

3D Composite Cell Printing Gelatin/Sodium Alginate/n-HAP Bioscaffold

Yuan Fan¹, Tingchun Shi¹*, Xiuyan Yue², Fangfang Sun¹ and Danyu Yao¹

¹College of Life Information Science and Instrument Engineering, Hangzhou Dianzi University, Hangzhou, Zhejiang, 310037, China
²Library of Hangzhou Dianzi University, Hangzhou Dianzi University, Hangzhou, Zhejiang, 310037, China
*Corresponding author’s e-mail: stc@hdu.edu.cn

Abstract. Hydrogel bioprinting has attracted much attention in the field of tissue engineering. However, due to their softness and tendency to shrink, 3D printed hydrogel scaffolds suffer from low printing accuracy and poorer mechanical properties, which makes it difficult to process them into materials with a certain structure. In this study, the gelatin/alginate hydrogel scaffolds were reinforced with nano-hydroxyapatite (n-HAP) and fabricated by CCA-II cell controlled assembly 3D machine. Compared with the pure gelatin sea/alginate scaffold, the addition of n-HAP markedly improved the stability and mechanical strength of the hydrogel scaffolds, and the printing accuracy was improved as well. The addition of n-HAP also adjusted the surface roughness of the scaffolds and improved the biodegradability of the scaffolds. The 3D printed composite hydrogel scaffold showed no cytotoxicity and supported the adhesion and growth of mouse chondrocytes. The printed cell-loaded bio-scaffold had high cell viability, and over 95% viable cells were detected after one week of culture.

1. Introduction

Tissue engineering research aims to repair the function of damaged tissues or organs by constructing alternative tissues or organs in vitro or in vivo[1]. Biological 3D printing aims to create complex shape structures that can be used to guide tissue regeneration[2]. The printed hydrogel is a highly hydrated polymer network that mimics the extracellular matrix (ECM) that supports cell attachment, proliferation and differentiation and is a favorable material for cell scaffolds. In order to prepare a 3D printed hydrogel scaffold, the bio-ink must have a high enough viscosity to allow printing into solid filaments while maintaining sufficient fluidity to ensure proper filament production during printing. Rapid gelation after extrusion is also necessary, which helps to maintain the shape of the printed filaments. In addition, the printed filaments should have sufficient mechanical properties to support the entire scaffold while providing good adhesion between adjacent layers to produce a robust hydrogel scaffold[3].

Gelatin and sodium alginate have good biocompatibility and hydrophilicity, and have been favored by tissue engineering researchers in recent years. However, the gelatin sodium alginate bio-scaffold suffers the problem of rapid degradation and poor mechanical properties[4]. With rapid degradation rate, the scaffold will not provide an effective environment for the cells to proliferate and grow and new tissues could not be reconstructed. Proper mechanical strength is also an important requirement for tissue engineering scaffolds. Ting Pan et al. selected gelatin and sodium alginate as printing
materials, and successfully printed 3D scaffolds with fine structure using 3D printing technology. Double cross-linking method was used to improve the mechanical properties and stability of scaffolds[5]. However, the residual glutaraldehyde crosslinker will affect the growth of cells on the scaffold[6]. Hydrogels with robust mechanical properties and good biocompatibilities are still to be developed.

As we all know, hydroxyapatite is one of the inorganic components in human bones, with good bio-affinity, biocompatibility, bioactivity and osteoconductivity[7], and has been used as an excellent biomaterial for tissue engineering research[8]. Compared with common hydroxyapatite, nano-hydroxyapatite (n-HAP) has higher solubility, greater surface energy and stronger adsorption, better biological activity, and tighter interface with other materials, which is beneficial to improving the mechanical properties of composites[9]. Therefore, based on this, this paper attempts to use n-HAP to enhance the stability and mechanical properties of hydrogels.

In this paper, gelatin, sodium alginate and n-HAP were selected to fabricate 3D printed scaffold. The purpose of this study was to develop a gel/sodium alginate/n-HAP bioscaffold which maintained the printability of the hydrogel while improving post-press shape fidelity and scaffold mechanical properties. Gelatin/sodium alginate/n-HAP hydrogel scaffolds were prepared using extrusion-based 3D printing techniques. The processing conditions, including material and printing parameters, were optimized to evaluate the rheological properties of different ratios of n-HAP bio-ink. The effect of n-HAP on printing accuracy, mechanical properties of the scaffold and degradation performance was quantified. Finally, mouse chondrocytes were used to assess cell compatibility and cellular interaction to support their potential application to cartilage repair.

