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Chitosan/Calcium Phosphate Composites Scaffolds Prepared by Membrane Diffusion Process

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Abstract

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) and Dicalcium phosphate dihydrate ($CaHPO_4.H_2O$, DCPD) were homogeneously hybridized into chitosan solution by membrane diffusion process. The three-dimensional scaffolds were obtained by freeze-drying process. The organic and inorganic contents of scaffold were determined by using thermogravimetry analysis (TGA). The surface morphology of the scaffold and characteristic of calcium phosphate were investigated by scanning electron microscope (SEM), energy dispersive X-ray spectrometer (EDX), and X-ray diffraction (XRD), respectively. Compressive modulus was also determined by dynamic mechanical analysis (DMA). The hybridized inorganic content ranges from 35-45%. Both chitosan/HAp and chitosan/DCPD scaffolds showed interconnected porous structure. The calcium to phosphorus ratios of chitosan/calcium phosphate composite scaffolds obtaining from SEM-EDX followed to the theoretical ratios of HAp and DCPC for chitosan/HAp composite and chitosan/DCPD composite, respectively. XRD pattern showed that the products of composite scaffold were HAp and DCPD. These results suggest that calcium phosphate can be hybridized into chitosan solution through membrane diffusion process, followed by forming the porous scaffolds by freeze-drying. Therefore, as prepared porous chitosan/calcium phosphate composite scaffolds can be considered as potential materials for tissue engineering.

Key words: Hydroxyapatite; Dicalcium phosphate dehydrate; Chitosan; Scaffold; Tissue engineering

Introduction

Tissue engineering has emerged as an important alternative approach to autografts and allografts. It has been defined as the application of biological, chemical and engineering principles towards the repair or regeneration of living tissues using biomaterials, cells and factors alone or in combination. Many tissue engineering methods involve usage of a biocompatible and biodegradable three-dimensional (3-D) composite scaffold as a temporary extracellular matrix for initial cell attachment and subsequent tissue formation. The scaffold should be porous, with interconnected pore networks for cell growth and transport of nutrients and metabolic wastes.

Nowadays, the composite materials of organic/inorganic origin are studied extensively. investigated for a variety of biomedical applications.[3] such as wound healing, bone and cartilage tissue engineering. Due to its excellent biocompatibility and biodegradability,[6, 2] chitosan is interesting as an organic phase in our studied composites.

Chitosan-based scaffolds possess special properties for use in tissue engineering. The interconnected-porous structures of chitosan can be easily formed by freeze-drying or lyophilizing from chitosan solution. The interconnected porous structure is very important, so that numerous cells can be seeded and migrated into the inside. Besides, sufficient amounts of nutrient can be passed through the interconnected pores in a consequence of enhancing cell proliferation or tissue regeneration.

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Due to the chemical compositions being similar to the inorganic component of bone, calcium phosphates (CaP) have been used as implant materials for bone repair and regeneration. Even though, the brittle and rigid nature of calcium phosphate limits its role in biomedical applications.[4, 9, 7] It must be highlighted at this point that the successful design of a bone substitute material requires an appreciation of the structure of bone composed of organic and inorganic materials. Thus, interconnected porous composites that are made up of chitosan and calcium phosphate mimic the morphology and properties of natural bone.

Thus, the aim of this work is to hybridize any kinds of calcium phosphate through membrane diffusion process. Two kinds of calcium phosphate, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) and dicalcium phosphate dihydrate ($CaHPO_4.H_2O$, DCPD), are homogeneously hybridized into chitosan solution. Subsequently, the three-dimensional porous structure was prepared by lyophilization. The organic/inorganic ratio of as prepared chitosan/AHp and chitosan/DCPD composites was investigated by TGA. The interconnected morphology was observed by SEM. The characterization of as hybridized calcium phosphate was determined by SEM equipped with EDX and XRD. The compressive strength of as prepared scaffolds was also reported in this article.

Materials and Experimental Procedures

Materials

Chitosan of high viscosity was supplied by Fluka Co., Ltd (Japan) with a degree of deacetylation of 85. Calcium chloride ($CaCl_2$), di–sodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) were supplied by Carlo Erba Co., Ltd (Italy). Acetic acid (CH_3COOH) was supplied by Bdh Laboratory Supply (England). Sodium acetate (CH_3COONa) and Tris–(hydroxymethyl)–aminomethane buffer substance (Tris) were supplied by Scharlua (Spain). The acetate buffer was prepared with 0.1 M CH_3COOH and 0.1 M CH_3COONa. Dialysis tube (MWCO 6000) was supplied by Membrane Filtration Products, Inc., (U.S.A.).

