A NOVEL COLLAGEN/HYDROXYAPATITE/MICROCRYSTALLINE CELLULOSE COMPOSITE FOR BONE TISSUE ENGINEERING

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A novel composite with collagen-hydroxyapatite and microcrystalline cellulose (MCC) was fabricated by biomimetic mineralization, sonication dispersion, dehydrothermal treatment (DHT), freeze-drying, and cold isostatic compaction technique. Fourier transform infrared spectroscope (FTIR), ultraviolet-visible spectrophotometer (UV), scanning electron microscope (SEM), and X-ray diffraction (XRD) were employed to analyze the structure and composition of the resultant composite. Swelling property, mechanical property and degradability of this novel composite were investigated. It is found that the composites prepared are hydrophilic and may swell in simulated body fluid. After being soaked in simulated body fluid for 40 days, it can still keep its original shape. Besides, the compressive strength of the composite is 99.05±1.74 MPa, reached the standards of artificial bone materials and is superior to some present used artificial bone materials. The workl may provide an efficient and alternative for bone tissue engineering.

Keywords: collagen; hydroxyapatite; microcrystalline cellulose

INTRODUCTION

With rapid development of biomedical engineering, chemical engineering, materials science and engineering, tissue engineering tends to flourish. This field is an interdisciplinary field that combines knowledge and technology of cells, biomaterials as well as suitable biochemical factors to fabricate artificial organs, tissues, or to regenerate damaged sites (Langer and Vacant, 1993). As the key branch of tissue engineering, bone tissue engineering potentially make alternative chances to bone substitudes instead of allograft, providing a framework for the cells to attach, proliferate, differentiate and form an extracellular matrix (ECM), and a carrier for cells, growth factors or other biofactors (Karen *et al.*, 2000; Molly, 2008).

Collagen and hydroxyapatite (HA) are the two major components of bone, seperately about 30% and 60% (Xu, 2011). Collagen is widely found in bone (Type I), cartilage (Type II) and blood vessel (Type III). It has excellent biocompatible biodegradable properties. It is also easily resorbed by the body and allows attachment to cells. Hydroxyapatite, with similar to chemical composition and morphology of bone apatite, can provide a good adhesion to the local tissue due to its surface chemistry and has been shown to enhance osteoblast proliferation and differentiation (Xie *et al.*, 2004). It also has high mechanical stiffness (Young's modulus) and a hard brittle surface. As such, collagen-hydroxyapatite composite has been inspiring interests of scientists and engineers (Du, 1978; Kikuch, 2001; Yu, 2004). However, there exist many disadvantages of collagen-hydroxyapatite composite that it can not meet requirements for artificial bone: (a) the discrepancy between degradation rate and osteogenesis progress; (b) the low surface activity; (c) the disagreement with nature human tissue about mechanical strength.

In order to overcome problems above, microcrystalline cellulose is introduced to collagen-hydroxyapatite composite and the structure and properties of the composite are investigated.

MATERIALS AND METHODS

Preparation of Collagen-Hydroxyapatite-Microcrystalline Cellulose (CHA-MCC) Composite

The CHA-MCC composites were synthesized by biomimetic mineralization method, sonication dispersion, dehydrothermal treatment (DHT), blending, freeze-drying and cold isostatic compaction technique. Briefy, collagen was dispersed in a 0.5 mol/L acetic acid aqueous solution. H_3PO_4 aqueous solution, $Ca(OH)_2$ aqueous solution were successively and gradually added into the reaction vessel with stirring frequently and starting materials for collagen-hydroxyapatite (Col-HA) composites were mixed at an 35/65 initial weight ratio by stoichiometry. The reaction temperature controlled using an oil bath and the pH of reaction solution controlled through NaOH aqueous solution by pH meter was set as 37°C and 7, respectively. Precipitates thus were obtained at the respective preparation temperature (37°C) for 48 h and were subsequently filtrated. After centrifugation, precipitates were freezed drying at −50°C under vacuum (GT2-Type-8, LYOTECH). The lyophilized samples were stabilized using dehydrothermal treatment (DHT). For DHT cross-linking, samples were placed in a vacuum oven at a temperature of 130°C for 24 h. The collagen-hydroxyapatite composites obtained were mixed in microcrystalline cellulose suspension with a weight ratio from 1/1 to 7/1. The mixture were ultrasonic dispersion for 48 h. Subsequently, the mixture were freezed dried at −50°C under vacuum (GT2-Type-8, LYOTECH). The product were cylindrically shaped upon a uniaxial pressure and consolidated further under an isostatic pressure of 200 MPa during 6 h.

