Citrate-based mussel-inspired magnesium whitlockite composite adhesives augmented bone-to-tendon healing

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Keywords: bone adhesive, bone-to-tendon healing, citrate, whitlockite, hemostasis, osteogenesis

1. Materials and methods

1.1 Materials

Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG-PPG-PEG, EPE, average Mn ~1100 Da), citric acid (CA), poly(ethylene glycol) (PEG, 200 Da), calcium carbonate ($CaCO_3$), were purchased from Sigma-Aldrich. Sodium (meta) periodate (PI) was from Alfa-Aesar. Dopamine hydrochloride and magnesium hydroxide ($Mg(OH)_2$) were provided by Aladdin Biochemical Technology Co., Ltd., China. Calcium hydroxide ($Ca(OH)_2$) was from Sinopharm Chemical Reagent Co., Ltd., China, and phosphoric acid (H_3PO_4) was from Macklin Biochemical Co., Ltd., China. All chemicals were used without further purification unless otherwise

specified.

1.2 Synthesis of whitlockite

Whitlockite (WH: $Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) was synthesized using a precipitation method according to previous literature [1]. Briefly, 500 mL $Ca(OH)_2$ (0.77 mol/L) and $Mg(OH)_2$ (0.23 mol/L) solution in distilled water was stirred homogeneously using an overhead stirrer at 70 ºC for 1 hour. Then, 500 mL H_3PO_4 (0.95 mol/L) was added dropwise into the $Ca(OH)_2$-$Mg(OH)_2$ mixed solution at a constant speed of 12.5 mL/min while vigorously stirred. The precipitates were aged for 24 hours, filtered, washed with distilled water, and then lyophilized.

1.3 Synthesis and characterization of iC-E-Ca^{2+} prepolymer

The iC-E-Ca^{2+} prepolymer was synthesized via a one-step polycondensation reaction of CA, EPE and DP (Scheme 1A). Briefly, CA and EPE (molar ratio = 1.2: 1.0) were placed in a one-necked round-bottom flask and the mixture was heated to 160 ºC under stirring until complete melting with nitrogen gas flow. Then, 0.3 eq. (to EPE) of DP was added to the mixture, and the temperature was reduced to 140 ºC, the reaction was continued under vacuum until the stir bar stopped turning at 60 rpm. The reaction mixture was dissolved in ethanol and slowly mixed with excess $CaCO_3$. After thoroughly stirring for 24 hours, the mixture was dialyzed. Then the precipitate was filtered, and the solution was collected, concentrated and freeze-dried to give purified iC-E-Ca^{2+} prepolymer as a soft and elastic solid. iCMBA prepolymer composed by CA, PEG200 and DP (iC-P) was also synthesized according our previous literatures [2,3], and used as control.

The prepolymers of iC-E-Ca^{2+} and iC-P were characterized by a Nicolet iS50 Fourier transform infrared spectroscopy (FTIR, Thermo Fisher Scientific, Waltham, USA) to analyze the functional groups of the prepolymers. Briefly, a certain amount of prepolymer solution in acetone was cast onto potassium bromide (KBr) discs, and the solvent was evaporated before FTIR tests, blank KBr disc was used as background. The ^1H NMR spectra of prepolymers were recorded with a Bruker Avance III 600 MHz NMR spectrometer (Bruker Biospin, Germany) using D_2O as solvent. The content of DP in prepolymer was determined by UV-vis spectrum by the absorbance of the prepolymer solutions (0.4 mg/mL) at 280 nm using Shimadzu UV-2600i UV-vis spectrophotometer (Shimadzu Corporation, Japan) with a minimum wavelength resolution of 0.1 nm. These results are shown in Figure 1.

1.4 Preparation of iC-E-WH composite adhesives and gel time tests

The iC-E-Ca^{2+} prepolymer was dissolved in distilled water to form a 33 wt% prepolymer solution, and then 0.5 mL 1, 2, 3, 4, or 8 wt% sodium (meta) periodate (PI) solution in distilled water, as a crosslinking initiator, was uniformly mixed with the prepolymer solution to give iC-E-XPI (X = 1, 2, 3, 4, or 8, representing the concentration of PI in wt%) adhesive/hydrogel. For composite adhesives/hydrogels, 30, 50, or 70 wt% (weight percent to the total weight of dry prepolymer plus WH) of ground WH powder was uniformly mixed with 33 wt% prepolymer solution to form a paste-like putty, and crosslinked by 2 wt% PI (the volume ratio of iC-E-Ca^{2+} prepolymer solution and PI solution was 2:1) to give iC-E-YYWH(-2PI) composite hydrogel (YY = 30, 50 or 70, representing the ratio of WH). The gel times of different samples were

measured using the tilting test method as reported in our previous literatures [2,4-6]. For each sample, tilting tests were conducted at least three times and the results were averaged. The gel time results are shown in Figure 2A and Table 1.

