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3D Printing Bioink Preparation and Application in Cartilage Tissue Reconstruction *in Vitro*

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Abstract: Three-dimensional (3D) bioprinting technology has great potential for application in the treatment of cartilage defects. However, the preparation of biocompatible and stable bioinks is still a major challenge. In this study, decellularized extracellular matrix (dECM) of soft tissue was used as the basic material to prepare the bioink. Our results showed that this novel dECM-derived bioink had good printing performance and comprised a large number of fine nanofibers. Biological characterization revealed that the bioink was compatible with the growth of chondrocytes and that the nanofibrous structure greatly promoted cell proliferation. Histological and immunohistochemical analyses showed that the *in vitro* printed cartilage displayed the presence of characteristic cartilage lacunae. Thus, a new preparation method for dECM-derived bioink with potential application in generation of cartilage was developed in this study.
Key words: bioink, cartilage, 3D printing, tissue engineering, biomaterials
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0 Introduction

Articular cartilage injury is a major research priority in orthopedic clinical practice due to its global prevalence and associated economic impact[1]. Articular cartilage covers the surface of synovial joints. Although it is a very thin layer, its structure and composition are relatively complex. Articular tissue comprises of water, collagen, proteoglycans, and specialized cells called chondrocytes[2-3]. The poor repair capacity of articular cartilage is due to its avascular nature and reduced cell migration. In clinical practice, the treatment of cartilage defects is also accompanied with the inherent challenges of poor prognosis and insufficient donors. Tissue engineering technology provides a repair approach that combines cells and materials for the management of cartilage defects.

Three-dimensional (3D) bioprinting is a recent technology that can fabricate different scaffolds by positioning living cells and biomaterials into the appropriate place, allowing for the generation of biological scaffolds with complex structure and composition for repairing tissue defects[4]. Currently, 3D bioprinting technology is widely used in tissue regeneration, such as in bone, cartilage, skeletal muscle, and skin. However, there are still many challenges to overcome in the application of 3D bioprinting to tissue engineering, especially regarding the printing efficiency of bioink material when used in combination with cells. Commonly used bioink materials in current research mainly include hydrogels such as collagen, sodium alginate, gelatin, and GelMA[5-7]. However, although these materials have good biocompatibility, they are not conducive to cell proliferation and growth after being combined with cells. Most bioink materials swell when exposed to water and exhibit relatively dense internal structures. Furthermore, the lack of fibrous structures, frequently found in natural extracellular matrix (ECM), limits the migration and proliferation of cells inside the bioink and also reduces its printing performance.

Decellularized extracellular matrix (dECM) has components similar to autologous tissue, and it is widely used in the field of tissue engineering[8]. Due to removal of cellular components and nucleic acid residues, dECM has relatively low immunogenicity[9]. In addition, the acellular matrix material has fluidity and printability, properties which can be exploited by 3D

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bioprinting for tissue engineering. In a study by Wolf et al.[8], dECM was first dissolved in pepsin in acidic conditions and then neutralized in base. The resulting dECM solution self-assembled into many nanofiber structures which better mimicked the ECM than conventionally used hydrogels, thus promoting cell proliferation, migration, and vascularization.

In this study, we used dECM as a bioink to study its printing performance and biological properties. We investigated the ability of the bioink to generate cartilage tissue by combining the bioink with chondrocytes and culturing it *in vitro* and studying the composition of the 3D-printed cartilage using histological and immunohistochemical analyses.

1 Materials and Methods

1.1 Preparation of dECM-Derived Bioink

Porcine skin was obtained from the market, followed by removal of the epidermis and other tissues to release the dermis. The dermis tissue was then ground into small pieces using liquid nitrogen. The crushed dermis tissue was decellularized using the following treatment: trypsin (2.5 g/L) for 6 h, deionized water washes for 15 min (three times), ethanol (70%, volume fraction) for 10 h, H_2O_2 (3%, volume fraction) for 15 min, deionized water washes for 15 min (three times), Triton X-100 (1 g/L) in EDTA (0.26 g/L)/Tris (0.69 g/L) for 6 h, deionized water washes for 15 min (three times), peracetic acid (0.1%, volume fraction)/ethanol (4%, volume fraction) for 2 h, three washes of phosphate-buffered saline (PBS) for 15 min, and final washes with deionized water for 15 min (twice). The obtained dECM was stored at −20 °C after freeze-drying.