Characterisation

FTIR spectra of CHA-MCC composites were obtained at room temperature using Bruker VERTEX 70 FTIR measurement (VERTEX 70, GER) in the range of 4000-400cm^{-1} using KBr pellets. And samples were subjected to X-ray diffraction (XD-3X, CHN) using CuKα radiation generated at 36 kV and 20 mA, the range of diffraction angle was 10°-70° 2θ. Micromorphology of samples were characterized using a scanning electron microscope (JSM-7100F, Japan) with an accelerating voltage of 3 kV.

The Atomic Ratio of Calcium to Phosphorous (Ca/P)

The calcium and phosphorous ratio of composites were determined according to the o-cresolphthalein complexon (OCPC) method and the molybdenum blue method respectively, combined with UV-Vis spectrometer (UV-2550, Japan).

$$C_{sample}(g/ml) = C * \left(\frac{A_{sample}}{A} \right)$$
$$\frac{Ca}{P}ratio = \frac{C_{sample(Ca)}}{C_{sample(P)}} \quad (1)$$

where C represents the calcium or phosphorous content of the respective standard solutions ($CaCO_3$ and KH_2PO_4), C_{sample} represents the composites'.

Porosity and Density Measurement

The density and porosity of scaffolds were measured by liquid displacement method using ethanol. Samples with a known weight (W_o) was immersed in a graduated

cylinder in a known volume of ethanol (V_o) for 24 h. The total volume of ethanol in the cylinder and ethanol -impregnated scaffold was recorded (V). The volume of scaffold was $V-V_0$ and the ethanol-impregnated scaffold removed from the cylinder was weighed (W). Each sample was measured in triplicate.

$$Porosity=\frac{W-W_0}{\rho_{ethanol}}\times\frac{1}{V-V_0}\times 100\% \quad (2)$$

$$\rho=\frac{W}{V-Vo} \quad (3)$$

where $\rho_{ethanol}$ is 0.789g/cm^3.

Swelling Tests

Swelling measurements were run in simulated body fluid (SBF, pH=7.4), at 37.0±0.5˚C with three parallel measurements using two different methods.

The first method was determined by measuring periodically the weight of swollen samples (W_{ws}) with hanging over until no dripping. In this case, we assessed the swelling ability of the composite structure with its pore system.In the second method, for a period of time, the same kind of swollen samples were removed the excess water in the pores by centrifugation within filter paper. Then, they were pressed between new filter papers to remove the residue water and then were weighed (W_{wm}). In this way the ability of the composite itself to absorb water was assessed.

$$Swellingratio(\%)=\left(\frac{W_w-W_d}{W_d}\right)\times 100\% \quad (4)$$

where W_w represents W_{ws} or W_{wm}.

Mechanical Tests

Mechanical testes were carried out with a constant pressure testing machine, YWE-300. The tests were carried out at a crosshead speed of 10 N/s. Each experiment was repeated three same samples and the average was reported.