Unless otherwise stated, all hydrogels were allowed to solidify for 24 hours and then freeze-dried for at least 2 days for further studies.

1.5 Characterization of the physical properties of iC-E-WH composite adhesives

The sol/gel contents of different hydrogels, as an indication of non-crosslinked/crosslinked fractions of the adhesives/hydrogels, were determined by washing the sol parts of the hydrogels with 1, 4-dioxane followed by freeze-drying, and calculated using equation (1). The results are shown in Figure 2B.

$$\text{Sol content (\%)} = \frac{W_i - W_d}{W_i} \times 100\% \quad (1)$$

Here, W_i represents the initial weight of the dried hydrogel, W_d means the dried weight of the hydrogel after the sol fraction being removed by solvent.

The swelling ratios of different hydrogels were measured by suspending the leached and dried hydrogels (W_d) in water for 24 hours, followed by blotted drying with filter paper, and weighed (W_s). The swelling ratios were calculated by equation 2. The results are shown in Figure 2C.

$$\text{Swelling ratio (\%)} = \frac{W_s - W_d}{W_d} \times 100\% \quad (2)$$

The mechanical property of dried and swollen iC-E-PI composite hydrogel films was measured on a universal material testing machine with a 50 N load cell (AMETEK, USA) according to American Society for Testing and Materials (ASTM) standard

D412A. Briefly, rectangle-shape samples (25 mm × 6 mm × 1.5 mm, length × width × thickness) were pulled to failure under a stain rate of 500 mm/min. The Young's modulus (calculated from the initial slope of the beginning 10% elongation of the curve), tensile strength and elongation at break were recorded for different samples. At least ten specimens were repeated for each sample, and the results were averaged. The mechanical test results of dried samples are shown in Figures 3A-D and Table 2. In order to evaluate the effect of hydration to the mechanical property of the composite adhesives, the mechanical tests were also conducted on samples after being hydrated in wet conditions when the film weight was increased to 110% of the original weight. The corresponding results are summarized in Table 2.

The degradation profiles of different samples were evaluated by *in vitro* degradation in phosphate buffered saline (PBS, pH 7.4) at 37 ºC. Cylindrical disc specimens (7 mm in diameter, 1 mm thick) were accurately weighed (W_0) and then were immersed in tubes containing 10 mL of fresh PBS and incubated at 37 ºC. The PBS solution was changed every other day. At a pre-determined time points, samples were collected, washed and freeze-dried (W_t) to determine the mass loss using equation (3), the results are shown in Figure 3E. The change of the pH values of the supernatants at each time points were also measured, the results are shown in Figure 3F.

$$\text{Mass loss (\%)} = \frac{W_0 - W_t}{W_0} \times 100\% \quad (3)$$

1.6 Release of magnesium and calcium ions and cytotoxicity study of magnesium ion

A certain mass of crosslinked composite hydrogels containing different proportions of

WH (30%, 50%, or 70%, w/w) was incubated in 10 mL PBS (pH 7.4) in a constant temperature shaker at 37 ºC. 0.1 mL supernatants were taken at preset time points each time, and the same amount of fresh PBS was supplemented. The contents of magnesium and calcium ions in the solutions were measured by inductively coupled plasma mass spectrometer (ICP-MS, NexION 2000, PerkinElmer) using diluted solutions. The results of the release of Mg and Ca ions from iC-E-30WH, iC-E-50WH, and iC-E-70WH, in parts per billion (ppb), are shown in Figures 4A and 4B.

