Preparation of collagen/hydroxyapatite/alendronate hybrid hydrogels as potential scaffolds for bone regeneration

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\section*{A B S T R A C T}

Development of biomimetic scaffolds represents a promising direction in bone tissue engineering. In this study, we designed a two-step process to prepare a type of biomimetic hybrid hydrogels that were composed of collagen, hydroxyapatite (HAP) and alendronate (ALN), an anti-osteoporosis drug. First, water-soluble ALN-conjugated HAP (HAP-ALN) containing 4.0 wt.\% of ALN was synthesized by treating HAP particles with ALN. Hydrogels were then formed from HAP-ALN conjugate and collagen under physiological conditions using genipin (GPN) as the crosslinker. Depending on the ALN/collagen molar ratio and GNP concentration, the gelation time of hydrogels ranged from 5 to 37 min. Notably, these hybrid hydrogels exhibited markedly improved mechanical property (storage modulus \(G^* = 38–187\, \text{kPa}\)), higher gel contents, and lower swelling ratios compared to the hydrogels prepared from collagen alone under similar conditions. Moreover, they showed tunable degradation behaviors against collagenase. The collagen/HAP-ALN hybrid hydrogels supported the adhesion and growth of murine MC3T3-E1 osteoblastic cells well. Such tough yet enzymatically degradable hybrid hydrogels hold potential as scaffolds for bone tissue engineering.

\section*{1. Introduction}

Repair of large-size bone defects resulted from trauma, resection, or congenital malformations remains challenging. Thus far, a variety of autografts, allografts, and alloplast materials have been used for the treatment of such defects in the clinical practices of orthopedic and craniofacial settings, each of which have considerable limits \cite{1–4}. Bone tissue engineering, through the use of osteoconductive and osteoinductive scaffolds together with osteogenic cells, represents a promising approach toward bone defect repair and regeneration. As a unique triphasic tissue, bone is composed of cells, a hydrated extracellular organic matrix (mainly Type I collagen), and an extracellular mineral phase (mainly hydroxyapatite) \cite{1,5,6}. In order to support cell adhesion and guide new bone formation in bone tissue engineering, it is critical to develop scaffolds that both chemically and structurally mimic the native extracellular matrix (ECM) of bone \cite{7–11}. Numerous scaffolds have been prepared from natural or synthetic polymers through various techniques such as electrosprining, self assembly, and salt leaching for guiding bone regeneration \cite{12–15}. Among them, hydrogels have gained special attention because they can hold a large amount of water while maintaining the structural integrity \cite{16,17}. More importantly, they allow adequate permeability of water, nutrients, metabolites or pharmaceuticals to promote bone formation \cite{18,19}. However, hydrogels generally do not possess sufficient mechanical strength for regenerating tough tissues such as bone. Improvement of mechanical properties of hydrogels can be achieved by physical, enzymatic, or chemical crosslinking \cite{20–22}. Genipin (GPN), a natural product derived from gardenia fruit extract which has little toxicity, functions as an efficient crosslinker by reacting with the amine groups of amino acids or proteins \cite{23–25}. The biocompatibility of materials or tissues crosslinked by genipin significantly exceeds those crosslinked by glutaraldehyde or epoxy compounds, yet the mechanical strength and enzymatic resistance of them are comparable \cite{26}.

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In this study, we aim to develop a type of biomimetic hydrogels for the applications as bone tissue engineering scaffolds. These hydrogels were designed as a hybrid system which contains hydroxyapatite (HAP), collagen, and alendronate (ALN) and was reinforced by crosslinking using genipin. Among them, HAP is one of the frequently used bioceramics for bone and dental tissue reconstitution. It has excellent biocompatibility and osteoconductivity despite its slow degradation, low mechanical strength and poor osteoinductive potential [27,28]. Collagen is the major ECM of bone tissue and can promote bone regeneration [29]. When associated with HAP particles to form a biocomposite, it results in an easily molded biomaterial and can prevent HAP dispersion in implants [30]. Alendronate (ALN), a potent nitrogen-containing bisphosphate for the treatment of osteoporosis, affects the activity of osteoclasts by inhibiting farnesyl dipiphosphate synthase (FDPS) in the mevalonate pathway and results in apoptosis of osteoclasts [31–33]. Meanwhile, it may also promote the activities of osteoblasts toward bone formation [34,35]. Therefore, we have prepared a series of GNP-crosslinked collagen/HAP-ALN hydrogels with various GNP concentrations and ALN/NH₂ molar ratios and studied their properties and biocompatibility.

