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Research Article

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Preparation, Microstructure and Cytotoxicity Research on Hydroxyapatite/Chitosan-Chondroitin Sulfate

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Abstract In this paper, the nano-hydroxyapatite/chitosan-chondroitin sulfate (Hap/Cs-Chs) composite materials with different mass ratios were prepared by liquid co-precipitation method. On the basis of the microstructure, phase and compressive strength of the composite materials were investigated respectively. The safety of composite materials was evaluated by MMT method. The results showed that Hap/Cs-Chs composite materials exhibited excellent mechanical performance and good biocompatibility. The nano-Hap particles were dispersed uniformly in the organic phase Cs and Chs with relatively weak crystallinity. It is combined with relatively closely between Hap particles and Cs-Chs. The particle size is about 40nm-100 nm with spherical shape. The crystallization, particle size, and dispersion of Hap are influenced directly by the mass ratio of Hap and Cs-Chs. The uniformity, compactness, and thermal stability can reach the optimum when mass ratio is 50/50. EDS analysis indicated that Ca/P ratio is around 1.80±0.03, belonging to the calcium-rich type of Hap. The Hela cell toxicity experiment confirmed that the composite material has good biocompatibility and safety, and the physical and chemical performance can meet the requirements of bone tissue engineering materials.

Keywords nano-hydroxyapatite, chitosan, chondroitin sulfate, composite materials, microstructure, cytotoxicity

1. Introduction

Hydroxyapatite (Hap) with rich surface properties, good bioactivity, compatibility and bone conductivity, which is the main inorganic component of human bones and teeth, has been widespread concern in recent years [1-2]. However, the application of Hap in bone tissue engineering has been limited due to its brittleness and low degradation rate [3-4]. Therefore, it is a hotspot for preparation of functional composite materials satisfying bone tissue engineering performance and evaluation of various properties of materials for the composite materials [5-6]. According to the present research, in order to overcome the shortcomings of Hap's brittleness and extremely low degradation rate, it is an effective means to improve the comprehensive performance of materials by the introduction of other phase material for modification, compound [7-8]. Among them, Hap/Cs composite is considered as the most promising bone tissue composite because of its good biocompatibility and biodegradability and other advantages [9-10]. However, the study found that hydroxyapatite/chitosan composite has the problems of low interfacial bonding between Hap and Cs, uneven dispersion of particles, poor brittleness and strength [11-12]. To overcome this limitation, more and more researchers introduce other substance for modifying Hap/Cs composite materials. Chondroitin sulfate (Chs) is a macromolecule classified as glycosaminoglycan found in natural cartilage and other tissues. Chs has a number of biological properties for tissue engineering including non-toxic, anti-inflammatory, adhesive, thickening, degradable, promoting cartilage regeneration and good biocompatibility [13-17], and has been widely used in the biomedical field. As the third phase, it can increase the interface combination between Hap and

Cs, while giving the composite material excellent mechanical, chemical and biological properties. Undoubtedly, it has an important theoretical significance and broad prospects. In recent years, many scholars have done a great deal of research on Hap/Cs-Chs composite materials, most of them mainly focus on their mechanical property, material preparation. However, there are few studies on microstructure and biological safety evaluation of materials. In this paper, nano-Hap/Cs-Chs composite materials with different weight ratios were prepared by a liquid co-precipitation method. Based on the analysis of phase, composition, microstructure and mechanical properties of composites, the safety of composites was evaluated by MMT method. Which provides a good experimental basis for the practical application of this series of composite materials.

2. Experimental Section

2.1. Reagent and Instrument

Main reagents: Hela cell line (Institute of Materia Medica, Chinese Academy of Sciences); RPMI-1640 medium (GIBCOcompany); RPMI-1640 culture medium powder and trypsin were purchased from Gibco company; fetal bovine serum (FBS) was obtained from Hyclone company, dimethyl sulfoxide was purchased from Sigma company; MTT was purchased from Amresco company; Chitosan (Cs) with 90 percent degree of the deacetylation, Chondroitin sulfate were obtained from Weifang Ruiguang Chemical Co., Ltd;. Analytical grade $Ca(NO_3)_2{\cdot}4H_2O$, $(NH_4)_2HPO_4$, NaOH, acetic acid (HAc).