Preparation of Chitosan/Calcium Phosphate Scaffolds

The chitosan/HAp composites and chitosan/DCPD composites were synthesized by membrane diffusion process as shown in Figure 1. HAp was synthesized simultaneously with the diffusion of 0.12 M Na_2HPO_4 to 0.02 M $CaCl_2$ in 1.75wt% chitosan solution. Similarly, DCPD was hybridized to chitosan solution by the diffusion of 0.02 M $NaH_2PO_4.H_2O$ to 0.02 M $CaCl_2$. At a given diffusion time, the as prepared chitosan/CaP composite solution was poured into the mold. The 3D porous scaffolds were obtained after freeze-drying.

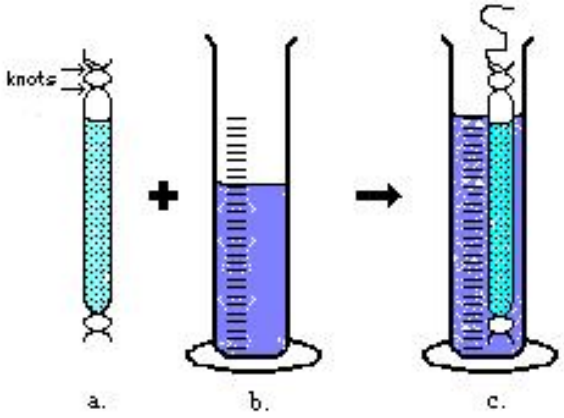

Figure 1. Schematic representation of preparation of chitosan/calcium phosphate composite (a) Chitosan and $CaCl_2$ mixed solution (b) Phosphate solution (c) Membrane Diffusion Process

Properties of Chitosan/Calcium Phosphate Composite Scaffold

The organic and inorganic content of chitosan/calcium phosphate composites were determined by thermogravimetric analysis (Mettler Toledo TGA/SDTA 851, Barcelona). Approximately 5 mg of chitosan/calcium phosphate composites were ground in a crucible pan for TGA analysis. The sample was heated from 50ºC to 700ºC at a heating rate of 20ºC/min and holding the final temperature for 10 mins. The measurement was made in an oxygen gas with a flow rate of 20 mL/min.

The porous morphology of the surface and cross-section of as prepared scaffolds were examined with SEM (JEOL JSM 5800LV, Japan). After being coated with gold in a sputtering device with condition of 15 mA for 3 mins 30 sec, the morphology was observed with an accelerating voltage of 15 kV and magnification of 200. The quantitative analysis of the composite scaffolds was examined by SEM-EDX technique. The average atomic percentage of Ca/P was calculated.

To investigate the characterization of the hybridized calcium phosphate, HAp or DCPD, the samples of as prepared chitosan/CaP scaffolds were analyzed using an XRD (Bruker AXS Model D8, Germany) with monochromatic CuK_{α} (λ=1.54060) radiation.

The compressive modulus was tested with a DMA (Model Mettler Toledo DMA/SDTA861^{e}, Barcelona) of compression clamp steel to compress the specimen (WxLxH = 5x5x3 mm^3) at a compress speed of resonance frequency 1670 Hz and spring constant 40 N/m on a computer-controlled machine. The test was conducted in air at room temperature. In the present study, chitosan scaffold, chitosan/HAp and chitosan/DCPD composite scaffolds were used as specimens. Comparative study of compressive modulus to sterilization solvent (70% v/v EtOH) and cell culture media (DMEM) was also investigated. The specimens were immersed in two different solvents before testing. The testing was conducted with five specimens of each scaffold.

Results and Discussion

Determination of Organic/Inorganic Ratio

The inorganic part of calcium phosphate hybridized into the composites can be determined by weight of the remaining ash after completed combustion at 700ºC in oxygen atmosphere. The organic/inorganic ratio of chitosan/calcium phosphate composites are shown in Table 1. The hybridized inorganic content ranges from 35-45%. The inorganic content percentage in chitosan/HAp composite scaffold was higher than chitosan/ DCPD composite scaffold.