2. Materials and methods

2.1. Materials

Hydroxyapatite nanocrystals (HAP, ca. 20 × 150 nm) and alendronate (ALN, 99%) were purchased from Nanjing Emperor Nano Material Co., Ltd. and Beijing JHYB Pharmaceutical Technology Co., Ltd., respectively. Collagen was purchased from Sichuan Mingrang Bio-Tech Co., Ltd. Genipin (GNP, 98%) was purchased from Linchuan letter Biological Technology Co., Ltd.

2.2. Synthesis of HAP-ALN

HAP (1.0 g) was dispersed through brief sonication in 150 mL aqueous NaOH (5 mM) and ALN (0.2 g) was dissolved in 50 mL aqueous NaOH (5 mM). After adjusting pH to 10 using 20 mM NaOH, the ALN solution was added to the HAP dispersion. The mixture was stirred for 3 days at 37 °C. The crude product was isolated through extensive dialysis (MWCO 1000) against water for 24 h at room temperature to remove free ALN and then freeze-dried.

2.3. Characterizations of HAP-ALN

1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz. The chemical shifts were calibrated against residual solvent (D₂O) signal. Fourier transform infrared spectrometer (FT-IR) was performed on a Thermo Scientific NICO-LET 6700 spectrophotometer. Powder X-ray diffraction (XRD) was performed on an X’Pert-Pro MPD X-ray diffractometer with a Cu tube anode. Thermo gravimetric analysis (TGA) was carried out on a Pyris 1 TGA (Perkin Elmer) under a nitrogen atmosphere at a heating rate of 10 °C/min. HAP and HAP-ALN nanocrystals were heated from 30 to 800 °C.

2.4. Preparation of collagen/HAP-ALN hydrogels

The synthetic route of the hydrogels is shown in Scheme 1. In brief, collagen and GNP were separately dissolved in phosphate buffered saline (PBS, pH 7.4, 10 mM) at concentrations of 2 wt.% and 1 wt.%, respectively, at 37 °C. The collagen/HAP-ALN hydrogels were prepared from HAP-ALN and collagen at molar ratios of ALN/NH₂ from 2.7/1 to 10.8/1 to ensure that collagen and HAP-ALN uniformly dispersed in the solution. After that, the obtained mixtures were crosslinked by GNP at concentrations ranging from 0.04 wt.% to 0.4 wt.% at 37 °C.

2.5. Characterizations of hydrogels

2.5.1. Rheological analysis

Rheological measurements of the hybrid collagen/HAP-ALN hydrogels were carried out using HAAKE RheoStress 6000 (Thermo Fisher, Germany). The sample was placed between parallel plates of 20 mm diameter and with a gap of 0.5 mm, a frequency of 1 Hz and a strain of 1% were applied to maintain the linear viscoelastic
2.5.2. Gel content measurement

To determine the gel content, 0.50 g of hydrogels samples were lyophilized and weighed \( W_s \). The dry hydrogels were extensively extracted with PBS to a constant weight. The remaining hydrogels were lyophilized and weighed \( W_d \). The gel content was expressed as \( W_s/W_d \times 100\% \). The experiments were performed in triplicates.

2.5.3. Swelling ratio determination

The hydrogel samples (about 0.50 g each) were incubated with 3 mL of PBS solution at 37 °C. At regular time intervals, the medium was removed carefully and the hydrogels were gently blot dried and weighed \( W_s \). The medium was replaced once a day. After \( W_s \) reached an equilibrium value \( W_{s,eq} \), the equilibrium mass swelling ratio was determined by the equation: \( (W_{s,eq}−W_d)/W_d \), where \( W_d \) is the initial solid weight of sample. The experiments were performed in triplicates.

2.5.4. Enzymatic degradation analysis

For enzymatic degradation studies, 3 mL 0.1 M Tris–HCl buffer (pH 7.4, 5 mM CaCl2) containing 0.02 mg/mL collagenase was added on the top of hydrogel samples (about 0.50 g), followed with incubation at 37 °C. The solution was replaced every 24 h to maintain enzyme activity. At different time intervals, the hydrogels were weighed after the surface water was gently blotted off. The percent residual mass of hydrogels was calculated according to the following equation: \( W_t/W_0 \times 100\% \), where \( W_0 \) is the initial weight of the hydrogel and \( W_t \) is the weight of the hydrogel at each time point. The tests were performed in triplicates.