RESULTS AND DISCUSSION

As shown in the FTIR spectra in Fig.1 , the appearance of peaks at 1058cm^{-1} and 964cm^{-1} is attributed to stretching mode of $PO4^{3-}$. And the distinctive peak in the region of 500~600cm^{-1} of hydroxyapatite, 568cm^{-1} is also observed. And the band at 1340cm^{-1}, ascribed to the wagging vibration of covalent bond between carboxylic acid groups of collagen and calcium ions of hydroxyapatite. Another red-shift of the band corresponding to C-O band, from 1658cm^{-1} in collagen to 1647cm^{-1} in CHA, can be observed. There are chemical reaction between collagen and hydroxyapatite. Rhee and other researchers have proved that carboxylic acid groups and carbonyl groups of collagen is the other nucleation site (Zhang, 2004; Zhai *et al.*, 2005). The additional -OH^- at 3343cm^{-1}, the C-H symmetrical stretching of methylene at 2900cm^{-1} of MCC and the C-O stretching within ether of cellulose molecule at 1112cm^{-1}, 1165cm^{-1} appear in FTIR spectrum of CHA-MCC composite. The band at 1340cm^{-1} of CHA, the peaks of C-O stretching vibration and -OH bending vibration between 1032cm^{-1} and 1430cm^{-1} disappear. It implies that there is chemical reaction between CHA and MCC owing to the increasing number of -OH from MCC.

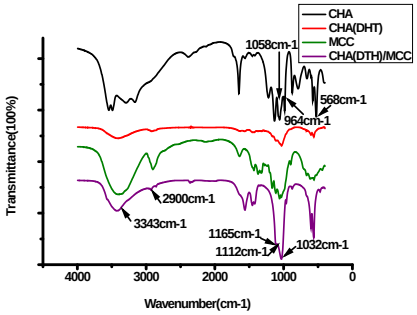

Figure 1. FTIR of collagen/hydroxyapatite/ microcrystalline cellulose composite

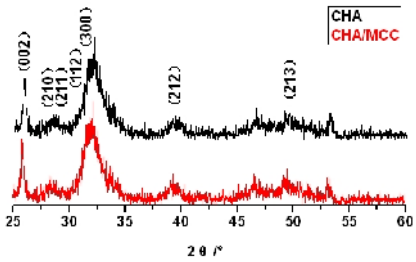

Figure 2. XRD of collagen/hydroxyapatite and collagen/hydroxyapatite/microcrystalline cellulose composite

XRD spectra of CHA and CHA/MCC composites are analyzed (Fig. 2). Diffraction patterns of samples show characteristic peaks of HA. The maxima peaks can be assigned to the HA included in the broad region of overlapping peaks corresponding to (002), (211), (112), (300) and (213), respectively. The peaks at 28.93° and 39.20° are also located between the positions expected for (210) peak and (210) peak of hydroxyapatite. The strong reflection intensity of (002) reflection indicates the occurrence of preferably orientated growth of apatite nanocrystals on organic collagen. X-ray diffraction indicates HA is poorly crystalline , which is similar to nature bone.

Futhermore, the average Ca/P ratio is 1.112±0.012, belonging to the range of stoichiometric hydroxyapatite (Ca/P=1.57±0.603) (Mathers and Czernuszka, 1991). A slight lower Ca/P ratio obtained in this study is probably due to the insolubility of some $CaCl_2$ in calcium solution through ionic interaction with negative charged groups. Besides, in reaction solution, the concentration of OH^- may be not enough to induce the complete reaction between calcium ion and phosphonium ion.

Figure 3 presents the morphology of CHA and CHA/MCC composites. These SEM photomicrographs show that the surface of composites are compact, microcrystalline cellulose disperses homogeneously within composites and combines with collagen-hydroxyapatite powder durably. By using ultrasonic dispersion, there are no agglomeration of microcrystalline cellulose or collagen-hydroxyapatite powder and delamination after isostatic compaction. And the composites form an irregular and interconnected porous network, with diameters on a nanoscale, about 150±50nm. The porosities observed in these composites suggest that they might be osteoinductive while their compositions should allow their eventual resorption.

All CHA/MCC composites with different ratios are found to be highly porous, retaining porosities above 70%. The relative density of the scaffolds was then calculated, as shown in table 1, approximately 0.7g/cm^3. Both porosity and density of composites meet needs of artificial bone, based on these of spongy bone (1.8-2g/cm^3, 5-30%) and compact bone (0.14-1.2g/cm^3, 30-90%) (Masoud *et al.*, 2010).