In order to prepare the bioink, the prepared dECM materials were added to pepsin (prepared in 0.1 mol/L HCl solution) and completely dissolved after stirring for 72 h at room temperature. Then, the dECM solution was neutralized using 1 mol/L NaOH and 10× PBS in an ice bath. The bioink was stored at 4 °C before further use.

The DNA, collagen, and glycosaminoglycan (GAG) contents of the natural dermis and 3D printing bioink were determined. The DNA of two samples was extracted using the TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) and determined using the PicoGreen dsDNA Assay Kit (Life Technologies Inc., Carlsbad, USA). The collagen content was determined by quantifying hydroxyproline (that makes up 14.3% of collagen mass) using the Hydroxyproline Assay Kit (Abcam, Cambridge, UK). The GAG content was determined using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor, Carrickfergus, UK). In addition, the morphology of the natural dermis and 3D printing bioink was observed using scanning electron microscopy (SEM).

1.2 Evaluation of Printing Performance of dECM-Derived Bioink

The printing performance of the bioink was studied by controlling the concentration of the bioink and varying the 3D printing parameters. The bioink at various concentrations, i.e., 3 g/L, 6 g/L, 9 g/L, and 12 g/L, was loaded into the plastic syringes on a 3D bioprinting machine (Regenove Bio-Printer-WS, Hangzhou, China). During the printing process, the bioink was injected through a 330-Teflon nozzle at 50 kPa of air pressure (at a printing temperature of 20 °C). The printing speed was varied at 6 mm/s, 8 mm/s, 10 mm/s, and 12 mm/s. Finally, the printed bioink was placed in humidified incubators maintained at 37 °C under a controlled atmosphere of CO_2 (5%, volume fraction) for 30 min to gelate and self-assemble. The final printing effect was used as a standard to evaluate the printing performance of the bioink.

1.3 Analysis of the Biological Properties of dECM-Derived Bioink

Primary chondrocytes from New Zealand white rabbits aged 2—4 weeks were isolated and cultured with the bioink to evaluate the biological properties of the bioink. The articular cartilage of the rabbits was removed and cut into small pieces before digesting them with trypsin (2.5 g/L) for 45 min followed by type II collagenase (0.1 g/L) for 3 h. After centrifuging at 1 000 r/min for 5 min, the primary chondrocytes were resuspended in DMEM containing fetal bovine serum (15%, volume fraction), penicillin-streptomycin (1%, volume fraction), and vitamin C (0.1 g/L) and allowed to proliferate for 2 d.

Cultured chondrocytes were collected and seeded on the bioink to determine cell proliferation and viability. The proliferation of cells was evaluated at different time points (1 d, 3 d, 5 d, and 7 d) after seeding using the CCK-8 assay according to manufacturer's instructions. Briefly, after replacing the medium, 10 μL of CCK-8 solution was added to each pore and incubated for 2 h; then, a micro-plate reader was used to measure the absorbance at 450 nm (A450). The chondrocytes cultured on tissue culture plate (TCP) were used as a control group. The cells cultured for 5 d were stained using the live/dead staining assay, and the staining results were used to analyze the cell morphology using fluorescence microscopy.

1.4 Cartilage Tissue Generation *in Vitro*

In this study, chondrocytes were combined with the bioink, and the cartilage scaffold was printed via 3D printing technology. The printed cartilage was prepared using the following method. First, a cylindrical model (14 mm diameter; 4 mm height) was designed and constructed. Then, the bioink (sterilized by filtration) was mixed with chondrocytes and loaded into plastic syringes. The distance between the needle and collector was set to 160 μm. The height of the printing layer was

160 μm and the printing temperature was maintained at 10 °C. After printing, the bioscaffold was placed in complete medium in an incubator at 37 °C for 30 min and cultured *in vitro* to characterize its ability to generate cartilage tissue.

After culturing for 5 d, Live/Dead Viability/Cytotoxicity Kit (Life Technologies) was used to determine the viability of cells in the printed cartilage. The samples stained with the Live/Dead Cell Assay Kit were analyzed to observe the cell growth behavior using 3D scanning with a fluorescence microscope.