2.6. Cell culture

2.6.1. Biocompatibility assays

The in vitro cytotoxicity of hydrogel was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay. First, the extracts of hydrogels were made in cell culture medium using the ratio of 1.25 cm²/mL between the surface area of the samples and the volume of medium and incubated at 37 °C for 24 h according to the ISO Standard 10993–12. The control collagen hydrogels were treated in the same way. Undiluted extracts were used for the tests. After that, MC3T3-E1 cells were seeded into 96-well plates at a density of 5.0 × 10³ cells/well in 100 µL α-MEM medium containing 10% FBS, antibiotics penicillin (100 IU/mL), and streptomycin (100 mg/mL). The cells were cultured for 24 h in a humidified incubator with 5% CO₂ at 37 °C. Afterwards, the medium was replaced by 90 µL fresh α-MEM medium and then 10 µL/well of hydrogel extract was added. The culture medium was replaced by fresh medium every 24 h. After culturing for 1, 3 and 5 days, 10 µL MTT stock solution (5 mg/mL in PBS) was added to each well and incubated for 4 h. The media was completely removed and 150 µL DMSO was added to each well to dissolve the formazan blue crystals. The absorbance of solution was measured using a microplate reader at 492 nm. The cell viability is determined as Cell viability (%) = A_sample/A_control × 100%, where A_sample and A_control are the absorbance values for the treated cells and the untreated control cells, respectively. The A_sample and A_control values were obtained after subtracting the absorbance of DMSO.

2.6.2. Proliferation of MC3T3-E1 cells on hydrogels

The radiation-sterilized collagen/HAP-ALN hydrogels (2 cm² × 2 mm) were placed in 24-well plates. MC3T3-E1 cells were seeded onto the gels (1.0 × 10⁴ cells/well) and then the cell/gel constructs were cultured in a humidified incubator at 37 °C with 5% CO₂ for 2, 4 and 6 days. After that, the medium was replaced by 100 µL fresh medium followed by adding 10 µL MTT.
solution. The cells were incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 400 µL DMSO, and the absorbance at 492 nm was measured using a microplate reader. The relative cell viability was determined by comparing the absorbance at 492 nm with control wells.

2.6.3. Cell morphology observation

The collagen/HAP-ALN hydrogels were coated onto microscope slides and placed in a 24-well plate. A MC3T3-E1 cell suspension was added to the well containing 1.0 × 10^4 cells/well. The medium was replaced every 24 h. After being incubated for 2 and 7 days, the cells were fixed with 4% paraformaldehyde in PBS for 30 min and permeated with 0.1% Triton X-100 for 10 min. Following that, the cytoskeleton of cells was stained with phalloidin-tetramethylrhodamine B isothiocyanate (Sigma) and the nuclei were stained with DAPI (Beyotime). The cells were observed using an inverted fluorescence microscope (Nikon ECLIPSE Ti-S).

2.7. Statistical analysis

All statistical analyses were performed using SPSS software. Kruskal-Wallis one-way analysis of variance (ANOVA) tests followed by Tukey post hoc tests were used. Unpaired student’s t-tests were also used where appropriate. Difference is considered statistically significant when p is less than 0.05.

3. Results and discussion

3.1. Synthesis of HAP-ALN

The modification of HAP with ALN was carried out at an HAP/ALN weight ratio of 1/0.2 in water at pH 10 and 37°C for 3 days. The resulting HAP-ALN conjugate was isolated by centrifugation, washed with deionized water to remove free ALN, and finally freeze-dried. Unlike HAP particles, the HAP-ALN conjugate could be dispersed well in water. The 1H NMR spectrum of HAP-ALN conjugate showed clearly signals attributable to ALN, including the peaks at δ 3.02 (a, -CH2-CH2-NH2), δ 1.74–2.11 (b, c, -CH2-CH2-CH2-NH2, -CH2-CH2-CH2-NH2) (Fig. 1A, B), confirming successful conjugation of ALN to HAP. In comparison, ALN has a low solubility.