To assess the cytotoxicity of magnesium ions against rat bone mesenchymal stem cells (rBMSCs, Cyagen RASMX-01001, passage 4-6), 100, 50, 20, 10, 5, and 2.5 mmol/L magnesium chloride ($MgCl_2$) solutions in complete alpha-minimum essential medium growth medium (MG, α-MEM, HyClone, USA, containing 10% (v/v) fetal bovine serum (FBS, CLARK, USA) and 1% (v/v) penicillin and streptomycin solution (HyClone, USA)) media were prepared, and the pH was adjusted to 7.4. For each well of 96-well plates, 200 μL rBMSCs solution in MG with a density of 5×10^4 cells/mL was added, and the cells were incubated at 37 ºC, 5% CO_2 for 24 hours. Next, the culture medium was replaced by 200 μL $MgCl_2$ solution with different concentrations and incubated for 1, 4, and 7 days before CCK-8 assay. The cytotoxicity testing results of magnesium ions against rBMSCs are shown in Figure 4C.

1.7 Effect of magnesium ion on alkaline phosphatase (ALP) activity during the osteogenic differentiation of rBMSCs

To evaluate the effect of magnesium ion on the osteogenic differentiation of rBMSCs, the effect of magnesium ion to the ALP activity during the osteogenic differentiation

of rBMSCs was studied. Briefly, $MgCl_2$ was dissolved into osteogenic (OG) media (Lifescience company, Guangzhou, China) with concentrations of 20, 10, and 5 mmol/L (OG-ZMg, Z= 20, 10, or 5, representing the concentration of Mg^{2+} in mmol/L), the pH of the solutions was adjusted to 7.4 and sterilized by filtering through 0.2 μm filters. The rBMSCs were seeded on different wells of 24-well plates at a density of 1×10^4 cells/well and cultured in OG, OG-ZMg or MG media for 3, 7 and 14 days. At each time point, semi-quantitative analysis of ALP activity was performed using the ALP assay kit (Beyotime, Shanghai, China). Briefly, the cells were lysed in 200 μL RIPA (P0013J, Beyotime, Shanghai, China) buffer without inhibitors, followed by sonication and centrifugation at 14, 000 rpm for 5 min at 4 ºC. The supernatant was collected to measure the ALP activity according to the protocol. Meanwhile, the total protein content of each specimen was also measured using a BCA protein assay kit (Beyotime, Shanghai, China). The ALP activity was normalized to the level of total protein. All experiments were repeated three times, with three specimens per sample in each experiment. The results are shown in Figure 4D.

1.8 *In vitro* cytocompatibility tests

rBMSCs were used to assess the cytocompatibility of the adhesive samples using CCK-8 (Cell Counting Kit-8, Invitrogen, USA) assay. For the prepolymer cytotoxicity test, different prepolymers were dissolved in MG in concentrations of 10, 1 and 0.1 mg/mL, sterilized by filtering through 0.2 μm filters, and the pH was then adjusted to 7.4. rBMSCs in MG were seeded in 96 well plates with a density of 5×10^4 cells/mL and 200 μL per well, which were then incubated for 24 hours at 37 °C, 5% CO_2. Next,

prepolymer solutions with different concentrations were added to the 96-well plates (20 μL/well) and incubated for another 24 hours. Then, 10 μL CCK-8 solution was added to each well and allowed to react for 1 hour in the incubator, followed by measuring the absorbance of every well at 450 nm with an Epoch™ microplate spectrophotometer (BioTek, USA). The results are shown in Figure 5A.

The *in vitro* cytocompatibility of iC-E-WH composite hydrogels was further assessed by testing the cell cytotoxicity of the sol contents (or leachable fractions) and degradation products of the crosslinked hydrogels, according to the processes described in our previous literatures [2-6]. The cytotoxicity of the crosslinked iC-E-WH composite hydrogels' sol contents was studied using CCK-8 assay against rBMSCs. The sol content solution of the hydrogel was obtained by incubating 1 g dried hydrogel specimen in 10 mL PBS (pH 7.4) for 24 hours at 37 ºC, followed by removing the flouting hydrogels and sterilizing the solutions with 0.2 μm filters to give 1× solutions. Next, 10× and 100× (10 times and 100 times diluted from 1× solution with PBS, respectively) dilutions were prepared. To each well of a 96-well cell culture plate, 200 μL of rBMSCs solution in MG with a density of 5×10^4 cells/mL was added and incubated for 24 hours. Then, 20 μL sol content solution with different dilution was added, and the cells were incubated for another 24 hours before conducting CCK-8 assay. The cytotoxicity of the crosslinked hydrogels' degradation products was also evaluated. Briefly, 1g dried iC-E-WH hydrogel sample or poly (lactic-co-glycolic acid) (PLGA, used as control, LA/GA = 50/50, Mw ~ 30 kDa, purchased from Guangzhou Boqiang Biotechnology Ltd.) was completely degraded in 10 mL 0.2 M NaOH solution.