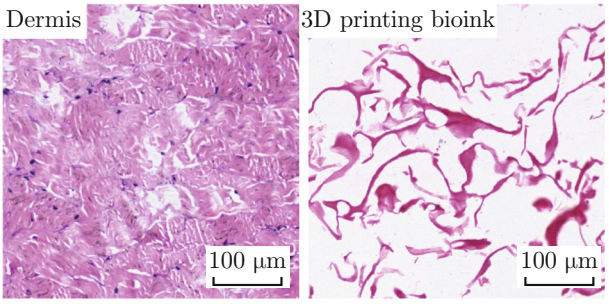
After culturing for 4 weeks, the *in vitro* generated cartilage was characterized using histological and immunological staining. The harvested and fixed specimens of 3D-printed cartilage (test) and natural cartilage (control) were embedded in paraffin and cross-sectioned. Tissue slices were stained with hematoxylin and eosin (HE) and picrosirius red (PR) and observed under a fluorescence microscope (DMi8, Leica, Germany). For immunohistochemistry, tissue samples were incubated with the appropriate primary antibodies (mouse anti-collagen type II, 1 : 200, Sigma-Aldrich, USA; mouse anti-collagen type X, 1 : 100, Abcam, UK) overnight at 4 °C. The tissue samples were then incubated with the standard IHC Kit. Finally, the immune-stained samples were observed with an inverted fluorescence microscope (DMi8, Leica, Germany).

2 Results and Discussion

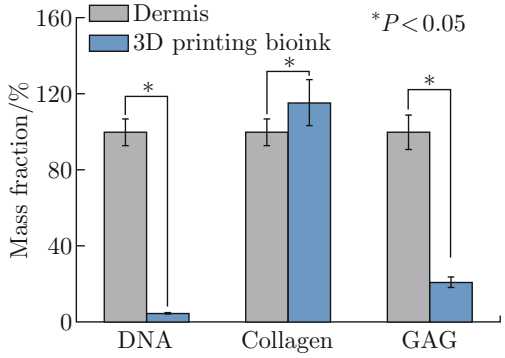
2.1 Characterization of dECM-Derived Bioink

First, the dermis was decellularized, a process important to ensure low antigenicity of the dECM. The effectiveness of the decellularization treatment was assessed using histological examination (Fig. 1(a)). HE staining of natural dermis and dECM showed absence of cell debris and a reduction in the number of cells in dECM after decellularization. In order to further characterize the effect of decellularization treatment, the DNA, collagen, and GAG contents of dECM were compared with those of natural dermis (Fig. 1(b)). Probability values of less than 0.05 ($P < 0.05$) were considered significant. It was observed that while the amount of DNA in dECM was significantly lower than that in natural dermis tissue, the collagen and GAG contents increased significantly after decellularization, thereby illustrating the effectiveness of this process.

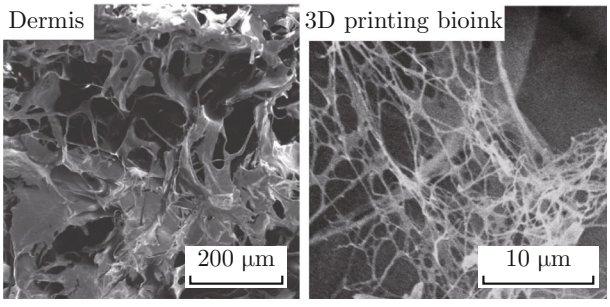
Second, the dECM was dissolved in pepsin in acidic conditions, followed by neutralization in basic conditions. The morphology of the obtained bioink was examined using SEM (Fig. 1(c)). It is known that the structure of the natural dermis is dense with many coarse fibers, whereas the structure of the dECM becomes loose after acellular treatment with the fiber thickness remaining unchanged. The isolated dECM undergoes enzyme-aided dissolution and subsequently self-assembles into nano-sized fibers, thus forming the fluid bioink. This simulated ECM structure is conducive to cell adhesion, proliferation, and migration[10].



(a) HE staining



(b) DNA, collagen and GAG contents



(c) SEM morphology

Fig. 1 Characterization of dECM-derived bioink

2.2 3D Printing Using the dECM-Derived Bioink

The printing performance of the dECM-derived bioink was investigated at different concentrations of bioink (3 g/L, 6 g/L, 9 g/L, and 12 g/L) and different printing speeds (6 mm/s, 8 mm/s, 10 mm/s, and 12 mm/s). The results showed that the printing performance was sub-optimal at extreme concentrations of the bioink (3 g/L, and 12 g/L), with the best result observed at a concentration of 9 g/L (Fig. 2). Furthermore, the highest printing fidelity was observed at 8 mm/s (Fig. 2). Importantly, printing performance was found to be temperature-dependent. At temperatures exceeding 10 °C, the bioink began to self-assemble

during the printing process, which further affected its rheological properties and subsequent printing performance. The printed scaffold was then placed at 37 °C for self-assembly.

