Citrate-Based Tannin-Bridged Bone Composites for Lumbar Fusion

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Conventional bone composites consistently fail to mimic the chemical composition and integrated organic/inorganic structure of natural bone, lacking sufficient mechanics as well as inherent osteoconductivity and osteoinductivity. Through a facile surface coating process, the strong adhesive, tannic acid (TA), is adhered to the surface of the natural bone component, hydroxyapatite (HA), with and without the immobilization of in situ formed silver nanoparticles. Residual functional groups available on the immobilized TA substituents are subsequently covalently linked to the citrate-based biodegradable polymer, poly(octamethylene citrate) (POC), effectively bridging the organic and inorganic phases. Due to the synergistic effects of the tannin and citrate components, the obtained citrate-based tannin-bridged bone composites (CTBCs) exhibit vastly improved compression strengths up to 323.0 ± 21.3 MPa compared to 229.9 ± 15.6 MPa for POC-HA, and possess tunable degradation profiles, enhanced biomineralization performance, favorable biocompatibility, increased cell adhesion and proliferation, as well as considerable antimicrobial activity. In vivo study of porous CTBCs using a lumbar fusion model further confirms CTBCs’ osteoconductivity and osteoinductivity, promoting bone regeneration. CTBCs possess great potential for bone regeneration applications while the immobilized TA additionally preserves surface bioconjugation sites to further tailor the bioactivity of CTBCs.

1. Introduction

Bone serves as the body’s support structure and mineral reservoir, protecting vital organs, producing hematopoietic derived cells, and helping maintain acid-base homeostasis in the body.[1–3] With ≈1.6 million procedures being performed annually in the United States alone, bone transplantation is one of the most common tissue transplant procedures.[4] Although autograft bone remains the gold standard for bone transplantation, the quality and quantity of autografts are greatly limited. Furthermore, the use of autografts necessitates additional surgery, extended recovery time and increased risk of postoperative complications.[5–7] Thus, the development of fully synthetic and biomimetic bone substitutes that restore the function of natural bone is in high demand.

To mimic the structure of natural bone, which is composed of 60–65 wt% hydroxyapatite (HA, inorganic) embedded in a collagen (organic) matrix,[5,6,8] extensive research effort has been made to develop...
organic/inorganic bone composites as bone substitutes.\cite{5,6,8,9} Calcium phosphates (CaPs), such as HA and β-tricalcium phosphate (βTCP),\cite{5,6,9,10} and bioactive glasses,\cite{11} are often used to approximate the inorganic components of bone. Organic polymers used in bone composites include biologically derived and biodegradable polymers, such as collagen,\cite{12} gelatin,\cite{13,14} chitosan,\cite{15} poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL) and their respective copolymers.\cite{10,11} Although promising, the compatibility of the organic and inorganic components is often far from ideal, leading to inorganic/organic phase separation, reduced mechanical properties, and failed bone integration and regeneration.\cite{5,6,9} To address these problems, HA has been surface modified with L-lactic acid (LA) oligomers,\cite{10,11} poly(amino acid)\cite{17} or poly(N-isopropylacrylamide) (PNIPAM).\cite{18} After surface “organic” modification, the organic/inorganic composites exhibited enhanced mechanical properties, uniform microstructures and prolonged mechanical durability during degradation.\cite{11} However, these surface modification strategies are greatly limited by the surface properties of inorganic particles,\cite{21} often making pre-treatment of the inorganic surface necessary to provide immobilized initiating sites.\cite{17,18} A universal strategy to bridge inorganic particles and organic polymers is urgently needed.

Marine creatures, such as the blue mussel, can strongly adhere to a range of non-specific surfaces under water via excreted proteinaceous fibers presenting catechol moiety L-DOPA (L-3,4-dihydroxyphenylalanine). Inspired by nature, in recent years the science community has achieved remarkable successes utilizing mussel-inspired and similar tannin-inspired adhesion strategies in tissue adhesive development.\cite{9,11,21,24} Although promising, the compatibility of the organic and inorganic components is often far from ideal, leading to inorganic/organic phase separation, reduced mechanical properties, and failed bone integration and regeneration.\cite{5,6,9} To address these problems, HA has been surface modified with L-lactic acid (LA) oligomers,\cite{10,11} poly(amino acid)\cite{17} or poly(N-isopropylacrylamide) (PNIPAM).\cite{18} After surface “organic” modification, the organic/inorganic composites exhibited enhanced mechanical properties, uniform microstructures and prolonged mechanical durability during degradation.\cite{11} However, these surface modification strategies are greatly limited by the surface properties of inorganic particles,\cite{21} often making pre-treatment of the inorganic surface necessary to provide immobilized initiating sites.\cite{17,18} A universal strategy to bridge inorganic particles and organic polymers is urgently needed.