![Fig. 2. The rheology measurements of collagen/HAP-ALN hydrogels. (A) Evolution profiles of storage modulus (G') and loss modulus (G'') of hydrogels prepared at ALN/NH2 molar ratio of 8.1/1 and crosslinked by 0.3 wt.% GNP; (B) G' of collagen and collagen/HAP-ALN hydrogels as a function of GNP concentration at ALN/NH2 molar ratio of 0 and 8.1/1; (C) G' of collagen/HAP-ALN hydrogels as a function of ALN/NH2 molar ratio at 0.3 wt.% GNP.]

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<tr>
<th>Table 1</th>
<th>Preparation of collagen/HAP-ALN hydrogels.</th>
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<tr>
<td>Sample</td>
<td>HAP-ALN (wt.%)</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>19.8</td>
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<td>9</td>
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† Gelation time, defined as the time when G' equals G'' as determined by rheology measurement.
Fig. 3. The gel contents of collagen/HAP-ALN hydrogels. (A) Gel content as a function of ALN/NH₂ molar ratio at 0.3 wt.% GNP; (B) Gel content as a function of GNP concentration at ALN/NH₂ molar ratio of 8.1/1.

Fig. 4. The equilibrium swelling ratios of collagen/HAP-ALN hydrogels as a function of ALN/NH₂ molar ratio (GNP, 0.3 wt.%) (A) or GNP concentration (ALN/NH₂ molar ratio, 8.1/1) (B). The hydrogels were immersed in PBS at 37°C during tests.

Fig. 5. The enzymatic degradation profiles of collagen/HAP-ALN hydrogels as a function of ALN/NH₂ molar ratio (GNP, 0.3 wt.%) (A) or GNP concentration (ALN/NH₂ molar ratio, 8.1/1) (B). The concentration of collagenase was 20 μg/mL.

3.2. Formation of collagen/HAP-ALN hydrogels

The collagen/HAP-ALN hydrogels were prepared from HAP-ALN and collagen and crosslinked by GNP. The ratio of HAP-ALN to collagen was characterized using the molar ratio of ALN/NH₂ which varied from 2.7/1 to 10.8/1. The concentration of GNP ranged from 0.04 wt.% to 0.4 wt.%. The influence of the ALN/NH₂ molar ratio and GNP concentration on gelation time was systematically investigated (Table 1). Clearly, at a constant ALN/NH₂ molar ratio, the gelation time markedly decreased with the increase of GNP concentration, likely due to the increase of crosslinking density. At a constant concentration of GNP (0.3 wt.%), the gelation time decreased with increasing molar ratio of ALN/NH₂ (from 2.7/1 to
10.8/1) as a result of the increase in the number of functional groups per volume of gel precursor solution. By changing GNP concentration or ALN/NH2 molar ratio, hydrogels with gelation times ranging from 5 to 37 min were obtained.

Fig. 2A shows the rheological profile of a mixture of HAP-ALN and collagen at an ALN/NH2 molar ratio of 8.1/1 crosslinked by 0.3 wt.% GNP. The G' and G'' increased rapidly and leveled off after about 20 min. The time point where G' equaled G'' was approximately 12 min. The mechanical properties of collagen/HAP-ALN hydrogels were examined as a function of GNP concentration and ALN/NH2 molar ratio at 37 °C. It was found that at a constant ALN/NH2 molar ratio (8.1/1), the G' of collagen/HAP-ALN hydrogels increased from 50 kPa to 150 kPa when GNP concentration was increased from 0.04 wt.% to 0.4 wt.% The G' values of collagen/HAP-ALN hydrogels were significantly higher than the G' of collagen hydrogels alone which did not contain HAP-ALN (Fig. 2B). At a constant GNP concentration (0.3 wt.%), the G' of hydrogels was improved from 1.2 kPa to 187 kPa when ALN/NH2 molar ratio was increased from 0 to 10.8/1 (Fig. 2C).

At a fixed GNP concentration (0.3 wt.%), the gel content of collagen/HAP-ALN hydrogels increased from 88.4% to 95.5% when ALN/NH2 molar ratios was increased from 2.7/1 to 8.1/1 (Fig. 3A). At a constant ALN/NH2 molar ratio (8.1/1), the gel content of hydrogels increased from 85.7% to 97% when GNP concentration was changed from 0.1 wt.% to 0.4 wt.% (Fig. 3B). Such high gel contents indicate efficient crosslinking between HAP-ALN and collagen upon GNP treatment.

