly higher than pure solid scaffolds (figure 3).

3.3. Biomechanical properties

The results of biomechanical testing for the three kinds of material are shown in figure 4. The stress–strain curves showed
Figure 4. Biomechanics of materials. (A) Representative stress–strain curves of the three scaffolds. (B) Elastic modulus derived from linear regions of stress–strain curves \((n = 3, ^*p < 0.05)\).

![Figure 4](attachment:figure4.png)

that the CS group exhibited a uniformly linear region from 0% to 25% strain (figure 4(A)). A toe-in region from 0% to 8% strain was observed in the curves of the CS-DBM and DBM groups, followed by a linear region between 8% and 25% strain (figure 4(A)). Determination of the linear modulus (10–25% strain) showed that the CS-DBM scaffolds had higher mechanical strengths than the other materials (figure 4(B)).

3.4. BMSC characterization

Figure 5(A) showed that the BMSCs expressed CD44 (99.69%) and CD90 (95.05%), but not CD34 (0.062%) and CD45 (0.026%). As shown in figure 5(B), the BMSCs exhibited a homogeneous phenotype after passage and expansion. A tri-lineage differentiation experiment was used to identify the multiple lineage differentiation capacity of the BMSCs. The results demonstrated that the BMSCs exhibited tri-lineage differentiation potential for adipogenesis (figure 5(C)), osteogenesis (figure 5(D)), and chondrogenesis (figure 5(E)). These results suggested that the cell source used in this study comprised identified BMSCs with multiple differentiation potential.

3.5. Cell viability, distribution and morphology in scaffolds

The LIVE/DEAD assay showed that all three kinds of scaffold could support cell activity (figure 6(A)). However, after three days in culture, most cells in the CS hydrogels showed a 2D distribution because of gravity and cell adhesion to the Petri dishes, which caused a cell-rich field at the bottom of CS hydrogels. CS-DBM scaffolds sustained equivalent amounts of cells as the CS gel, but with a 3D distribution. Moreover, compared with DBM alone, the CS-DBM composite encapsulated uniformly many more cells. This result indicated that the cell retention of the CS-DBM group was more efficient and uniform than the other groups.
Cytoskeleton immunostaining images were consistent with LIVE/DEAD assay (figure 6(B)). After one week of *in vitro* culture, a significantly lower number of BMSCs were attached to pure DBM scaffolds compared with the other groups. BMSCs encapsulated in DBM scaffolds showed a typically fusiform MSC morphology. By contrast, cells in CS or CS-DBM materials were transformed into a chondrocyte-like shape. Moreover, in the CS-DBM materials, BMSCs exhibited an aggregating tendency towards the surface of the demineralized matrix.

### 3.6. *In vitro* proliferation and cartilage matrix production of encapsulated BMSCs

The proliferation and chondrogenesis assay results of BMSCs cultured in the different biomaterials are shown in figure 7. The Cell Counting Kit-8 test showed that the BMSCs in the three groups showed an increasing proliferative tendency during the culture period of 1–7 days (figure 7(A)). In addition, the OD values of the DBM group were significantly lower than the other groups at each time-point because of poor cell retention (figure 7(A)). Similar to the CCK-8 results, assessment of DNA content showed that pure DBM scaffolds had significantly lower initial DNA contents compared with the CS-DBM group (figure 7(B); *n* = 3, *p* < 0.05 versus CS-DBM at day 2). DNA contents in CS and CS-DBM groups significantly increased compared with results at day 2 (figure 7(B); *n* = 3, **p** < 0.01 versus CS-DBM at day 2). Moreover, after three weeks in culture, the hybrid scaffolds have significantly more deposited glycosaminoglycan compared with the other groups (figure 7(C); *n* = 3, *p* < 0.05 versus CS and DBM). An ELISA assay was performed to confirm the absence of osteogenesis or mineralization of BMSCs within the scaffolds. As shown in figure 7(D), there was no significant change of ALP synthesis in all three groups at all three time points during *in vitro* chondrogenic culture. Although there was significant change between CS and DBM groups at day 21, the synthesis of ALP in all three groups were very low compared to that in the cell with osteogenic differentiation [28, 31, 32]. These results suggested that the three kinds of scaffolds did not promote osteogenesis during chondrogenic culture, and the hybrid scaffolds showed superior BMSC proliferation and cartilaginous matrix production compared with pure gel or solid scaffolds.