Main instruments: Y-2000 Automatic X-ray diffraction (Dandong Radiative InstrumentGroup Co. Ltd); JSM-7500F (Field emission scanning electron microscope) with a Thermo Noran X-ray energydispersive spectrometer(EDS);Material compressive strength was measured using JWE-50 universal testing machines; Cytotoxicity tests were performed by BD FACS Calibur flow cytometry (US BD Corporation).

2.2. The Preparation of Compound Materials

In this experiment, cthe solution of $Ca(NO_3)_2{\bullet}4H_2O$ and $(NH_4)_2HPO_4$aqueous solution were prepared with near 1.67 Ca/P stoichiometric ratio. The mass ratio of Cs and Chs were fixed at 5:1. Hap/Cs-Chs composite materials with quality ratio 70/30, 50/50, 40/60 and 30/70 were prepared.

The specific methods are as follows: Cs powder and Chs were dissolved in 2 wt% HAc and deionized water respectively and mixed completely stirring for 1h. $Ca(NO_3)_2$ solution was dispersed in the above solution to form a homogeneous solution.. Then $(NH_4)_2HPO_4$solution was dropped into the mixture with vigorous stirring at 40 °C for 6 h, and the pH was adjusted to 10 with NaOH solution. The obtained white slurry was aged for 24 h at room temperature, and then the precipitate was filtered, washed, and then put them into the mould to prepare composite samples of different proportions.

2.3. Cytocompatibility evaluation

Hela cells were cultured by adherent culture methods in RPMI1640 cell culture medium with 10% heat-inactivated fetal bovine serum, 0.2% $NaHCO_3$, and 1% penicilli–streptomycin. The cells were maintained at 37°C under 5% CO_2 with culture medium changed every 2 days. When cell density reached up to 80%, the cells were cultured with trypsin enzyme-digesting technique and passaged with the ratio of 1□2. Cells were subcultured every 3 to 4 days. Cells in logarithmic growth phase were used in MTT assay. The preparation of the material leaching solution was carried out according to the Biological Evaluation of Medical Devices (ISO10993.5) and related literature [18-21]. The absorbance (OD) value was measured by MTT method at a wavelength of 490 nm under the automatic microplate spectrophotometer (ELX-800). The survival rate of the blank experimental cells was set at 100% and used as a reference. The cell viability of the samples under different concentrations of samples (100, 50, 25, 12.5, 6.25mg/mL) was calculated by following formula:

Cell Relative Growth Rate RGR (%) = (Test Group OD / Negative Control Group OD)×100 %

Toxicity of cells was assessed by the cell relative growth rate. When RGR≥80%, the sample showed no toxic effects on cell proliferation .Otherwise, it has a toxic effect on cell proliferation

3. Results and Discussion

3.1. XRD Analysis

Figure 1 shows the XRD patterns of Hap/Cs-Chs composite materials with different mass ratio. As can be seen from Figure 1, the characteristic diffraction peaks of Hap appear at 2θ=26°, 31.8°, 32.3°,32.9°, 39°, which meets with standard ($Ca_{10}(PO_4)_6(OH)_2$, PDF file No: 72-1243). The base-line of spectra is high, and the peak shape is wide, no other crystalline phases could be detected in the XRD patterns. It shows that Hap is a weak crystalline state in the composite, which is similar to the natural bone [14]. With the content of amorphous Cs-Chs increasing, the relative intensity of the Hap diffraction peak decreases gradually and the diffraction peak of chitosan appeares at 2θ=20°.This indicates that there is interaction between Hap and Cs Chs. The change of Cs-Chs amount does not affect the formation of hydroxyapatite phase in the composite material, but it affects the crystallinity and particle size of Hap.in composite materials. The characteristic diffraction peaks of chondroitin sulfate did not appear in Figure 1, because Chs is noncrystalline phase and less amount in the composite.

