Preparation and Characterization of Porous Collagen/Hydroxyapatite/Gum Arabic Composites[#]

Wenpo Feng[1], Keyong Tang[1], Xuejing Zheng[1], Yuanming Qi[2], Jie Liu[1]*

[1] College of Materials Science and Engineering, Zhengzhou University, Zhengzhou 450052, Henan, P.R. China.

[2] Bioengineering Department, Zhengzhou University, Zhengzhou 450052, Henan, P.R. China.

Abstract: The aim of the present study was to synthesize collagen/hydroxyapatite (Col/HA) composite with similar composition and structure to that of natural bone. Collagen and analyzed grade $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ were used as the raw materials to synthesized Col/HA composite in situ. Gum Arabic, a kind of plant-polysaccharides, was used to improve the property of the artificial bone matrix. Solid-liquid phase separation method was used to shape 3D porous structure for cell growing. Influences of the preparation conditions on the microstructure of composite and the bonding formation of HA particles with collagen fibrils were characterized by X-ray diffraction and scanning electron microscopy (SEM). The cytotoxicity was tested on co-cultures of fibroblast in the tissue culture laboratory. The structure evaluation of the Col/HA/Gum Arabic composite shows that the HA particles homogenously existed on the skeleton of the collagen-gum Arabic network and bonded tightly to the fibrils. The test of contact cytotoxicity shows a very good biocompatibility of the biomaterial. Higher cohesivity of the biomaterial may find a potential application as bone implant material.

Key words: collagen; hydroxyapatite; gum Arabic; artificial bone

1 Introduction

Bone is the most implanted tissue after blood, and its major solid components are collagen, hydroxyapatite, glycoprotein, and proteoglycan et al [1]. Hydroxyapatite (HA: $Ca_{10}(PO_4)_6OH_2$) is widely used for bone implant and bone cement applications due to its compositional and biological similarities to native tissues[2, 3]. Collagens are probably the most abundant proteins in the vertebrate body [4]. However, the biocomposites of collagen and hydroxyapatite alone do not have adequate mechanical properties for various biomedical applications including scaffolding tasks. Compounding of collagen and hydroxyapatite with polymeric binders may improve the durability and mechanical properties.

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[*] Corresponding author. Tel.: +86-371-67763216. E-mail: keyongtang@yahoo.com.cn

Gum Arabic (Gum A), a kind of plant-polysaccharides, is a complex mixture of calcium, magnesium, potassium salt of Arabic acid. There are usually little other cations, such as Na^+, Mn^{2+}, Fe^{2+}, Zn^{2+} etc present in it. The gum macromolecular structure is based on a highly branched framework of β-1,3-and β-1,6-linked D-galactopyranose unites, to which are attached short side-chains of galactose and arabinose, with these terminated, in turn, by D-glucuronic acid (in neutralized salt form) and/or L-rhamnose. This gum can interact with some proteins at certain pH values to give complex co-acervates [5]. In the present paper, we used it as a cross-linking agent to improve the property of the artificial bone matrix.

2 Materials and Procedures

2.1 Materials

Collagen was extracted from rabbitskin with the method of acid dissolution and enzyme digestion. Gum A was obtained from Tianjin Guangfu fine chemical research institute, China. Acetic acid glacial, $Ca(NO_3)_2 \cdot 4H_2O$ and Ammonia solution (25%) were purchased from Tianjin Tianli Chemical Reagents Ltd. China. $(NH_4)_2HPO_4$ was obtained from Zhengzhou Paini chemical reagent factory. NaOH was purchased from Tianjin No.3 chemical reagent factory, Tianjin, China. $Na_2HPO_4 \cdot 12H_2O$ was from Shantou Jinsha chemical plant Ltd., Shantou, China. All the reagents were analytical pure.

2.2 Preparation of Collagen Neutral Solution

Collagen was dissolved in 0.5mol/Lglacial acetic acid, $Na_2HPO_4 \cdot 12H_2O$ was added to 0.02 mol/L at last. And then, the pH was adjusted to 7.2 using NaOH. All the processes were carried out under 10℃.

2.3 Synthesis of Collagen/ Hydroxyapatite (Col/HA) biocomposite in situ

Collagen neutral solution was added to 0.72M $(NH_4)_2HPO_4$, 1.2M $Ca(NO_3)_2 \cdot 4H_2O$ solution (1:1, v/v) slowly and the pH was adjusted to 7.2 using $NH_3 \cdot 2H_2O$. In the process, it was stirred under 10℃ for 2 hours, and incubated at 35℃ for 20 hours. After being deionized water washed, collected, and centrifuged, the Col/HA mixture, as a hydrogel, was obtained. A solid-liquid phase separation technique was used to generate the highly porous Col/HA composite scaffolds. The mixture was cooled to -20℃ for a day to conduct solid-liquid phase separation, and then the solvent was removed by freezing-drying.