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nanoparticles (Ag NPs) can also be immobilized onto the surface of HA during the one-step reaction process to produce HA modified with TA and silver (AgTHA) in order to confer antimicrobial activity (Scheme 1). Both THA and AgTHA preserve unreacted hydroxyl groups on immobilized TA molecules which were chemically reacted with the carboxylic acid groups available in the prepolymer of the citrate-based biodegradable polymer, poly(octamethylene citrate) (POC), creating a stable tannin bridged inorganic/organic matrix. The efficiency of this strategy to enhance the compatibility between inorganic (HA) and organic (POC) phases was investigated. The mechanical properties, degradation profiles, biomineralization performance, biocompatibility and antibacterial capability were studied in vitro. Furthermore, in vivo study of porous POC-HA, POC-THA, POC-HA/THA and POC-THA/AgTHA composite scaffolds was conducted using a lumbar fusion model in rabbits to further evaluate the bone regeneration performance of citrate-based tannin-bridged bone composites (CTBCs).

2. Results and Discussion

2.1. HA Modification

In traditional HA surface modification strategies, most of the coupling agents including organic isocyanates, LA oligomers, silane coupling agents, and polyacids are grafted onto HA surfaces through covalent bonding with surface hydroxyl groups. However, both the amount and the reactivity of the active hydroxyl groups on the HA surface are limited, greatly hindering the application of these strategies. In contrast, mussel-inspired and tannin-inspired surface coating strategies are universally applicable to nonspecific surfaces, such as underwater rock, inorganic microparticles, or polymeric microspheres, regardless of surface properties.

After a facile one-step reaction, two peaks at $1750$–$1550 \text{ cm}^{-1}$ appeared in the FTIR spectra of THA and AgTHA (Figure 1A) which were not found in the FTIR spectrum of HA and can be assigned to the characteristic absorbance of the ester bonds in

Figure 1. A) FTIR, B) XRD, and C) XPS spectra, as well as D) TGA curves of HA, THA, and AgTHA. Characteristic peaks are inserted as enlarged spectra in (A) and (C) for the comparison of different samples.
TA molecules. The characteristic peaks (38.1°, 44.2°, 64.4°, and 77.3°) of silver nanoparticles (Ag NPs) presented in the X-ray diffraction (XRD) spectrum of AgTHA (Figure 1B), confirming the successful immobilization of Ag NP on the surface of the modified HA. There is nearly no difference detected from the XRD spectra of HA and THA, implying that the surface modification of HA with TA had no influence on the crystal structure of HA (Figure 2B). The presence of Ag 3d_{3/2} and Ag 3d_{5/2} peaks at ≈373 and ≈366 eV, respectively,[48] in the X-ray photoelectron spectroscopy (XPS) spectrum of AgTHA further confirmed the successful immobilization of Ag NPs onto HA (Figure 1C). The presence of carbon in THA and AgTHA as well as the presence of Ag in AgTHA samples detected through energy dispersive spectroscopy (EDS) analysis further supported the success of surface conjugation (Table S1). During thermogravimetric analysis (TGA) testing (25–700 °C, N₂, 10 °C min⁻¹), the differences of the weight loss percentages of HA (3.5% weight loss), THA and AgTHA (both ≈8% weight loss) indicated that ≈4.7 wt% of TA was successfully coated onto THA and AgTHA (Figure 1D). Although the modification is conducted in a heterogeneous manner, if the reaction pH is controlled and vigorous stirring is applied, the modification process is controllable and repeatable.

