Surface Modification of Chitosan-Grafted Hydroxyapatite and Biocompatibility of Its Composite Hydrogel Postprint

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Abstract

The HA surface was modified with chitosan to prepare chitosan-grafted nano-hydroxyapatite (HA-g-CS), which was then blended with chitosan to fabricate CS/HA-g-CS composite hydrogels. FTIR, TGA, and XRD analyses demonstrated that CS had been successfully grafted onto the HA surface with a grafting rate of 15.8%; SEM analysis revealed that the dispersibility of HA-g-CS in the CS matrix was significantly improved compared to HA, and the compressive strength of CS/HA-g-CS was 43% higher than that of CS/HA composite hydrogels. Biocompatibility evaluation results of CS/HA-g-CS indicated that both the cytotoxicity and implantation safety of the material met the requirements of national standards. This demonstrates that CS/HA-g-CS composite hydrogels can be applied as excellent scaffold materials in the field of tissue engineering.

Full Text

Surface Modification of Hydroxyapatite-Grafted-Chitosan and Biocompatibility Evaluation of CS/HA-g-CS Composite Hydrogel

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Abstract
To improve the compatibility between chitosan (CS) and hydroxyapatite (HA), surface modification of HA was performed using chitosan to prepare hydroxyapatite-grafted-chitosan (HA-g-CS) nanoparticles. FTIR, TGA, and XRD analyses confirmed that CS was successfully grafted onto the HA surface with a grafting ratio of 15.8%. SEM observations revealed that the dispersibility of HA-g-CS in the CS matrix was significantly improved compared to unmodified HA. The compressive strength of CS/HA-g-CS composite hydrogel was 43% higher than that of CS/HA composite hydrogel. Biocompatibility evaluation demonstrated that both the cytotoxicity and implantation safety of the material met national standard requirements. These results indicate that CS/HA-g-CS composite hydrogel can serve as an excellent scaffold material for tissue engineering applications.

Keywords: organic polymer materials, chitosan, hydroxyapatite, surface modification, composite hydrogel, biocompatibility

Introduction

Chitosan is a natural polymer material possessing inherent antibacterial properties, biodegradability, and excellent tissue compatibility, making it widely applicable in tissue engineering and regenerative medicine. In 2000, Chenite et al. developed a temperature-sensitive chitosan hydrogel by mixing acidic chitosan solution with β-glycerophosphate (β-GP). This hydrogel remains liquid at room temperature, allowing for the incorporation of therapeutic drugs, cells, and growth factors. Upon injection into the body, it forms a gel in situ as temperature increases, thereby exerting its therapeutic effects. Furthermore, due to its structural similarity to glycosaminoglycan (GAG) and hyaluronic acid found in articular cartilage, such chitosan hydrogels have been successfully applied in cartilage repair with promising clinical outcomes.

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA] constitutes the primary mineral component of human bone and teeth, accounting for approximately 60% of bone mass and over 90% of tooth enamel. HA exhibits excellent tissue compatibility and osteoinductive properties, making it the preferred inorganic material for bone repair. Composite materials combining HA with CS integrate the advantageous characteristics of both components—antibacterial activity from CS and osteoinductivity from HA—showing great potential as clinically applicable bone substitutes and repair materials. Consequently, CS/HA composites have

attracted considerable attention in the biomaterials field. However, as a filler material, HA nanoparticles possess high polarity and surface energy, leading to aggregation and poor compatibility with polymer matrices. Additionally, the weak interfacial bonding between HA and CS causes premature failure at the HA-CS interface upon implantation, resulting in rapid particle detachment and deterioration of hydrogel strength. To address this issue, we first modified HA with 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde, then grafted chitosan onto the surface (HA-g-CS). The CS/HA-g-CS hydrogel was prepared through physical crosslinking, and the modification effects were characterized using XRD, FT-IR, TGA, and SEM. The biocompatibility of CS/HA-g-CS hydrogel was systematically evaluated.