After sterilizing the solutions with 0.2 μm filters and adjusting the pH to 7.4, 1× degradation solutions were obtained. The 1× solutions were further diluted into 10× and 100× solutions using PBS (pH 7.4). The three dilutions of degradation products were then used for cell culture (with the same process used in the sol content cell cytotoxicity study described above) and CCK-8 assay. The results are shown in Figures 5B and 5C.

1.9 Effect of iC-E-WH degradation products on rBMSCs' osteogenic differentiation process

The effect of the iC-E-WH composite hydrogels' degradation products on the proliferation (cell viability) and osteogenic differentiation (ALP activity and calcium deposition) of rBMSCs was studied, and the cell morphology was observed after staining. Briefly, 0.1g iC-P-8PI, iC-P-70HA and iC-E-70WH composites were directly degraded in 10 mL OG media at 37 ºC to produce OiC, $OiC_{200}H70$ and OiCW70 media. To investigate the effect of WH particles on cell differentiation, 0.22 μm filter was used to filter OiCW70 medium to obtain OiCW medium. rBMSCs were then cultured in different media for 14 days using MG as a negative control. The culture media were replaced every other day. The ALP activity tests were conducted at day 3, 7 and 14. The results are shown in Figure 5D. Furthermore, Acridine orange/Ethidium boromide (AO/EB) fluorescent staining (Sigma-Aldrich) and fluorescence microscope (Leica, Germany) observation was conducted to assess the cell viability and morphology of the differentiated rBMSCs. The results are shown in Figure 5E.

As an indicator of extracellular matrix mineralization, alizarin red (ARS, Solarbio, China) staining was conducted after rBMSCs (1×10^4 cells/well in 24-well plates) being

cultured in OG, OiC, OiCW, and OiCW70 media for 7, 14 and 21 days. At each time point, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min followed by washing with distilled water and being stained with ARS (1%, pH=4.2) for 30 min at room temperature. After being washed with PBS until there was no non-specific staining, the attaining images were captured under a phase contrast inverted optics microscope (Leica DMI4000 B; Leica Microsystems GmbH, Wetzlar, Germany). The results are shown in Figure 5E.

1.10 Hemostatic performance of iC-E-Ca^{2+} prepolymer and iC-E-WH composite adhesive

The standardized strip rat liver injury model was used to further evaluate the hemostatic capacity of iC-E prepolymer (without $CaCO_3$ treatment) synthesized according our previous literature [20], iC-E-Ca^{2+} containing calcium ions, and iC-E-70WH. SD rats (female, 200g-300g) were anesthetized by injecting pentobarbital (0.1mL/10g), and then they were fixed on a surgical corkboard. The liver of the rat was exposed by an abdominal incision, and the serous fluid around the liver was carefully removed to prevent inaccuracies in the estimation of the blood weight obtained from the hemostatic samples. A pre-weighed filter paper on a paraffin film was placed beneath the liver. An 8 mm wound was cut in the liver by a surgical scalpel with the corkboard tilted at approximately 30º. The bleeding time and blood loss were recorded until the wound stopped bleeding. The wound without treatment was used as the negative control. The results are shown in Figure 6.

1.11 *In vivo* study

Because of its strong mechanical strength and favorable biocompatibility *in vitro*, iC-E-70WH composite adhesive was chosen for the following *in vivo* studies, iC-E-2PI and iC-P-70HA were used as controls to investigate the effect of WH.

1.11.1 Creation of ACL reconstruction animal model

72 male Sprague-Dawley rats (250-300 g) were purchased from the Laboratory Animal Center of Southern Medical University, China, and used for the creation of animal model. All surgical procedures as well as perioperative handling were conducted following the "guidelines for the ethical review of laboratory animal welfare" and the "guidance on the well-treatment to laboratory animals", in accordance with a protocol approved by the Southern Medical University Ethics Committee of Animal Care and Use (Guangzhou, China). The animals were generally anesthetized, and a longitudinal incision was created on the medial of the distal leg and ankle. The full length flexor digitorum longus tendon (average length 25 mm) was cut from the distal to the ankle and harvested. A second incision was made over the knee, and a medial parapatellar arthrotomy was performed, and the native ACL was excised. Bone tunnels (1.2 mm in diameter) were constructed starting at the intra-articular portions of the proximal tibia and the distal femur corresponding to the attachment sites of the ACL. A 4-0 ethibond suture (Ethicon, Somerville, NJ) was passed through each end of the previously harvested tendon grafts, and the grafts were wrapped with the developed adhesives. The adhesive treated graft was then passed through the bone tunnels to reconstruct the ACL, with untreated tendon as control (CON). Both ends of the tendon were secured to the surrounding periosteum using 4-0 ethibond sutures at the level of the extra-articular

bone tunnel exit sites of the distal femur and proximal tibia. The wounds were closed with sutures, and the animals were allowed free activity post-operation. Five groups were set for subsequent analysis: 1) CON, 2) iC-P-2PI, 3) iC-E-70WH, 4) iC-P-70HA.