2. Experimental materials and methods

2.1. Preparation of printing inks

The gelatin and sodium alginate and n-HAP were mixed at a mass ratio of 8:2:0, 8:2:1 and 8:2:4, respectively, and printed as follows. Gelatin (AR, Kernel, China) was dissolved in ultrapure water and stirred at 50 °C for 2 h using a water bath thermostatic magnetic stirrer. Then the solution was cooled to 37 °C, and sodium alginate (BR, Aladdin, China) was added in proportion and stirred. A certain proportion of n-HAP nano-hydroxyapatite (BR, Aladdin, China) was added to the prepared gelatin/sodium alginate solution, and stirred at 37 °C overnight. The hydrogel solution was obtained as a print material for the hydrogel scaffold (unloaded cells) for subsequent experiments. The proportion of each hydrogel solution is shown in table 1.

| Scaffolds | Gelatin(g) | Sodium alginate(g) | n-HAP(g) | Gelatin / Sodium alginate /n-HAP ratio(w/w/w) |
|-----------|------------|--------------------|----------|---------------------------------------------|
| 0% n-HAP  | 4          | 1                  | 0        | 8:2:0                                       |
| 9.1% n-HAP| 4          | 1                  | 0.5      | 8:2:1                                       |
| 30% n-HAP | 4          | 1                  | 2        | 8:2:4                                       |

Bio-ink preparation: gelatin, sodium alginate, and n-HAP sterilized under ultraviolet light for 24 hours were prepared into a hydrogel solution as described above. The mouse chondrocytes ATDC-EGP-5 cultured to cover about 80% of the culture flask were decomposed into individual cells using trypsin to obtain a cell suspension. The cell suspension was counted using a hemocytometer, and mixed into the prepared hydrogel solution at a final cell concentration of 2×10² cells/ml.

2.2. Scaffolds printing

A 3D gelatin/sodium alginate/n-HAP bioscaffold was prepared using a CCA-II cell controlled assembly printer (Figure 1). First, a 10mm × 10mm × 5mm solid model was designed using 3D modeling software SolidWorks. The hierarchical model of the 3D printer was used to layer-cut the
solid model to obtain the CLI model, and then the layered CLI model was loaded into the printer-assisted control software Cark. The syringe carrying the hydrogel ink was mounted to the printing unit of CCA-II and the nozzle size was 400 μd. The stepping motor of the printing unit pushed the syringe piston at a speed of 0.60 mm/s to feed, layer by layer, for a total of 25 layers. The wire space was set as 800μm. In the printing process, in order to ensure the shape of the printed wire and the overall structure of the bracket, according to practical experience, the temperature of the printing unit was controlled at 25 °C to ensure that the printed material could solidified in time.

The bio-ink printing bracket was used to open the UV lamp of the cell-controlled assembly machine for sterilization for 24 hours, and the bio-ink-loading syringe was mounted to the CCA-II printing unit. The print parameter setting and print model selection were the same as above.

\[ \text{Figure 1. Schematic diagram of the printing method. (a) Scaffold forming system schematic; (b) Bio-ink; (c) Scaffold printing process.} \]

2.3. Morphology of the scaffolds
The morphology of the three scaffolds were observed under an optical microscope after printing. The printed scaffold was placed in a vacuum freeze dryer (LGJ-12, Beijing Songyuan Huaxing) for 24 hours at a temperature of -20°C. The surface morphology and microstructure of the scaffold were observed under a scanning electron microscope (Phenom prox, Phenom, Netherlands).

2.4. Degradation of the scaffolds
The dried scaffolds were pre-weighed and then immersed in a modified SBF simulated body fluid (pH 7.4~7.5, Xi'an Hurt Biotech, China), incubated in a constant temperature incubator at 37°C, 5% CO₂. At different time intervals, the scaffolds were taken out and rinsed with distilled water. After the liquid was blotted using chemical analysis filter papers, the scaffolds were placed in a constant-temperature drying oven for drying. After completely dried, they were weighed and recorded, and the weight loss of the scaffold is calculated. Co-culture for 28 days. The calculation formula of the degradation rate is as follows:
%100m

\[ Scaffolds\ degradation(\%) = \frac{m_0 - m_1}{m_0} \times 100\% \quad (1) \]

Where \( m_0 \) and \( m_1 \) represent the initial and final weight, respectively.

2.5. Mechanical properties of the scaffold

The WDX-100 electronic universal testing machine (Zhejiang Wenzhou Weidu Electronics Co., Ltd.) was used to test the compressive stress and strain of the scaffolds. The compressive stress application rate was 0.3 mm/min. The Young's modulus was obtained from the regression of the linear portion of the stress and strain curves. Each measurement was performed in triplicate and the results were averaged.