Experimental Methods

Materials and Reagents

The experimental reagents included: nano-hydroxyapatite with particle size of 100$±$20 nm (analytical grade, Aladdin); chitosan (molecular weight 100 kDa/400 kDa, deacetylation degree 85%, Sigma-Aldrich); 3-aminopropyltriethoxysilane (APTES) (Aladdin); glutaraldehyde aqueous solution (50%) (Aladdin); β-glycerophosphate sodium (Sigma-Aldrich); 0.25% trypsin (HyClone); 10% fetal bovine serum (HyClone); DMEM medium (HyClone); methyl thiazolyl tetrazolium (MTT, Sigma); L-929 mouse fibroblast cells; clean-grade SD rats (180-200 g); and Kunming mice (20-25 g).

Preparation and Characterization of HA-g-CS

Preparation of HA-g-CS: The synthesis proceeded in three steps. First, 1.4 mL of APTES was added dropwise to an ethanol-water solution (9:1 v/v) and hydrolyzed for 0.5 h, followed by addition of 3 g HA and reaction at room temperature for 8 h. The product was centrifuged, washed, and dried, designated as HA-APTES. Second, 140 mL of 1% glutaraldehyde aqueous solution was prepared, and 2.5 g HA-APTES was added and reacted at room temperature for 6 h. The resulting product was centrifuged, washed, and dried, designated as HA-APTES-GD. Third, 100 mL of 1% CS solution (molecular weight 100 kDa) was prepared, and 2 g HA-APTES-GD was added and reacted at room temperature for 8 h. After completion, the product was washed with dilute acid solution and deionized water, then freeze-dried to obtain HA-g-CS. The reaction mechanism is illustrated in [Figure 1: see original paper].

Characterization of HA-g-CS: X-ray diffraction analysis was performed using a D8 Focus X-ray spectrometer (Bruker, Germany) at 36 kV tube voltage and 20 mA tube current, with a scanning rate of 2°/min over a range of 5-80°. Fourier transform infrared spectroscopy was conducted using an EQUINOX FTIR analyzer (Bruker, Germany) with KBr pellet method to analyze chemical

composition and structure. Thermogravimetric analysis was carried out using a TG209F3-ASC thermogravimetric analyzer (NETZSCH, Germany) from 30°C to 800°C at a heating rate of 10°C/min to determine the amount of CS grafted onto HA.

Preparation and Characterization of Composite Hydrogels

Hydrogel Preparation: A 10 mL solution of 5% CS (molecular weight 400 kDa) was prepared. One gram of HA and HA-g-CS was separately added to the CS solution, followed by addition of 1 mL of 1 g/mL β-glycerophosphate sodium solution. After thorough mixing, the solutions were poured into molds with 1 cm diameter and placed in a 37°C water bath to form gels, yielding CS/HA and CS/HA-g-CS hydrogels respectively.

Hydrogel Characterization: For microstructural analysis, the two types of gels were freeze-dried using a freeze dryer (FD-1A-50, Shanghai Bilon Instruments), then cut into small pieces. The cross-sections were sputter-coated with gold for SEM observation. For mechanical testing, the gels were allowed to fully gel for 24 h before compressive strength measurement using a universal electronic testing machine (AG-1, Shimadzu, Japan) at a loading speed of 2 mm/min. Each sample group was tested in triplicate, and the compressive strength was calculated using the formula where Rc represents compressive strength, Nmax is the maximum load (N), and S is the surface area of the gel under force (m^2).