2.2. Mechanical Properties and Degradation Profiles of POC-HA and CTBC Composites

Mechanical studies of POC-THA, POC-HA/THA, and POC-THA/AgTHA composite cylinders thermally crosslinked at different conditions were conducted with POC-HA as control. For all groups, mechanical strength increased with longer crosslinking time, higher temperature and vacuum application (Figure 2A–D). Regardless of crosslinking conditions, the compression strengths of POC-HA cylinders were lower than
eral crystal layers were observed. The thickness and density of controls, respectively. As shown in Figure S2 in the Supporting Information, the effect of the TA coating was reflected in the results of the mineralization study of different composite disks (Figure 3). After incubation for one and seven days, more mineral deposition was observed on the CTBC composite disks than on POC-HA disks (Figure 3 insets), which can also be deduced from the change in densities and crystal sizes shown in the SEM images (Figure 3). These results agree well with previous literature indicating that polydopamine or natural polyphenol coatings can promote HA crystallization or biomineralization.[32,33]

2.4. In Vitro Biocompatibility

The cytocompatibility of the composites was estimated by the cytotoxicity study of the degradation products of the composites using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against human mesenchymal stem cells (hMSCs) (Figure 4A). Although the cell viabilities of the 1× degradation products of the composites (~30%) were significantly lower than that of the degradation product of PLGA (52.8 ± 4.08%) at 1× dilution, the cell viabilities of the degradation products of these composites at 10× and 100× dilutions were higher than that of PLGA degradation products at the same dilutions (Figure 4A). Cytotoxicity of the CTBCs and POC-HA were similar (Figure 4A). The cell proliferation of hMSCs on composite films was also evaluated by the MTT assay and SEM imaging using commercially available tissue culture treated plates (Costar, USA) as control (Figure 4B,C). The cell numbers on the composite films containing THA and/or AgTHA were higher than on the POC-HA films as early as one day after seeding (Figure 4B) indicating better cell adhesion on the films containing TA. After the initial cell adhesion, hMSCs grew better on these composite films relative to the POC-HA films as indicated by higher cell counts 7 days post cell seeding on the composite films (Figure 4B) coinciding with the SEM images on day 7 (Figure 4C). These results suggest that the inclusion of TA and Ag NP did not induce significant cytotoxicity, and provide further evidence that the inclusion of a polyphenol or polydopamine compound can promote cell adhesion and proliferation as reported previously.[11,29,31]

2.5. Antibacterial Performance

The antibacterial performance of THA and AgTHA particles (HA as control) as well as POC-HA, POC-HA/THA and POC-THA/AgTHA composites (POC-HA as control) was tested against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) as representative Gram-positive and Gram-negative bacteria, respectively.

The minimal inhibitory concentrations with 100% bacterial inhibition (MICs) of HA, THA, and AgTHA particles were determined using the agar dilution method.[22–24] As shown in Figure 5A,B and Figure S2 (Supporting Information), unmodified HA had nearly no antibacterial effect against S. aureus.
and *E. coli* with bacterial survival around 100% for all tested concentrations, and even > 100% for some concentrations (5, 10, 20 mg mL\(^{-1}\) HA against *E. coli*). Both THA and AgTHA exhibited antibacterial activities against *S. aureus*, with MICs of 5 mg mL\(^{-1}\) (Figure 5A; Figure S3, Supporting Information). For tests against *E. coli*, THA exhibited very weak antibacterial activity where 20 mg mL\(^{-1}\) of THA dispersed in agar induced only \(\approx 20\%\) of *E. coli* death (Figure 5B; Figure S3, Supporting Information). The inclusion of Ag NPs led to greatly improved antibacterial activity against *E. coli*; the MIC of AgTHA against *E. coli* was 2.5 mg mL\(^{-1}\) (Figure 5B; Figure S3, Supporting Information). The SEM images of the bacteria taken after 1 day of incubation on agar gels containing THA, AgTHA and HA particles (10, 5, and 10 mg mL\(^{-1}\), respectively) are shown in Figure 5C. These results suggest that the inclusion of both TA and Ag NP are necessary in order to confer considerable innate antibacterial properties against both Gram-positive and Gram-negative bacteria.