2.6. Cell toxicity

The printed scaffold was immersed in DMEM cell culture medium containing 10% fetal calf serum and 1% penicillin-streptomycin solution, and cultured in a constant temperature incubator at -37°C, 5% CO₂ for 24 hours to obtain an extract. The scaffold was tested for toxicity using CCK 8. The wells in the blank group 1 were the cell culture medium, the wells in the blank group 2 were the scaffold extracts, the wells in the experimental group were the cells and the extract, and the wells in the control group were the cells and the cell culture medium. Set 5 replicate wells in each group, and add 10% volume of CCK 8 reagent to each well. After incubating for 0.5 h in a carbon dioxide incubator, the absorbance of each well was measured using a microplate reader, and the average of the absorbance of each set of duplicate wells was taken as the absorbance of each group. The cell viability calculation formula is as follows:

\[ \text{Cell viability(\%) =} \frac{A_s - A_{b1}}{A_c - A_{b1}} \times 100\% \quad (2) \]

Where \( A_s \) was the absorbance of the experimental group, and \( A_c \) was the absorbance of the control group, and \( A_{b1} \) and \( A_{b2} \) were the absorbances of the blank groups 1 and 2.

2.7. Cell survival rate test

The printed cell-loaded biological scaffold was transferred into a 24-well culture plate, and 1 ml of DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution was added to each well, and placed in a constant temperature incubator (37°C, 5% CO₂). Change the medium every other day. Cell morphology was observed under an inverted light microscope (CKX41, Olympus, Japan) every other day during the culture. Fluorescence staining was performed using Calcein-AM/PI live cell/dead cell double staining kit (Yeasen, China) and cell viability and proliferation rate were calculated.

2.8. Statistical analysis

All experiments were repeated at least three times independently, and the data were expressed as \( \bar{x} \pm s \). The experimental data were analyzed by one-way ANOVA. \( P < 0.05 \) was considered to be statistically significant.

3. Results and discussion

3.1. Morphological analysis of gelatin / sodium alginate / n-HAP scaffold

Under the same printing conditions, the surface of the hydrogel scaffold without n-HAP was rough and the printed pores were slightly deformed (Figure 2(a)). The diameter of the printed fiber widened during printing and there were broken or collapsed wires, which might own to the insufficient load-bearing capacity of the printed hydrogel fibers when printing the next layer, and gravity led to severely deformation of the hydrogel fibers. The 9.1% n-HAP hydrogel scaffold showed a good molding effect with flat surface, uniform printing fibers, and printing pore size e, and the overall shape is better.
The 30% n-HAP hydrogel scaffold had a smooth but uneven surface, and some of the printed pores were blocked (Figure 2(c)). The uneven printing might be caused by the agglomeration of n-HAP. The agglomeration phenomenon affected the binding efficiency of n-HAP to the surface of the hydrogel material[10], which would affect the microstructure and properties of the scaffold, and thus affecting the growth and proliferation of cells on the scaffold. After printing, the hydrogel scaffold was cross-linked with 4% (w/w) calcium chloride solution for 5 min, and no obvious deformation occurred after the cross-linking process.

The printed scaffold was placed in a vacuum freeze dryer (LGJ-12, Beijing Songyuan Huaxing) for 24 hours, and the surface morphology and microstructure of which were observed under a scanning electron microscope (phenom prox, Phenom, Netherlands). Compared with the wet scaffold, the
morphology of the dried scaffold was changed (Figure 3). The gelatin sodium alginate material had a large water swellability, and the solvent sublimation in the drying process caused the significant shrink and deformation of the scaffolds. With the increase of n-HAP content, deformation of scaffolds decreased obviously. Among them, the 0%n-HAP scaffold printing fiber shrank severely, the pores were deformed and pore size became larger (Figure 3(a)). The pore size of the 9.1%n-HAP scaffold became larger slightly, and the printed fibers shank a little (Figure 3(b)); There was little change of the morphology of 30%n-HAP scaffold (Figure 3(c)). The result indicated that the addition of n-HAP would affect the water absorption performance of the scaffolds, and n-HAP played a key role in morphology stability of the scaffolds under the action of external force. From the internal structure of the scaffold material (Figure 3(d)-3(f)), it was obvious that the internal structure became tighter as the content of n-HAP increased. There were a large number of connected microporous structures inside the 0%n-HAP scaffold, of which the surface was smooth and the pore size was 10μm~300μm (Figure 3(d)); For the 9.1%n-HAP scaffold, there were a large number of connected microporous structures, of which the surface was rough and the pore size was 20μm~200μm (Figure 3(e)). The interconnected microporous structure was barely visible in the 30%n-HAP hydrogel scaffold (Figure 3(f)), indicating that excessive addition of n-HAP did not improve the overall performance of the scaffold. In addition, with the addition of n-HAP, the surface of the scaffold became rough, which also contributed to cell adhesion in a free state.