Biocompatibility Evaluation

Cytotoxicity Test: The MTT assay evaluates cytotoxicity through cell culture, reflecting molecular-level interactions between materials and cells with high sensitivity, quantitative capability, and operational simplicity. L-929 fibroblast cells were used in this study. Cell suspension at a density of 1$×10^{5}$ cells/mL was seeded into 96-well plates at 100 L per well. The experimental group consisted of CS/HA-g-CS gel mixed with cell suspension at a 2:1 ratio, the negative control group received 100 L of corresponding control solution, and the positive control group received 100 L of 0.64% phenol solution, with six replicate wells per group. After incubation at 37°C with 5% CO_2 for 24 h, MTT assay was performed on six wells from each group. The original culture medium was removed, 20 L of MTT solution was added, and incubation continued at 37°C for 4 h. After removing the supernatant, 150 L of dimethyl sulfoxide (DMSO) was added to each well, followed by 10 min of shaking. Absorbance at 492 nm (A_{492}) was measured using a microplate reader, with three measurements per well averaged to obtain the final absorbance value. The relative growth rate (RGR) was calculated as $(A/A_0) \times 100\%$, where A is the experimental group absorbance and A_0 is the negative control absorbance. Toxicity grading was as follows: RGR 100% = Grade 0; 75-99% = Grade I; 50-74% = Grade II; 25-49% = Grade III; 1-25% = Grade IV; 0% = Grade V.

Muscle Implantation Test: Six clean-grade SD rats were used. Twenty-four

hours before surgery, 1.5 cm × 1.5 cm areas of fur were shaved on both sides of the spine, avoiding skin damage. The CS/HA-g-CS gel was processed in a laminar flow hood into cylindrical shapes measuring 3 mm in diameter and 10 mm in length with smooth edges. Anesthesia was induced by intraperitoneal injection of 3% pentobarbital sodium at 30 mg/kg. Under sterile conditions, two implantation sites were selected approximately 1 cm from the spinal midline on both sides. Muscle tissue was separated to create muscle pockets, with one implant placed on each side. The left side received the experimental material while the right side served as a blank control (incision without implantation). Gentamicin was administered intramuscularly for three consecutive days post-surgery to prevent infection. Evaluation included monitoring animal activity, feeding, and wound healing. Rats were sacrificed at 1, 4, and 12 weeks post-implantation (three rats per time point). Tissue samples were collected, processed into routine histological sections, stained with hematoxylin-eosin (HE), and examined under light microscopy to assess inflammatory reactions and capsule formation around implantation sites.

Results and Discussion

XRD Analysis of Hydroxyapatite-Grafted-Chitosan (HA-g-CS)

[Figure 2: see original paper] presents the XRD patterns of HA and HA-g-CS. The diffraction peaks of HA-g-CS align with those of HA, indicating that grafting CS did not alter the crystal structure of HA. However, the diffraction peak intensities of HA-g-CS decreased compared to HA, resulting in reduced crystallinity. According to literature, HA crystals in human bone tissue exhibit relatively weak crystallinity. The surface modification approach described here reduces the crystallinity of HA by grafting CS, making HA-g-CS more similar to the crystalline characteristics of HA in human bone and thereby promoting favorable osteoconductive properties for bone defect repair. Additionally, CS possesses excellent biodegradability, tissue compatibility, and antibacterial properties. Grafting CS onto the HA surface enhances the overall performance of HA-g-CS, particularly by significantly improving interfacial compatibility between HA-g-CS and the CS matrix, which facilitates preparation of HA-g-CS/CS composites.

FT-IR Spectroscopy of HA and HA-g-CS

As shown in [Figure 3a: see original paper], the FT-IR spectrum of HA displays -OH stretching and bending vibrations at 3426 cm^{-1} and 1637 cm^{-1}, respectively, and PO_4^{3-} antisymmetric stretching at 1094 cm^{-1}. In the HA-g-CS spectrum ([Figure 3b: see original paper]), the -OH stretching peak at 3400 cm^{-1} broadened significantly compared to HA, representing the superposition of -OH and N-H stretching vibrations. A new methylene stretching vibration peak appeared at 2925 cm^{-1}, indicating successful $-NH_2$ modification of the HA

surface in the first reaction step. Furthermore, as shown in [Figure 3d: see original paper], a doublet emerged at 1640 cm^{-1} after CS grafting, corresponding to -OH bending vibration and the characteristic C=N absorption of Schiff base structures. These results confirm successful grafting of chitosan onto the HA surface.