The antibacterial performance of composite disks was evaluated by directly exposing composite disks (Φ15 mm × 1 mm) to *S. aureus* and *E. coli* suspensions (1 mL) with initial bacteria concentrations of \(\approx 1 \times 10^6\) CFU mL\(^{-1}\) (CFU: colony-forming units). The bacterial inhibition ratios were between 20–85% after 24 h (Figure 5D). The contact killing effect of these disks after 24 h was also evaluated by imaging the bacteria attached on the disks with SEM after 24 h (Figure 5E). After 24 h of material exposure, 60–85% of *S. aureus* was inhibited in the POC-THA, POC-HA/THA and POC-THA/AgTHA groups, which was higher than that for POC-HA and PLGA controls (\(\approx 22\%\) and \(\approx 3\%\), respectively), indicating antibacterial activity against *S. aureus* (Figure 5D). The antibacterial property of CTBCs against *S. aureus* is also supported by the contact killing test results (Figure 5E). The weak antimicrobial activity of POC-HA against both *S. aureus* and *E. coli* could be attributed to the innate antimicrobial property of POC.\(^{[49]}\) Against *E. coli*, the POC-THA and POC-HA/THA groups exhibited a slight improvement of antibacterial performance relative to POC-HA (Figure 5D). Consistent with the MIC studies, the inclusion of Ag NPs provided synergy with TA containing groups to increase the bacterial inhibition ratio from \(\approx 10\%\) for POC-HA alone and \(\approx 20\%\) for both POC-THA

Figure 3. SEM images of mineral deposited on POC-HA, POC-THA, POC-HA/THA 50/50, POC-THA/AgTHA 50/50 composites crosslinked at 80 °C for 3 days plus 120 °C under vacuum for another 3 days in 5 times concentrated simulated body fluid (SBF-5×). Insets are photos of the gross morphology of mineralized composites.
and POC-HA/THA to ≈40% for POC-THA/AgTHA (Figure 5D). The enhanced antibacterial activity in the Ag NP containing group is also shown in the contact-killing SEM images shown in Figure 5E. The composite antibacterial tests further supported the importance of including both TA and Ag NP in the development of ideal CTBCs with innate antimicrobial properties.

### 2.6. In Vivo Lumbar Fusion

To assess the in vivo biocompatibility and bone regeneration performance of CTBCs, porous POC-HA/THA and POC-THA/AgTHA scaffolds with a cuboid shape (10 × 10 × 20 mm, porosity: 65%, pore size: 250–425 µm) were fabricated and evaluated using a lumbar fusion model in rabbits. POC-HA scaffolds and autograft bone (AB) were used as material comparison and positive control, respectively, and a blank negative control group (Control) without any implantation in the bone defect was also used for comparison. The surgical procedure for the lumbar fusion defect is described in the experimental section of the Supporting Information. The application of the implanted scaffold as well as the assembly of the fixation tools is portrayed in Figure 6A with the corresponding X-ray images in Figure 6B.

After 8 and 12 weeks post-surgery, the treated bone tissue sections were harvested for visual observation and micro-computer tomography (micro-CT) analysis. Histological examination of the decalcified tissue sections was conducted by hematoxylin and eosin (H & E), Masson’s trichrome, Safranin O/Fast green staining and osteocalcin (OCN) immunohistochemical staining. Visual observation on the harvested tissue sections at 8 and 12 weeks after surgery (Figure S4, Supporting Information) indicates sufficient fixation of all the implanted grafts. Grafts were surrounded with newly-grown soft tissues, and no signs of infection were observed. In the 2D and 3D images reconstructed by micro-CT analysis (Figure S5, Supporting Information), the in-growth of newly-formed bone tissue from the edge of the bone implantation bed can be observed at 8 weeks after surgery, and increased at week 12 where the boundary between the implantation beds and the implanted bone grafts was less profound. Both visual observation and micro-CT reconstruction suggested enhanced bone tissue regeneration of the three composite treatment groups compared to the blank control group that contained distinct cavities not observed in other groups at week 8 and 12 (Figures S4 and S5, Supporting Information). Quantitative analysis on bone mineral density (BMD, Figure 6C) was consistent with the qualitative observations (Figures S4 and S5, Supporting Information). Although the BMDs of POC-HA/THA and POC-THA/AgTHA groups at week 8 and week 12 were much lower than that of the AB group, there was a significant increase in BMD compared to the blank...
control and POC-HA groups, especially at week 12 (Figure 6C). These results support the favorable osteoconductivity of CTBCs.