### 3.7. Chondrogenic differentiation of encapsulated BMSCs

All three kinds of materials were assessed for their effects on gene expression of chondrogenic differentiation or dedifferentiation, including the expression of cartilaginous...
Figure 7. *In vitro* proliferation and cartilage matrix production of BMSCs in scaffolds. (A) CCK-8 value of BMSCs cultured in different scaffolds ($n = 3$, *p* < 0.05). (B) Changes in DNA content of BMSCs for different scaffolds ($n = 3$, *p* < 0.05). (C) Glycosaminoglycan deposited into the scaffolds, as analysed by the DMMB assay ($n = 3$, *p* < 0.05, **p** < 0.001). (D) The ALP products deposited in the scaffolds were determined using an ELISA assay ($n = 3$, *p* < 0.05).

genes (AGC and COL2), key dedifferentiation or osteogenesis markers (COL1 and ALP) and a cell hypertrophy maker (COL10). The expression level of cartilage-specific genes (AGC and COL2) was significantly upregulated in the CS-DBM group during the culture period (figures 8(A) and (B)). While COL2 gene expression was not detected in DBM group although the experiment was repeated several times, this was probably caused by its low cell retention. In contrast to the expression of cartilage-specific genes, there was no significant change in the expression of osteogenic genes (COL1 and ALP) in all three groups throughout the induction period, although the ALP expression in the DBM group was higher than that in the pure CS hydrogel group (figures 8(C) and (D)). We also checked the expression of a hypertrophic marker (COL10), there was no significant change between day 10 and day 21 in all three groups (figure 8(E)), although a high level expression was observed in the CS group at day 21. The results suggest that the hybrid scaffolds could promote robust BMSC chondrogenic differentiation without osteogenesis or hypertrophy.

Immunofluorescence of type II collagen was matched with relative mRNA expression data such that the CS-DBM showed the strongest intensity of immunostaining for type II collagen, which was similar to the positive control cartilage tissue (figure 8(F)). These results indicated that BMSC chondrogenesis and cartilaginous collagen deposition in this composite scaffold are superior to pure gel or solid scaffolds.

4. Discussion

Recently, mesenchymal stem cells (MSCs) have been studied extensively for tissue engineering because of their convenient availability, high expansion capacity, lack of significant immunogenicity and multipotent differentiation capability [1, 33, 34]. The prerequisite for using MSCs in cartilage tissue engineering is appropriate chondrogenesis and maintenance of the chondrocytic phenotype [1, 34–36]. In the literature, many factors are recognized as effective cues for inducing MSC chondrogenic differentiation, including 3D environment, growth factors, and chemical and biophysical stimuli [1, 34–36].

Growth factors have been used widely to induce chondrogenic differentiation of MSCs; however, the degree of chondrogenesis depends on the scaffold structure or culture environment to a considerable extent [37]. Providing MSCs with a 3D environment that mimics the biological and structural properties of the native ECM has been proven to encourage cell proliferation and differentiation [36, 37]. In native cartilage, chondrocytes account for a mere 5% of the total volume, the rest is a dense extracellular matrix comprising two main macromolecules, type II collagen and the aggregating proteoglycan [36]. The structure of native cartilage is a proteoglycan gel reinforced with a network of type II collagen fibres [38]. In this study, we have poured hydrogel (CS) into the collagen network (DBM), mimicking the structure of native cartilage (figure 1). Therefore, CS-DBM can absorb equivalent amounts of liquid because of the high water content of chitosan gel within the collagen network (figure 2), which allowed appropriate nutrition exchange and penetration of growth factors. This biomimetic scaffold improved the proliferation and chondrocytic differentiation of MSCs, based on combining advantages of hydrogel and solid ECM.

For cartilage tissue engineering material, cell interaction is believed to be important for proliferation and chondrogenic differentiation. High cell retention can improve cell–cell contact within 3D scaffolds, which promotes chondrogenesis
of MSCs for producing hyaline-like cartilage repair tissue [36, 39, 40]. Of the three kinds of scaffolds tested, DBM had a significantly larger pore size (300–600 μm), leading to obvious cell escape during in vitro culture or possible poor attachment to the surrounding tissues during repair of cartilage defects in vivo. This poor cell retention led to DBM exhibiting lower levels of MSC proliferation and chondrogenic differentiation compared with the other materials (figures 7 and 8). In contrast, the CS-DBM group sustained more cells homogeneously, which contributed to increased proliferation and chondrogenic profiles of MSCs without osteogenesis or hypertrophy (figures 6–8).

The biophysical properties of 3D scaffolds affect cell proliferation and differentiation, ultimately determining the performance of a tissue-engineered construct. It has been reported that degradation-mediated cellular traction can direct stem cell differentiation in a 3D microenvironment between osteogenesis and adipogenesis [41]. Moreover, stimulation of proliferation and prevention of early apoptosis improves MSC chondrogenesis [42]. In this study, the rapid degradation of CS-DBM in the early period (the first week) produced a potential cellular traction to further improve MSC chondrogenesis. Meanwhile, this period was accompanied by the cell proliferation in the CS-DBM group, which implied the rapid degradation provided a micro-space suitable for proliferation (figures 3, 7(A) and (B)). The influence of matrix degradation on stem cell differentiation into chondrocytes requires further study.

We have noticed that there are some studies suggesting that bone-derived matrix can drive MSC progression towards an osteogenic lineage [43, 44]. However, we did not observe significant osteogenic changes during the culture. Although the synthesis of ALP, a marker of osteogenesis, is higher in the DBM group than that in CS group at day 21, there is no significant change compared with day 2 and day 10 in the DBM group. We suppose that this is probably because of the application of chondrogenic differentiation medium in our culture system so the chondrogenic differentiation is dominant.