Thermogravimetric Analysis of HA and HA-g-CS

Thermogravimetric curves for HA and HA-g-CS are presented in [Figure 4: see original paper]. Pure HA exhibited minimal weight loss of approximately 3%, likely due to evaporation of adsorbed water. In contrast, HA-g-CS showed significant weight loss between 250-550°C ([Figure 4b: see original paper]), attributed to decomposition of grafted CS. This result further corroborates successful CS grafting onto the HA surface. Based on the weight loss difference between HA-g-CS and HA, the grafting amount of CS on HA surface was calculated to be 15.8%.

SEM Observation of CS/HA and CS/HA-g-CS Xerogels

[Figure 5: see original paper] shows SEM images of CS/HA and CS/HA-g-CS composite xerogels. In CS/HA ([Figure 5a: see original paper]), HA exhibited non-uniform dispersion within the CS gel matrix with block-like agglomerations exceeding 20 mm in diameter, resulting from the high surface energy of nano-hydroxyapatite. Conversely, CS/HA-g-CS showed no obvious HA agglomeration, with HA-g-CS particles distributed uniformly throughout the CS matrix. This improvement arises because the CS molecular chains on the HA surface prevent particle aggregation, while the grafted CS enhances compatibility between HA-g-CS and the CS matrix, achieving uniform dispersion.

Mechanical Strength of Hydrogels

The compressive strengths of CS/HA and CS/HA-g-CS gels are listed in . The CS/HA gel exhibited a compressive strength of 8.57 kPa, whereas the CS/HA-g-CS hydrogel showed a compressive strength of 12.27 kPa, representing a 43% improvement. In CS/HA gels, HA particles should theoretically facilitate stress transfer and energy dissipation. However, the high surface energy of HA leads to agglomeration and defect formation, which cause stress concentration during compression and reduce composite strength. After modification, HA-g-CS demonstrates good compatibility with the CS matrix, enabling uniform distribution without aggregation. This allows the composite gel to absorb external energy uniformly during compression, resulting in superior mechanical performance that prevents premature strength loss during application.

Biocompatibility Evaluation

Cytotoxicity Test: [Figure 6: see original paper] illustrates L-929 cell morphology after 24 h culture. Both the experimental group and negative control

showed good cell adhesion and uniform morphology, with spindle-shaped or polygonal cells exhibiting strong refractivity and visible cell division. In contrast, the positive control group displayed shrunken cells with reduced volume and partial cell lysis. MTT test results in revealed that CS/HA-g-CS gel exhibited Grade 0 cytotoxicity, meeting the requirements for biomaterials and demonstrating non-cytotoxic characteristics.

Implantation Safety Evaluation: In vivo implantation tests evaluate local toxic reactions to implanted materials. Postoperative observations revealed normal feeding and activity in all rats, with no inflammatory signs such as redness or exudate at incision sites, indicating good healing without implant rejection. Histological examination ([Figure 7: see original paper]) showed: at 1 week post-implantation, lymphocyte infiltration and macrophage presence were observed around the implant material without significant fibrous encapsulation, likely due to foreign body reaction and surgical trauma; at 4 weeks, mild chronic inflammation was evident with few lymphocytes, multinucleated giant cells, and fibroblast proliferation with fibrous capsule formation; at 12 weeks, minimal inflammatory cells remained with no progression of inflammation and established fibrous encapsulation, indicating stable material-tissue integration.

Conclusion

Hydroxyapatite was modified using 3-aminopropyltriethoxysilane and glutaraldehyde as an intermediate to prepare chitosan-modified hydroxyapatite (HA-g-CS). CS was successfully grafted onto the HA surface with a grafting ratio of 15.8%. The introduction of CS onto HA surface reduced crystallinity without altering the HA crystal structure, making HA-g-CS more similar to HA in human bone tissue and enhancing osteoconductive properties. The CS/HA-g-CS composite hydrogel exhibited 43% higher compressive strength compared to CS/HA gel. Furthermore, both in vitro cytotoxicity and implantation safety evaluations of CS/HA-g-CS hydrogel met national standards, demonstrating its potential as a scaffold material for tissue engineering applications.

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