2.4 Synthesis of Collagen/ Hydroxyapatite/Gum Arabic (Col/HA/Gum A) biocomposite in situ

This procedure was the same as 2.3 except the addition of some Gum A solution, Na_3PO_4 and $Ca(NO_3)_2 \cdot 4H_2O$ to the collagen neutral solution. The hydroxyapatite thus synthesized in situ in the Col/Gum A binder.

3 Characterization

3.1 Scanning electron microscopy (SEM)

The samples were sputter coated with gold and viewed under an FEI-quantum 200 SEM operated at 20 kV for morphology and microstructure evaluation.

3.2 X-ray diffraction (XRD)

X -ray diffraction analysis was performed using a Philips Diffractometer at room temperature. In all these cases, Cu K_α radiation from a Cu X-ray tube (run at 40 mA and 40 kV) was used. These samples were scanned in the Bragg angle, 2θ, rang from 20- 70°. A natural bone (from chicken) was observed as a control.

3.3 Cytotoxicity analysis

Cytotoxicity analysis was carried out according to GB/T 16886.5-2003/ISO 10993-5: 1999 Biological evaluation of medical devices- Part 5: Test for in vitro cytotoxicity and GB/T16175-1996 Organic silicon material for medical use- Biological evaluation test methods, the cytotoxicity potential of the biocomposite was evaluated by co-culturing MEF-WT cells (wild type mouse embryonic fibroblasts) for a week utilizing an indirect contact method; cell viabilities were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay at 2, 4, and 7 days[6, 7]. This assay is based on the reductive cleavage of MTT (a yellow salt) to formazan (a purple compound) by mitochondrial dehydrogenase of living cells.

3.3.1 Sample preparation for cytotoxity assays

Samples were sterilized by brief treatment with 70% ethanol. Appropriate samples were immersed in the Dulbecco's minimum essential medium (DMEM, Gibco) (sample/medium: 0.2g/mL) and were maintained in incubator (MCO-15AC, SANYO) at 37 ℃ with 5% CO_2 for 24 h. Then the resultant solutions were removed and the pH was measured.

3.3.2 Cell cultivation

MEF-WT cells, conventional laboratory-based preservation projects, were trained in a culture media with 100U/mL of streptomycin, 100U/mL of penicillin G, 25mg/mL of ascorbic acid, 4.5g/L glucose-including DMEM plus 10% fetal calf serum(FCS; Sijiqing, Hangzhou). All cultivation was maintained at 37℃ in an incubator (MCO-15AC, SANYO) containing 5% CO_2.

3.3.3 Cellular viability/activity by MTT assay

Check 200μL cell suspension (5000 /mL, join the 96-well plates in a normal training day; continue to train with leaching liquor. After 1 day, 3 days, 6 days of incubation, the supernatant in each well was replaced with 200 μL fresh DMEM medium supplemented with L-glutamine and 10% FBS, and 25 μL MTT (5 mg/mL in PBS). After 4 hours of incubation with CO_2 at 37℃, the reaction solution was carefully removed from each well and 200 μL dimethyl sulfoxide (DMSO, Tianjin No.3 Chemical Reagent Factory) was added. The plates were gently agitated until the formazan precipitate was dissolved. The absorbance was measured with a spectrophotometer (Bio-Rad Mode 680) at a wavelength of 570 nm. The background (control with no cells) was subtracted from all samples. Normal control group was a normal training, and the other containing 0.64% phenol is a negative control group.

The relative growth rate (RGR) = (OD value of experimental group / OD value of normal control) × 100%. The evaluation of cytotoxicity of composite materials is in accordance with the provisions of GB/T16175-1996, ISO 10993-5:1999 evaluation methods.

4 Results and discussion

The morphology of the composite scaffolds were examined with SEM. Fig. 1 illustrates a cross sectional view of the microstructure of scaffolds. The HA particles are mechanically interlocked with Collagen or Col/Gum A fibrils. The fibrils surround the HA particles to fully or partially cover the particle surface. The HA crystals deposited in the biopolymer, reunion into a beam, formed a porous structure.