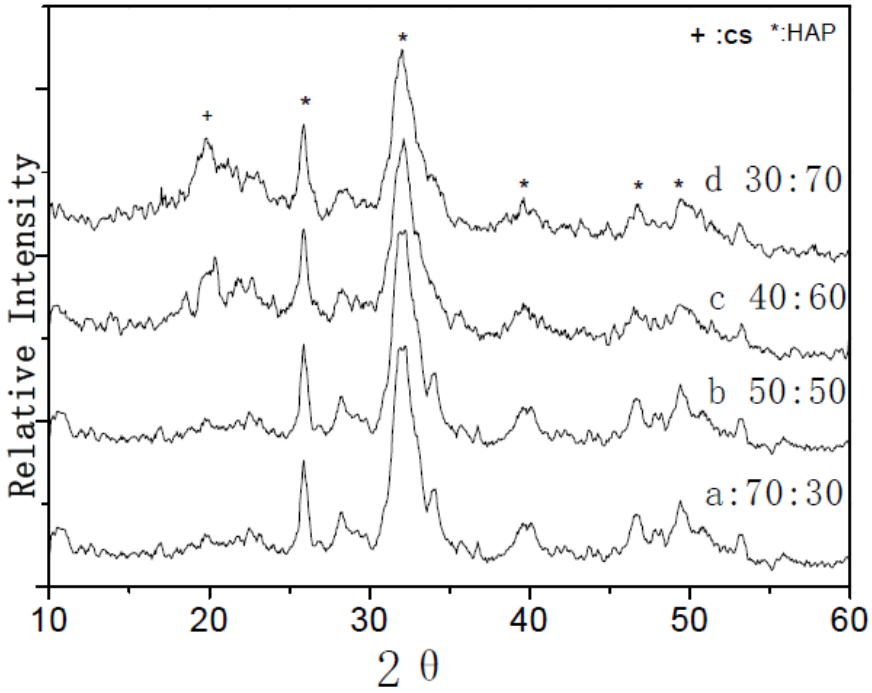

Figure 1: XRD patterns of n-Hap/Cs-Chs composites materialsat different mass ratios

3.2. SEM and EDS analysis

The microstructure and chemical composition of the composites with different mass ratio were observed by SEM and EDS. As shown in Figure 2(a) and 2(b), the Hap particles can be homogeneously dispersed with Cs and Chs matrix with good density, and the inorganic phase Hap bonding with organic phase in composite material. There are a lot of small pores in the composites, which are very helpful for bone cell metabolism of nutrients and moisture transfer. Figure 2 (c), (d), (e), (f) show the microstructure of different mass ratio of Hap/Cs-Chs composites materials. The uniform distribution, good crystallinity and near-spherical shape of Hap particles in the composite are showed by observing SEM. Moreover, the different mass ratio of Hap/Cs-Chs has direct influence on Hap size and distribution. When the mass ratio of Hap/Cs-Chs is 70/30, the composite particles are relatively loose and the size is about 40nm. When the mass ratio of Hap/Cs-Chs is 50/50, the particles were featured of good dispersion, high porosity and the particle size of Hap is about 45nm. It found that microstructure of the composite becomes more dense as Cs-Chs mass ratio increased, while the porosity and the number of Hap particles decreased. The reasons may be that during the co-precipitation process, nano hydroxyapatite particles were firstly formed, and Cs-Chs had

relate to the adhesive effect of the nano hydroxyapatite particles, resulting in the increase of particle size in microstructure. Especially, the pH value of the compound system was changed instantaneously when the Chs was added, which affected hydroxyapatite particles formation and crystal growth .The specific reasons and composite mechanism need further research.

Furthermore, elementary analysis (EDS) results show an average Ca/P atomic ratio of 1.85 and it can be observed the presence of trace elements Na in composites materials from Figure 2. It belongs to the rich calcium type of Hap and the existence of sodium is good for natural bone..