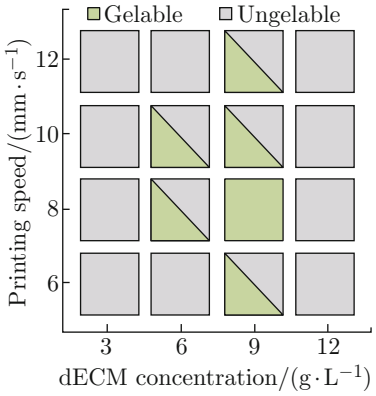
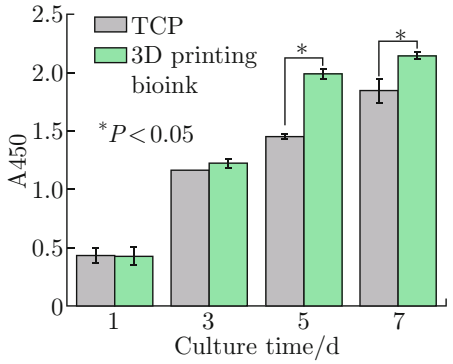


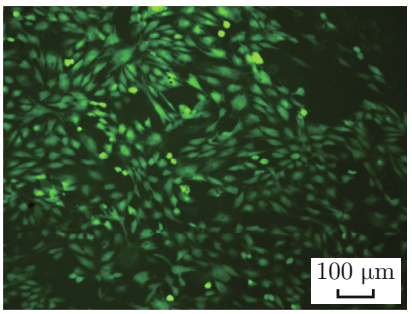
Fig. 2 Printing properties of the bioink

2.3 Biological Properties of dECM-Derived Bioink

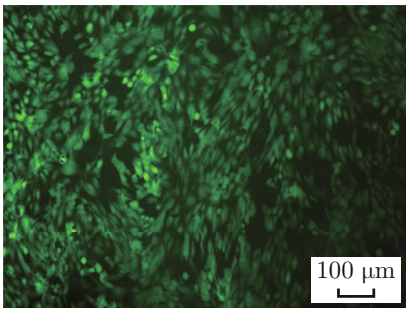
Chondrocytes were seeded on the surface of the printed scaffold to study the biological properties of the bioink. After culturing for 1 d, 3 d, 5 d, and 7 d, cell viability was determined using the CCK-8 assay. The result of this assay showed that chondrocytes grew well on the surfaces of 3D printing bioink and control groups, indicating that the bioink was biocompatible (Fig. 3(a)). In addition, chondrocytes exhibited improved cell proliferation on bioink when compared with that on TCP, possibly due to the nanofibrous structure of the bioink that facilitated cell migration and proliferation. Results of the live/dead staining assay also revealed successful cell adhesion and spreading on the surface of the bioink material (Figs. 3(b) and 3(c)) and enhanced cell proliferation on bioink when compared with that on TCP, thus complementing the findings of the CCK-8 assay.