Further, the swelling behaviors of collagen/HAP-ALN hydrogels were studied. Fig. 4A displays the influence of ALN/NH2 molar ratio on the swelling ratio of hydrogels at 0.3 wt.% GNP in PBS at 37 °C. Clearly, the equilibrium swelling ratio decreased with an increase of ALN/NH2 molar ratio. Hydrogels formed at ALN/NH2 molar ratio of 8.1/1 exhibited the lowest equilibrium swelling ratio (109 wt.%). Similarly, when ALN/NH2 molar ratio was fixed (8.1/1), the equilibrium swelling ratio of collagen/HAP-ALN hydrogels decreased as a result of increase in GNP concentration (Fig. 4B).

The in vitro degradation behaviors of collagen/HAP-ALN hydrogels upon collagenase treatment were investigated by monitoring the weight loss of hydrogels for 3 weeks. At fixed GNP concentration, increasing ALN/NH2 molar ratio significantly slowed down the degradation of hydrogels (Fig. 5A). Similarly, at a constant ALN/NH2 molar ratio (8.1/1), increasing GNP concentration resulted in reduced hydrogel degradation (Fig. 5B). These findings, again, show that by increasing ALN/NH2 molar ratio or GNP concentration, the crosslinking density of hydrogels was increased, which blocked collagenase activity and slowed down the degradation of hydrogels.

3.3. Cell culture

In order to determine the biocompatibility of collagen/HAP-ALN hydrogels, MC3T3-E1 cells were cultured in medium supplemented with extracts from collagen/HAP-ALN hydrogels. As measured by MTT assays, the number of cells kept increasing within 5 days of culture (Fig. 6). In addition, there was no apparent difference among the viability of cells cultured on collagen/HAP-ALN hydrogels, collagen hydrogels, and control tissue culture plates, indicating the decent biocompatibility of collagen/HAP-ALN hydrogels.

To further check the adhesion and proliferation capacity of MC3T3-E1 cells on the hydrogels, MC3T3-E1 cells were directly cultured on them. As seen, the cells on collagen/HAP-ALN hydrogels were elongated and adequately aligned at day 2, while the cells on collagen hydrogels were chaotic and randomly aligned (Fig. 7A). By day 7, the cells significantly proliferated on both collagen/HAP-ALN and collagen hydrogels. There was no significant difference in the number of cells between them, again showing the considerable biocompatibility of collagen/HAP-ALN hydrogels (Fig. 7B). This was likely due to the biomimetic nature of these hybrid hydrogels which were composed of HAP and collagen, the major components of native bone tissue [37].

Fig. 6. The viability of MC3T3-E1 cells after being incubated with the extracts from collagen/HAP-ALN hydrogels and collagen hydrogels. The hydrogels were prepared at ALN/NH2 molar ratio of 8.1/1 and 0, respectively, following with crosslinking by 0.3 wt.% GNP. The untreated cells were used as control. The cell viability was determined by MTT assays.

Fig. 7. The adhesion (A) and proliferation (B) of MC3T3-E1 cells on hydrogels after 2 or 7 days of culture. The hydrogels were prepared at ALN/NH2 molar ratio of 8.1/1 and 0, respectively, following with crosslinking by 0.3 wt.% GNP. Rhodamine-labeled phalloidin and DAPI were used for staining F-actin and nuclei, respectively. *p < 0.05. Scale bars, 100 μm.
4. Conclusions

We have developed a type of enzymatically degradable hybrid hydrogels based on collagen and HAP-ALN conjugates using GNP as the crosslinker. Simply by adjusting the ALN/NH₂ molar ratio or GNP concentration, the mechanical characteristics, gel content, and swelling behavior of collagen/HAP-ALN hydrogels could be readily controlled and markedly improved compared to those of hydrogels prepared from collagen alone. These hydrogels also showed tunable degradation behaviors against collagenase. In addition, they had excellent biocompatibility and promoted the adhesion and proliferation of MC3T3-E1 cells well. Such tough yet enzymatically degradable hybrid hydrogels hold potential as scaffolds for bone tissue engineering. Our following studies will explore the potential of these hydrogels in promoting bone defect repair and bone tissue regeneration through in vivo implantation in animal models.

Acknowledgments

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