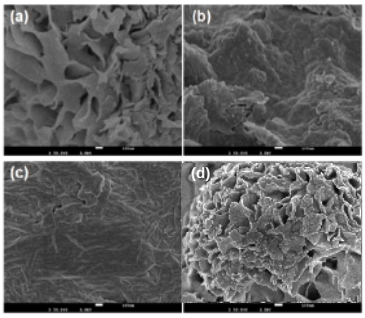

Figure 3. SEM micrographs of collagen/hydroxyapatite/microcrystalline cellulose composite: (a) CHA, (b) CHA(DHT), (c-d) CHA/MCC(1:1) (a-c: surface, d: fracture surface)

Table 1. Porosity of collagen/hydroxyapatite/microcrystalline cellulose composite, spongy and compact bone

Sample	Ratio of weight (collagen/hydroxyapatite to microcrystalline cellulose)	ρ(g/cm^3)	Porosity(%)
CHA/MCC	1:1	0.661±0.002	78±0.69
CHA/MCC	2:1	0.669±0.013	79±0.45
CHA/MCC	3:1	0.674±0.004	77±1.02
CHA/MCC	5:1	0.662±0.009	78±0.92
CHA/MCC	7:1	0.656±0.005	72±0.34

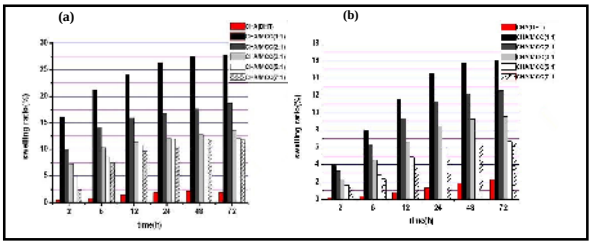

Figure 4. Swelling ratio of collagen/hydroxyapatite (DHT) composite and collagen/hydroxyapatite/microcrystalline cellulose composite(different weight ratios of collagen/hydroxyapatite to microcrystalline cellulose)

Figure 4(a) and 4(b) showed that the swelling properties were associated with the swelling ability of the composite structure with its pore system and the ability of the composite itself to absorb water. It is obvious that swelling equilibrium is reached at approximately 24 h. Swelling ratios of CHA/MCC materials are much higher than CHA, caused by the presence of MCC. The results clearly revealed that MCC has an important effect on the swelling behavior of CHA/MCC materials and makes a control of it.

The CHA composite cross-linked by DHT has a higher compressive strength compared with CHA samples. The increase content of MCC in composite gives rise to an increase in the stiffness of the material. The composite with a CHA/MCC weight ratio of 1/1 shows the highest compressive strength. And the compressive strength of all samples meet the requirement of artificial bone, being considerably higher than cancellous bone (1-20MPa) and near to compact bone (100-200MPa) (Gibson, 1985).

Table 2. Compressive strength of collagen/hydroxyapatite, collagen/hydroxyapatite (DHT) and collagen/hydroxyapatite/microcrystalline cellulose composite

Sample	Ratio of weight (collagen/hydroxyapatite to microcrystalline cellulose)	Compressive strength / MPa
CHA/MCC	1:1	99.05±1.74
CHA/MCC	2:1	73.56±2.05
CHA/MCC	3:1	58.94±1.49
CHA/MCC	5:1	50.23±1.92
CHA/MCC	7:1	48.92±1.85
CHA	—	36.50±1.37
CHA(DHT)	—	49.19±0.91

CONCLUSIONS

A novel composite with collagen-hydroxyapatite and microcrystalline cellulose (MCC) was fabricated by biomimetic mineralization, sonication dispersion, dehydrothermal treatment (DHT). Hydroxyapatite in composite is poorl crystalline, which is similar to nature bone. And material has an irregular and interconnected porous microstructure, with diameters on a nanoscale, about 150±50nm. retaining porosities above 70% and density approximately 0.7g/cm^3. CHA-MCC material shows high hydrophilicity, good swelling property and superior mechanical property. The rate of hydrophilicity, swelling and mechanical properties of the material can be modified by DHT and the microcrystalline cellulose concentration.

Acknowledgements

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