For clinical applications, cartilage tissue-engineered structures should possess certain mechanical properties to meet operational handling during surgery and mechanical loading after implantation. DBM is a 3D collagen network with pliable, but strong mechanical properties. DBM has favourable biocompatibility without biotoxicity and immunogenicity because of the absence of cellular antigens [45]. It has been reported that there are several growth factors and other cytokines reserved in the DBM material, which have a proven stimulatory effects on the osteochondrogenic activity of MSCs when implanted in vitro or in vivo [45–47]. The greater strength and chondrogenic induction of DMB has led to it being used in tissue engineering for cartilage repair, where neo-cartilage tissue is needed to meet the mechanical requirements [45, 46].
We demonstrated the efficacy of a DBM-based composite to support and promote cartilage repair in a previous study [22], thus providing further evidence of the potential of DBM for incorporation into cartilage tissue engineering. However, as a solid scaffold with macroporosity (300–600 μm), DBM was subject to increased cell escape through its large pores during in vitro culture, or poor attachment to the surrounding cartilage and subchondral bone in cartilage repairing in vivo. Moreover, in previous studies [45], researchers cultured cells on the surface of DBM, which led to a distribution of cells similar to a monolayer in 2D culture. In this study, CS-DBM composite scaffolds have a collagen network on the surface of DBM, which led to a distribution of cells similar to a monolayer in 2D culture. The CS-DBM scaffolds showed higher mechanical strength than its precursor materials (figure 4). This composite scaffold can absorb adequate fluid and retain more cells homogeneously because of chitosan’s sol–gel transition. The cationic nature of chitosan in a neutral environment also allows for electrostatic interactions with anionic glycosaminoglycan in the extracellular matrix, which may facilitate adherence of chitosan-based constructs to most human tissue sites [8, 48]. Moreover, the additional chondrogenic factors preserved in the DBM material further improved MSC chondrogenesis in this composite scaffold (figure 8).

In addition, the mechanical strength of the constructs played a critical role in MSC chondrogenesis for implantation in cartilage regeneration [36]. Even a subtle alteration in the mechanical properties of the extracellular matrix in vivo or the surrounding microenvironment in vitro may affect the lineage-specific fate selection of resident MSCs in a similar fashion to chemical changes [36, 49, 50]. In this study, CS-DBM scaffolds, with a relatively high strength, showed a significantly increased sGAG deposition or expression within the scaffolds or medium after 21 days in culture (figures 4 and 6), implying that structural strength is an effective stimulus for matrix production. The results of mRNA expression also are consistent with previous articles: scaffolds with kPa-level modulus support the expression of cartilage markers, such as AGC and collagen II [27, 29]. However, compared with native cartilage (0.4–0.8 MPa), CS-DBM scaffolds showed a lower strength. Increasing scaffolds’ stiffness prompted a shift in MSC differentiation towards fibrous phenotypes, or increased matrix calcification [29, 51]. Furthermore, preclinical studies have provided evidence that neo-cartilage tissue regeneration and remodelling can be promoted by a mechanically soft material [27]. In this study, one purpose of integrating DBM with hydrogel was to allow convenient handling and to provide a temporary mechanical shield for neo-cartilage tissue regeneration after implantation, instead of bearing articular stress as a permanent substitute. On the other hand, the biphasic of this hybrid scaffold may provide sufficient options for functional modification, in which the hydrogel may strengthen the mechanical or viscoelastic properties by control of cross-linking densities or adding gelation agents [3, 21, 52–55]. Meanwhile, the solution–gelation transition may facilitate the delivery of factors or/and cells after implantation into a joint environment [4, 5, 9, 12, 20, 48]. Moreover, the demineralized matrix provides a solid phase for tailoring substrate elasticity or surface functionalization, such as architectures processing (size, shape, porosity or surface microstructure), biological moieties coupling or ligand–surface-immobilization, which have a profound impact on the biological function of a biomaterial [22, 56–59]. Future work will focus on functionalizing this biomaterial framework and evaluating the effects of osteochondral repair in vivo.

5. Conclusions

In this article, combining a chitosan thermogel and demineralized bone matrix produced a solid-supported hydrogel, which can retain more cells homogeneously with chitosan’s sol–gel transition and provide sufficient strength for cartilage tissue engineering. Moreover, the differences in degradation kinetics between the two compositions provided a 3D microenvironment that was temporally and spatially suited for MSC proliferation and chondrogenesis within this hybrid scaffold. To the best our knowledge, this is the first time that a thermosensitive gel and demineralized ECM has been integrated into one 3D scaffold. This solid-supported thermogel appears to be a promising platform for cartilage tissue engineering studies. Future work will focus on functionalizing this biomaterial framework and evaluating the effects of osteochondral repair in vivo.

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