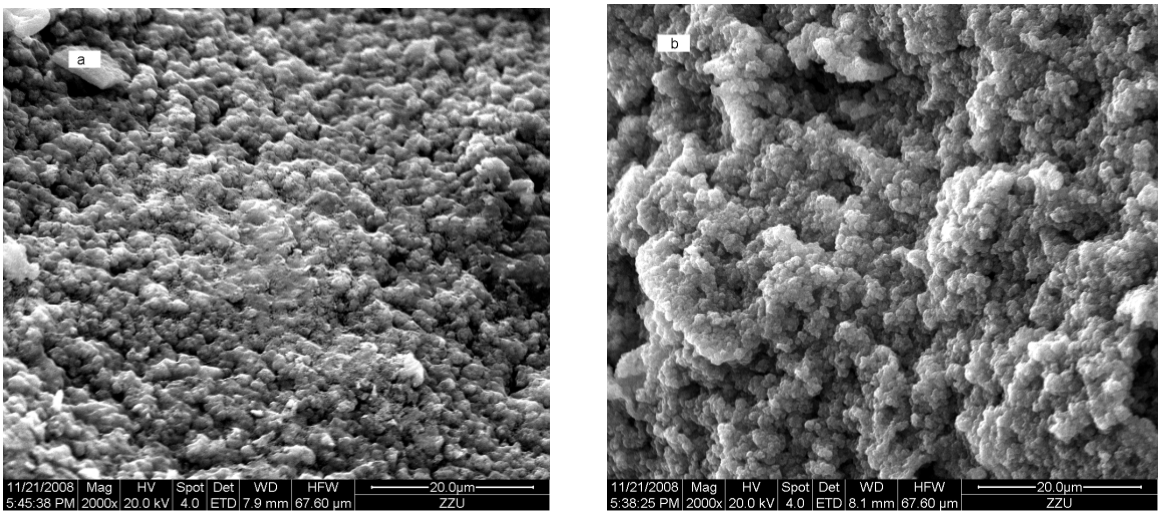

Fig. 1 Cross-sectional views of the interior of Col/HA (a) and Col/HA/Gum A (b) scaffolds

The crystallographic structure of the HA crystals was investigated by X-ray diffraction. Fig. 2 is a XRD

pattern of a chicken bone (natural bone) and the composite materials. Diffraction peak in accordance with the location and d value of XRD crystal check card showed that the standard diffraction peaks of complexes are in line with the characteristic peaks of HA crystals. Inorganic phases of the composite materials were mainly of HA (about 35%), while there might be a number of hydroxyl replaced by carbonate (about 33%) and chlorine (about 30%). Meanwhile, there is some iron (about 2%) in the materials. All of them are natural components of bone [1]. Carbonate might be added during mixing; iron might come from Gum A and chlorine from collagen solution. Collagen was extracted from rabbitskin with the method of acid dissolution and enzyme digestion. NaCl was employed in the extraction. It also can be found that the positions, quantity of composite materials diffraction peak are similar to natural bone [8]. Shown in the figure are mainly hydroxyapatite's crystal planes.

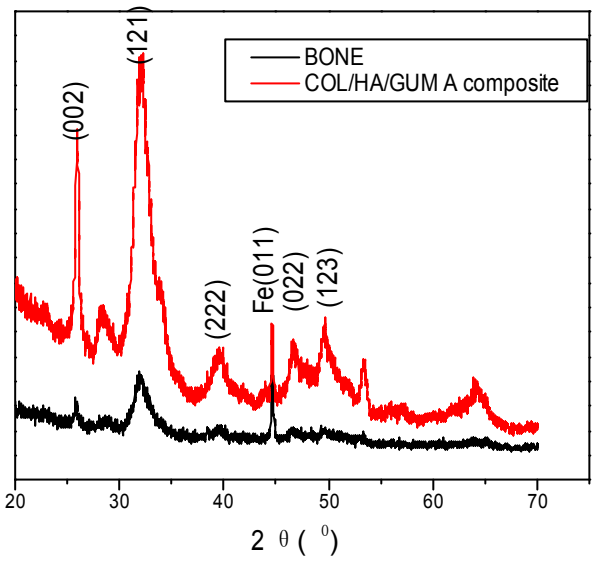

Fig. 2 The XRD patterns of composite materials and the natural bone

In 7 days of cell cultivation, cells grew in a better state and had a higher proliferation. OD value of MEF-WT cell culture was shown in Tab. 1. It indicated that the largest number of cells appeared on the fourth day, followed by the lower. This was probably because the cells had been covered, but there was no passage, resulting in the death of some cells. At the same time, we also found that the RGR was more than 75%, and no cytotoxicity was found.

Collagen is the major component of extracellular matrices, such as tendons, ligaments, skins and scar tissues in vertebrates [2]. HA is one of the major constituents of the inorganic component in human hard

tissues (bones and teeth), and it is one of the most common biomaterials studied in bone tissue engineering because of its good biocompatibility. Gum A is a kind of plant-polysaccharides, widely used for pharmaceuticals, cosmetics, lithography, food industries, miscellaneous purposes, etc. In the synthesis of composite, pH value was 7.2, similar to the human body to avoid excessive NH_4^+. After synthesis, the full cleaning eliminated the effects of NH_4^+.

Tab. 1 OD value of MEF-WT cell culturing for 2, 4 and 7 days

Group	OD value		
	2d	4d	7d
Normal control	0.1058±0.006	0.4200±0.053	0.3857±0.058
Positive control	0.1178±0.006	0.4905±0.018	0.4520±0.025
Composite [a]	0.0807±0.006	0.3433±0.025	0.3267±0.006
Composite [b]	0.0943±0.018	0.3897±0.056	0.3267±0.006

[a] 100% composite reaction solution, [b] 50% composite reaction solution

5 Conclusions

A composite scaffold of Col/HA/Gum A was prepared and investigated in the paper. Col/Gum A and HAP were combined homogenously through the in situ synthesis of nano-HA using the wet chemical method. The nano-HAP particles bonded to the organics tightly. The composite scaffolds showed better biocompatibility. Cells grown were in a better state and had a higher proliferation. The results indicated that the composite material was expected to find applications for bone repairing.

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