(c) CCK-8 assay results



(b) Live/dead staining of TCP



(c) Live/dead staining of bioink

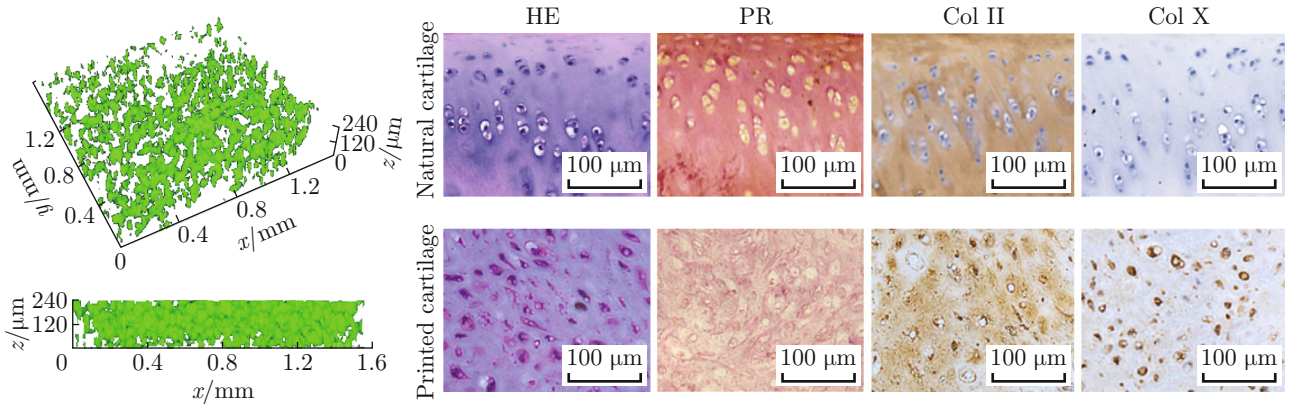
Fig. 3 Characterization of the biological properties of 3D printing bioink

2.4 dECM-Derived Bioink Application in Cartilage Generation *in Vitro*

We sought to examine the ability of dECM-derived bioink to generate cartilage *in vitro*. Chondrocytes were mixed with the bioink before using it to print cartilage. After printing, the viability of chondrocytes using the live/dead staining was found to be 98%, which persisted even after culturing for 7 d. On the 7th day of culturing, fluorescence microscopy-coupled 3D scanning was used to study the adhesion and spreading of chondrocytes in the whole printed cartilage (Fig. 4(a)). Scanning electron micrographs of the 3D-printed cartilage revealed that the chondrocytes were almost completely encapsulated within the printed cartilage with no observable defects in proliferation or changes in cell shape.

After culturing for 4 weeks, the *in vitro* generated cartilage was harvested and stained with HE and PR to examine its morphology (Fig. 4(b)). HE staining revealed an abundance of chondrocytes and a morphologically intact cartilage tissue in the 3D-printed cartilage. In particular, the printed cartilage also possessed cartilage lacunae characteristic of natural cartilage. As collagen is an important component of cartilage, forming up to 50% of its mass (dry weight), histological staining of collagen fibers is important for the evaluation of cartilage formation. PR is a strong acid anionic dye that specifically stains collagen in the ECM, and different collagen fibers are stained with different colors due to the phenomenon of double refraction. PR staining of printed cartilage showed highly evident collagen staining (in red and yellow), while the structure of collagen fiber resembled that of natural cartilage.

In order to investigate the differences in collagen composition between 3D-printed and natural cartilages, immunological staining was performed to assess expression of two collagen subtypes, collagen type II (Col II) and collagen type X (Col X), as shown in Fig. 4(b). Immunohistochemistry and the corresponding statistical analysis showed a comparable amount of Col II in 3D-printed cartilage as well as natural cartilage. However,



(a) 3D fluorescence (b) Histological and immunological staining

Fig. 4 Cartilage tissue generation performance of dECM-derived 3D printing bioink

Col X exhibited lower expression in 3D-printed cartilage than in natural cartilage. Col X is known to form gradually during the process of cartilage ossification. Our results suggest that natural cartilage may be maturer than *in vitro* 3D-printed cartilage, likely due to different gene/protein expression profiles. The main component of bioink is collagen, and the content of Col X is still unclear, which may lead to the difference between the two groups. Additionally, despite similarities in the composition of natural cartilage and 3D-printed cartilage as observed in this study, their micro structures may still be different. This can be because in addition to chondrocytes and ECM, a variety of growth factors, including external mechanical stimulation, and cell-cell interactions, influence formation of natural cartilage. Therefore, greater in-depth research is needed for discovering methods to improve the generation of cartilage tissue.

3 Conclusion

In this study, we successfully prepared a bioink that could self-assemble into nanofibers and that could be used for 3D printing, using an acellular matrix of soft tissue as the basic material. The printing properties of the bioink were thoroughly characterized and its biocompatibility with chondrocytes was assessed. Combined with chondrocytes, dECM-derived bioink was successfuly used to print cartilage tissue. The cartilage tissue printed *in vitro* was similar in composition to natural cartilage tissue. Thus, a novel bioink preparation method for 3D printing in biomedical applications is described. In addition, a direction toward potential application of dECM-derived bioink in treatment of cartilage defects is provided.

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