3.2. Degradation performance of gelatin/sodium alginate/n-HAP scaffold

The statistical results of degradation rates of different scaffolds were shown in Figure 4. The results(Figure 4(a)) showed that there was a certain degree of weight loss in each scaffold within one week. At the beginning of degradation, both 0%n-HAP scaffolds and 9.1%n-HAP scaffolds had a significant weight loss, and 30%n-HAP scaffolds had a much less weight loss. With the increase of time, all scaffolds showed increased weight loss. The degradation rate of the scaffold decreased with the increase of n-HAP within 7 days. Interestingly, 30%n-HAP scaffolds showed the fastest degradation in the next 7 days, which might be caused by the uneven distribution of n-HAP.

From the long-term weight loss observation(Figure 4(b)), the 0%n-HAP scaffold had the fastest weight loss rate of 60% in the first week, the weight loss was basically unchanged in the second week, and the weight loss began to increase after the second week. The 9.1%n-HAP scaffold had the weight loss rate of 45% within one week, but the value was between the other two scaffolds, and then the weight loss rate remained lowest in the second and third weeks, after which rapid degradation began; The 30%n-HAP scaffold had the lowest weight loss rate within one week, and the weight loss rate remained basically unchanged, and gradually increased from the second week. The n-HAP hybridized into the gelatin alginate hydrogel system could significantly improve the degradability of the hydrogel.

In theory, with a high rate of weight loss at the beginning, hydrogel scaffolds could provide enough space for cell growth, which was conducive to cell growth and proliferation[11]. Then, maintaining a low degradation rate for a certain period of time, the scaffold could provide sufficient attachment space and support for the cells, which was conducive to the growth of new tissue. With the formation of new tissue, the scaffold would gradually degrade, providing enough space and nutrients until it was completely degraded, and new tissue was formed[12]. According to the experimental results, the 9.1%n-HAP scaffold had a more suitable degradation performance, so we chose it as a optimized scaffold printing material for composite cells.
3.3. Mechanical properties of gelatin / sodium alginate / n-HAP scaffold

Obtaining proper mechanical strength is an important requirement for tissue engineering scaffolds[13]. It is generally accepted that it is essential to adjust the mechanical properties to the tissue. For example, soft tissue strength is typically between 0.4 and 350 MPa, while hard tissue regeneration requires an intensity of 10-1500 MPa[14]. After testing with the WDX-100 electronic universal testing machine, a stress-strain curve was obtained. The Young's modulus of each group was 21.62±0.98, 24.16±1.09, 32.26±1.05 MPa for 0% n-HAP scaffold, 9.1% n-HAP scaffold and 30% n-HAP scaffold, respectively. The results showed that the combination of n-HAP with the gelatin alginate hydrogel system could improve the mechanical properties of the hydrogel to a certain extent.

3.4. Toxicity test of gelatin/sodium alginate/n-HAP scaffold material

The cell viability value obtained by CCK 8 for cytotoxicity assay was 133.2% ± 3.3% as shown in Figure 6. Within one week, the number of cells of the extract group continued to increase, but the proliferation rate gradually decreased, and the proliferation rate was the fastest from the first day to the
third day, which might owned to the limited living space of cells. The scaffold material toxicity test showed that the hydrogel material did not significantly inhibit cell growth or kill cells, which can be used as a bioprinting material for loading cells[15].

![Figure 6. OD values of experimental group and control group at each time point.](image)

3.5. Cell activity and cell proliferation assay

The mouse chondrocytes in the adherent state (before mixing) and the cells on the bioscaffold (after printing) were respectively fluorescently stained using a living cell/dead cell double staining kit, and the cell survival rate and proliferation rate were calculated. It could be seen from living cell/dead cell double staining test (Figure 7) that the cells encapsulated in the hydrogel were evenly dispersed within the bioscaffold. The number of living cells gradually increased within 7 days after printing, and the number of dead cells gradually decreased. On the fifth day, it was obvious that the ratio of the two cells changed greatly, which was basically consist with the results of scaffold toxicity test. Compared with the cell viability (70% ± 5.3%) on the first day after printing, the cells proliferated rapidly within one week, and the cell survival rate reached 95% ± 2.1% after one week, which was in line with our expected results. On the tenth day, cell viability can reach 99% ± 0.3% (table 2). This indicated that the biological scaffold printed by cell controlled assembly technology could provide a good living environment and sufficient nutrients for cell growth and proliferation. In addition, the printed biological scaffold could maintain a good shape within one week, indicating that the bioscaffold were well prepared by the method provided in this paper.