Both H & E and Masson's trichrome staining (Figure 7A,B) show new bone and fibrous tissue in-growth into the pores of the composite scaffolds, indicating that all the three kinds of scaffolds can induce de novo trabecular bone formation consistent with the micro-CT analysis. In the AB group, newly formed trabeculae can be found at week 12 (Figure 7B) confirming the favorable bone regenerative effects of autograft bone as the gold standard. Compared to POC-HA and blank control groups, more new bone formation was found in the POC-HA/THA and the POC-THA/AgTHA groups. In the blank control group, the bone defect cavity was mostly filled with fibrous tissue (Figure 7A,B). No significant inflammatory cell infiltration into the surrounding tissue and implanted materials was found at all tested time-points (Figure 7A). Cartilage was detected in the newly formed bone tissue of all five groups (Figure 7B), which is most evident by the Safranin O and Fast Green staining images where cartilage appears orange while bone tissue stains green (Figure 7C). For the AB group, cartilage

Figure 5. Bacteria survival on agar plates with different concentrations of HA, THA, and AgTHA against A) S. aureus and B) E. coli. C) Bacterial growth images on blank agar plates and agar plates with HA (10 mg mL$^{-1}$), THA (10 mg mL$^{-1}$) and AgTHA (5 mg mL$^{-1}$); D) Bacterial inhibition ratios (against S. aureus and E. coli after incubating 1 mL bacteria solution with one circular disk (diameter: 15 mm, thickness: 1 mm) of POC-HA, POC-THA, POC-HA/THA, POC-THA/AgTHA, or PLGA for 24 h and images of bacteria (E) attached on respective disks after 24 h. **p < 0.01.
tissue surrounded the implanted autograft bone at week 8 but was less evident by week 12 (Figure 7C) indicating that cartilage formation is an inherent part of early bone formation in the lumbar fusion process. More cartilage tissue was observed in the POC-HA/THA and POC-THA/AgTHA groups than in the POC-HA group (Figure 7C), suggesting that CTBCs may promote lumbar fusion through endochondral ossification. Osteocalcin (OCN) is a characteristic indicator for mature osteoblasts. Thus, OCN immunohistochemical staining can be used to reflect the osteoinductivity of bone implants by highlighting the number of mature osteoblasts in a given sample. At week 8, a large number of OCN-positive osteoblasts in the AB group was observed on the surface of the newly formed trabeculae between the edge of the bone defect and the graft, indicating the high presence and potential activity of osteoblasts (Figure 7D). In contrast, in the blank control group, very few OCN-positive osteoblasts were found relative to the treatment groups and were confined to the edges of the defect (Figure 7D). OCN-positive osteoblasts were similarly observed on the surface of the new trabeculae in the three composite bone grafts, suggesting that both CTBCs and normal citrate-based bone composites can promote the differentiation of osteogenic precursor cells, exhibiting favorable osteoinductivity.

The in vivo study results further confirm the biocompatibility of CTBCs as well as indicate favorable osteoconductivity and osteoinductivity of CTBCs to promote osteogenesis in lumbar fusion compared to blank control and POC-HA groups.

3. Conclusion

In conclusion, a family of citrate-based and tannin-bridged bone composites (CTBCs) was developed by reacting tannic acid (TA) with hydroxyapatite (HA) particles to strongly
adhere TA to the HA surface followed by chemical binding with poly(octamethylene citrate) (POC), the representative citrate-based biodegradable polymer, during the subsequent thermal crosslinking process. TA effectively bridged the inorganic and organic composite phases, improving the compression strengths of the TA containing composite scaffolds up to 325 MPa, exceeding the maximum strength of native bone. The surface coating of TA and in situ formed silver nanoparticles (Ag NP) on HA endowed the modified HA and CTBCs with considerable antimicrobial performance, enhanced biomineralization, favorable biocompatibility, and increased cell adhesion and proliferation. In vivo lumbar fusion using porous CTBCs further confirmed their enhanced osteoconductivity and osteoinductivity relative to blank and POC-HA controls. Immobilized TA also preserves multifunctional reactive groups for surface bioconjugation to further improve the bioactivity of CTBCs. The tannin-mediated adhesion and chemical reaction strategy provides a new paradigm to mimic the integrated organic/inorganic structure of natural bone, and to utilize the cell adhesion, biomineralization and antimicrobial functions of polyphenols for the promotion of bone regeneration lacking in currently available bone-mimetic materials. This versatile application paradigm may be widely expanded into other bio-related applications to develop high performance composites.

Acknowledgements

J.G., X.T., and D.X. contributed equally to this work.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomimetics, bone regeneration, citrate-based biodegradable polymers, composite materials, tannin-mediated adhesion

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.