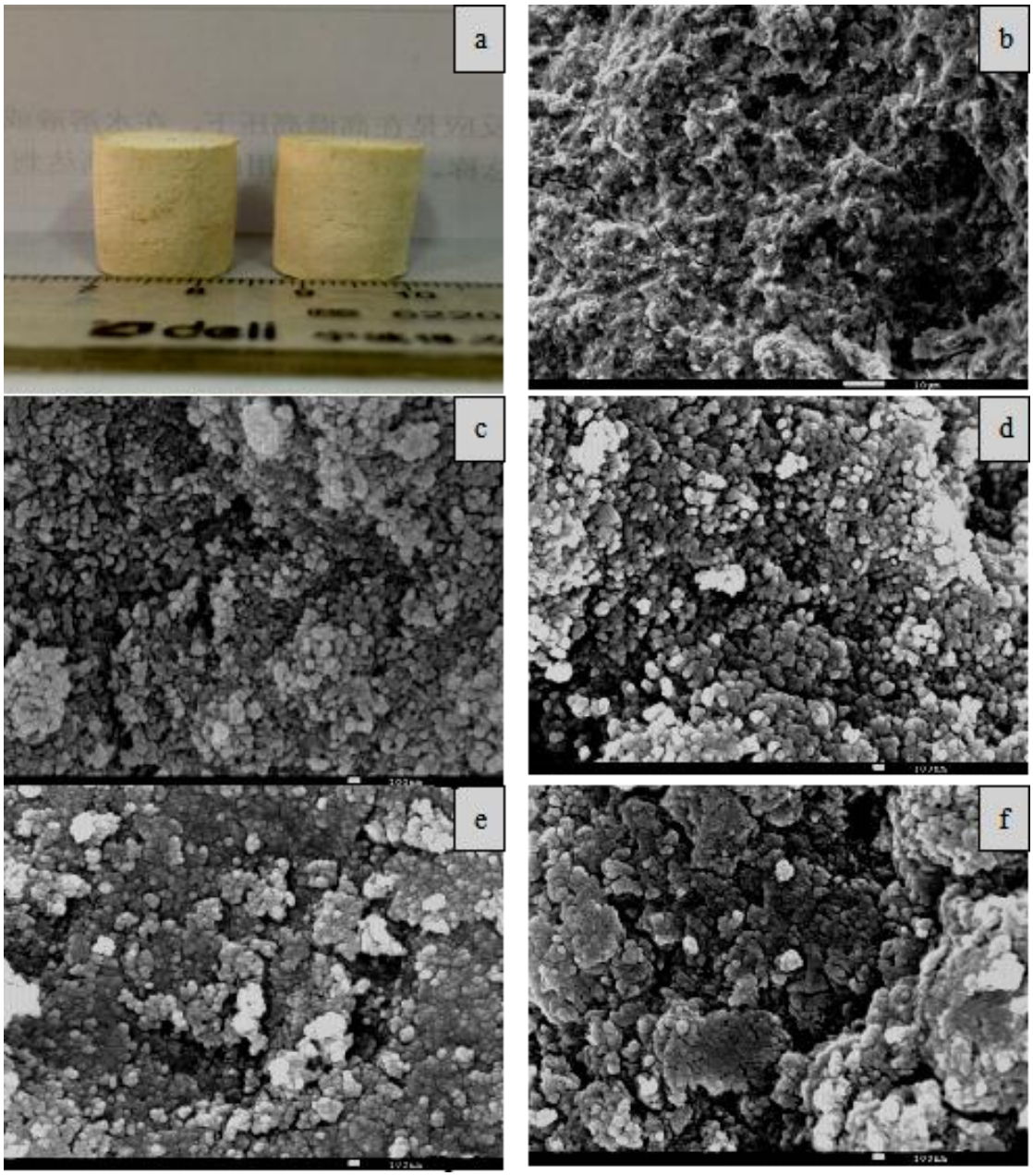

Figure 2: SEM photos of composites materials
(a: actual photo; b: cross section; c:70/30; d:50/50; e:40/60; f:30/70)