| Bio-scaffolds culture days | Cell viability at each time point |
|----------------------------|----------------------------------|
|                            | 1 d | 3 d | 5 d | 7 d | 10 d |
| Cell survival rate (%)     | 70± 5.3 | 77± 5.1 | 86± 3.2 | 95±2.1 | 99± 0.3 |

![Table 2. Cell survival rate on the scaffold](image)
4. Conclusion

In this paper, several properties of tissue engineering bioscaffolds were used to determine the properties of gelatin sodium alginate n-HAP cartilage bioscaffolds prepared by 3D printing. The results showed that the combination of n-HAP with gelatin/alginate produced a composite bio-ink system with excellent printable properties, and had better post-print shape retention ability compared with pure gelatin sodium alginate. The compressive modulus increased with the increase of n-HAP content. And the addition of n-HAP could adjust the degradation properties of the scaffold, as well as the surface roughness and pore structure of the micropores of the scaffold. Scaffold toxicity and cell viability experiments confirmed that the material did not have strong toxic side effects on cells and the scaffolds supported cell adhesion and cell growth. In addition, n-HAP/gelatin alginate composite cell printing showed increased cell activity and stability compared to gelatin sodium alginate scaffolds. Therefore, n-HAP reinforced hydrogels offer promise as materials for soft tissue engineering where stability and cytocompatibility are required.

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References

[1] Leong, K.F., Cheah, C.M., Chua, C.K. (2003) Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. Biomaterials, 24: 2363-2378.

[2] Yeong, W.Y. (2004) Rapid prototyping in tissue engineering: challenges and potential. Trends in Biotechnology, 22: 643-652.

[3] Zhang, J. (2018) 3D Printing of Silk Particle-Reinforced Chitosan Hydrogel Structures and Their Properties. ACS Biomaterials Science & Engineering, 4: 3036-3046.

[4] You, F., Wu X., Chen X. (2016) 3D printing of porous alginate/gelatin hydrogel scaffolds and their mechanical property characterization. International Journal of Polymeric Materials & Polymeric Biomaterials, 66: 299-306.

[5] Pan, T. (2016) 3D Bioplotting of Gelatin/Alginate Scaffolds for Tissue Engineering: Influence of Crosslinking Degree and Pore Architecture on Physicochemical Properties. Journal of Materials Science & Technology, 32: 889-900.

[6] Pan Z., Yan Z., Gao P. (2018) Comparison of the effects of two cross-linking agents on the physicochemical and biological properties of β-tricalcium phosphate gelatin composite bone scaffold. Chinese Journal of Tissue Engineering, 22: 833-839.

[7] Wang, L.L. (2010) Preparation of Porous Hydroxyapatite-Zirconia Composite Scaffolds by Combination of Gel-Casting and Polymer Sponge Methods. Advanced Materials Research, 105-106: 616-619.

[8] Cui Y. (2013) Preparation and properties of hydroxyapatite whisker material artificial bone scaffold. Kunming University of Science and Technology.

[9] Zhang Y. (2014) Research on preparation and properties of metal organic framework materials and their composites. Anhui University.

[10] Wang, F. (2014) Biocompatibility and osteogenic activity of nano-hydroxyapatite/chitosan/polylactide scaffolds. Chinese Journal of Tissue Engineering, 18: 1198-1204.

[11] Piasecki, D.P. (2003) Intraarticular injuries associated with anterior cruciate ligament tear: findings at ligament reconstruction in high school and recreational athletes. An analysis of sex-based differences. Am J Sports Med, 284: 601-605.

[12] Du, J.H. (2007) Research on the degradation rate of poly-β-hydroxybutyrate tissue engineering scaffolds. Northwestern Polytechnical University.

[13] Zhuang, H., Han, Y.A. (2008) Feng, Preparation, mechanical properties and in vitro biodegradation of porous magnesium scaffolds. Materials Science and Engineering: C, 28: 1462-1466.

[14] Hollister, S.J. (2005) Porous scaffold design for tissue engineering. Nature Materials, 4: 518.

[15] Yu H.Y., Ma D.D., Wu B.L. (2017) 3D printed gelatin sodium alginate gel scaffold adhesion to human dental pulp cells. Journal of Southern Medical University, 5: 668-672.