1.11.2 Tissue harvest and biomechanical tests

At preset time points, the rats were euthanized by overdose CO_2. For biomechanical testing, the femur-graft-tibia complexes were harvested at week 4, 8 and 12, and the samples were quickly prepared as described in the biomechanical assessment section below. For histochemical and immunohistochemical staining, the femur-graft-tibia complexes were harvested, quickly embedded in the optimal cutting temperature (OCT) compound (Miles Scientific, Elkhardt, IN), frozen in liquid nitrogen, and then stored at -80 ºC.

1.11.3 Biomechanical assessment

To assess the adhesion and healing effect of the adhesives to the reconstructed ACL, the biomechanical strengths of the treated ACLs were tested post-operatively at week 4 and 12 (n = 6 from each group). Before the biomechanical tests, the hind limbs were completely freed of all soft tissue except the ACL graft, which was carefully dissected. The femur and the tibia were cut 20 mm from the joint line, and both femur and tibia were fixed in a cylinder hole of a specially designed aluminum plate using super glue. The isolated femur-ACL-tibia complex was mounted in a conventional mechanical tester (DS2, Imada Inc, Northbrook, IL). The applied load was directed along the longitudinal axis of the ACL graft. Prior to the tests, ten cycles of 1 N longitudinal loads were applied to the knee specimen for preconditioning. After preconditioning, each

femur-ACL-tibia complex was elongated at a crosshead speed of 1.3 mm/min until gross failure of the ACL occurred. In addition to the tests performed on the reconstructed ACL knee specimens, biomechanical testing was also performed on healthy ACL specimens and served as control. The specimens were kept moist with PBS throughout the preparation and test processes. The results are shown in Figure 7.

1.11.4 Computer tomography analysis for implants.

Micro-computer tomography (Micro-CT) analysis was conducted using a Micro-CT imaging system (ALOKA LCT200, HITACHI, Japan) following standard and validated precise protocols. Briefly, the scanning system was set to “Isol”. Bone scan mode: pixel size of 24 um, slice thickness of 48 um, slice pitch of 48 um, rotation angle of 360 deg, low X-ray voltage. A quantitative 3D histomorphometric evaluation (i.e., determination of bone mineral density and bone mineral content) was then performed on a rectangular volume of interest (VOI) using well-recognized methods. The mean bone volume/total volume fraction (BV/TV) and bone mineral density (BMD) of a 1.5 mm diameter circular region of interest within the 6 mm bone tunnel were measured and the data were processed and analyzed using CTAn image analysis software based on the micro-CT images. The micro-CT results are shown in Figure 8.

1.11.5 Histological examination

The harvested ACL samples treated with adhesive wrapped tendon were decalcified with ethylenediaminetetraacetic acid (EDTA) decalcifying fluid (pH 7.4, Solarbio, China) at 37 ºC for 1 month. Histological examination was performed at pre-determined time points (4, 8, 12 weeks post-operation) according to the standard protocol. The 5

mm longitudinal sections were cut at the diaphysis of interest using a SP2500 microtome (Leica Microsystems, Wetzlar, Germany). Then hematoxylin and eosin (H & E) and Masson's trichrome staining, as well as collagen II (COL2) and Runt-related transcription factor 2 (Runx2) immunohistochemical staining of the sliced sections were conducted. The stained sections were observed by a photo microscope (DM 5500B, Leica, Germany), the results are shown in Figures 9A-E. The semi-quantitive analysis of the COL2 and Runx2 positively stained cells in the images were also conducted using ImageJ, and the results are shown in Figures 9F and 9G.

1.12 Statistical analysis

All quantitative results were expressed as mean ± standard deviation (SD). The significance of differences between results was evaluated by One-Way Analysis of Variance (ANOVA), with the significant level set as $p < 0.05$.

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