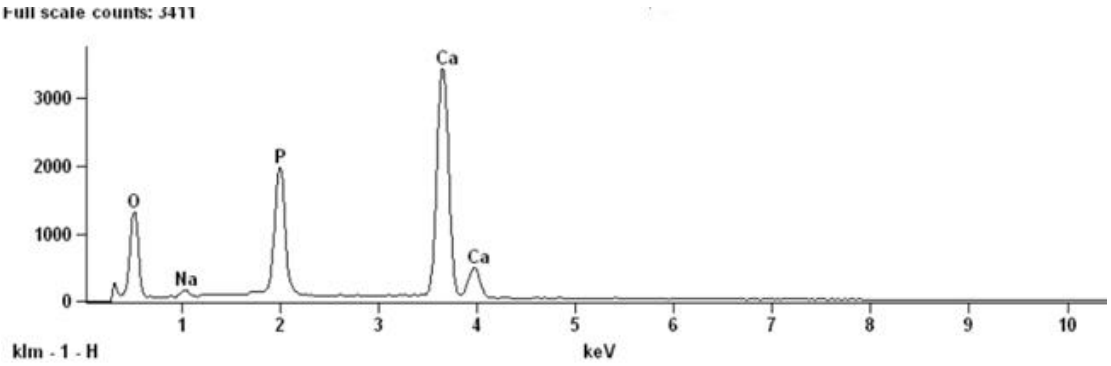

Figure 3: EDS spectrum of Hap/Cs-Chs composites materials

3.3 Mechanical Testing

The microstructure of materials is closely related to the macroscopic physical properties. The compressive strength of the composites was measured using a computer-controlled Universal Testing Machine (JWE-50). Each group with different mass ratio was tested 3 times, then calculating the average error. The results are shown in table 1. As can be seen from table 1, the mechanical properties of composite materials is directly affected by the ratio of Hap/Cs-Chs. When Hap content is 70%, the compressive strength of composite is the smallest (35.2 Mpa). When Hap/Cs -Chs is 50/50, the compressive strength can be achieved to peak value about 45.3MPa. The toughness of the composite is improved with a further increase in Cs-Chs, but the compressive strength of the composite decreases gradually due to the decrease of the inorganic phase. The results are agreement with the results of microstructural analysis.

Table 1: Compressive strengths of composites materials at different mass ratios

Hap/Cs-Chs	Compressive strength (MPa)
70/30	35.2±0.2
50/50	45.3±0.1
40/60	40.6±0.3
30/70	38.2±0.2

3.4. Cytotoxicity tests

Table 2 shows experimental results of Hela cytotoxicity test by MTT method after 24, 48 and 72 hours. As can be seen from the table, the extract of the composite had no obvious toxic effect on the cells and showed high cell proliferation rate (RGR>90%). The toxicity graduation of the Hap/Cs-Chs tends to 0 with the extract concentration decreasing and culture time increasing. According to cell relative growth rate and toxicity grading standards [22-24], the toxicity rating of the experimental materials ranged from 0 to1, indicating that the Hap/Cs-Chs materials were not toxic to the Hela cells. The results show that the composite has good cell compatibility and safety to fit the biological requirements for bone tissue engineering materials.

Table 2: Toxicity experimental results of Hap/Cs-Chs composite materials on Hela cell

Extract Mass Concentration (mg/mL)	Absorbance OD			Cell Relative Growth Rate (RGR %)			Toxicity Grade		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
100	0.3244±0.0014	0.3918±0.0017	0.4805±0.0017	96	95	96	1	1	1
50	0.3245±0.0009	0.396±0.0009	0.4756±0.0012	96	96	95	1	1	1
25	0.3447±0.0014	0.4248±0.0017	0.5406±0.0013	102	103	108	0	0	0
12.5	0.3616±0.0021	0.4537±0.0010	0.5606±0.0016	107	110	112	0	0	0
6.25	0.3684±0.0011	0.4536±0.0021	0.5607±0.0012	109	110	112	0	0	0
Control Hela	0.3380±0.0010	0.4125±0.0008	0.5006±0.0013	100	100	100	0	0	0

4. Conclusions

- The Nano Hap/Cs-Chs composite materials with different mass ratio were prepared by liquid co-precipitation method. The noncrystallization structure of hydroxyapatites in the composite material is similar to that of the natural bone.
- The Hap particle size and dispersion are affected by adding different weight percentages of Cs-Chs, which exhibits that the particle become more compact with increasing Cs-Chs in composition. The nano-Hap were dispersed uniformly in the organic phase Cs and Chs with relatively good crystallinity. The particle size of Hap is about 40-100 nm with a spherical shape. When Hap/Cs-Chs is 50/50, the uniformity, compactness and compressive strength achieve the best performance.
- Composites containing trace amounts of sodium that is beneficial to the growth of natural bone. The Ca/ P ratio of composite material is around 1.85, which close to the theoretical value of Hap and belongs to the rich calcium type of Hap. Cytotoxicity test showed that the Hap/Cs-Chs composite materials had good biocompatibility and safety. Therefore, the physical and chemical performance of the composite material can satisfactorily meet the requirements of bone tissue engineering material.

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