Table 1. Organic and inorganic content of chitosan/calcium Phosphate composite scaffolds

Chitosan/CaP	Chitosan(%)	CaP(%)
Chitosan/HAp	57.50	42.50
Chitosan/DCPD	65.55	34.45

Morphology of Chitosan/Calcium Phosphate Composite Scaffold

The SEM images (Figure. 2) shows that the composite scaffolds have complicated and three-dimensional irregular porous structures together with good interconnection between the pores either surface or cross section. The pore size ranged from 50 μm to 400 μm. Comparing the chitosan/HAp and chitosan/DCPD composite scaffolds, it can be seen that the porosity of the chitosan/DCPD composite is higher than the chitosan/HAp composite. The picture showed chitosan/HAp has a pore size range from 200 μm to 400 μm and chitosan/DCPD has a pore size range from 50 μm to 100 μm. The reason may be that the higher percentage of calcium phosphate occupied less space of ice in the frozen mixture resulting in the lower porosity in chitosan/HAp composite scaffold. In addition, between the two scaffolds, the chitosan/HAp composite scaffold had the better ideal porous structure, the majority being the size of from 200 μm to 400 μm, which may be helpful to promote cell adhesion and conductive tissue in-growth with a good nutrient delivery to the site of tissue regeneration.[8] Additionally, its porosity can also meet the requirement for bone tissue engineering scaffold material.

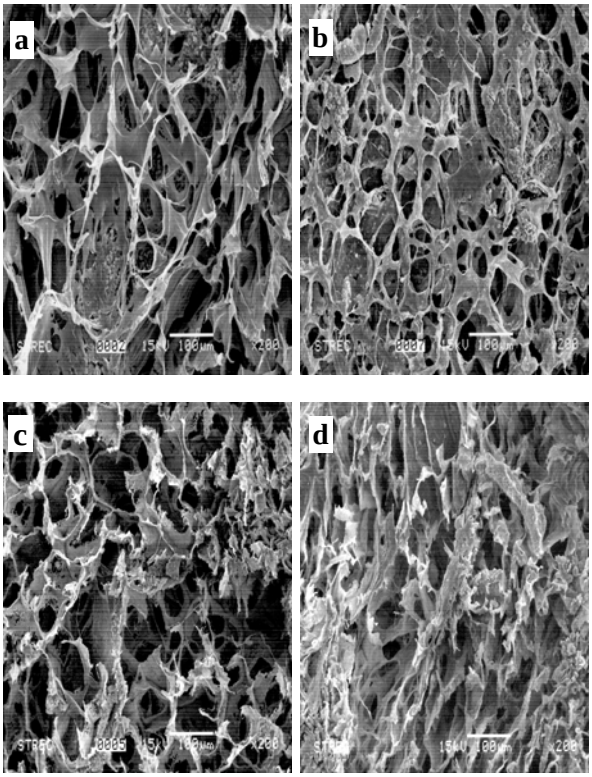

Figure 2. SEM images of chitosan/calcium phosphate composite scaffolds with magnification by 200. (a) chitosan/HAp (Surface) (b) chitosan/ DCPD (Surface) (c) chitosan/HAp (inside) (d) chitosan/DCPD (inside)

Calcium Phosphate Composition Analysis

Top surface and cross section compositional analysis had been conducted on samples using SEM-EDX and the percentage of calcium and phosphorus are presented in Table 2. As synthesized HAp and DCPD showed calcium to phosphorus ratios following to the theory.

Table 2. Percentage of calcium atom and phosphorus atom

Type of samples	*% Atomic*		*Calculate Ca/P*	*Ideal theory Ca/P*
	Ca	P		
Chitosan/HAp (surface)	1.41	0.89	1.58	1.67
Chitosan/HAp (cross section)	15.36	9.34	1.64	
Chitosan/DCPD (surface)	3.66	4.05	0.90	1.00
Chitosan/DCPD (cross section)	1.35	1.44	0.93	

XRD Pattern

Figure. 3 shows the X-ray diffraction patterns of chitosan, chitosan/HAp and chitosan/DCPD composite scaffolds. In Figure. 3a, the main diffraction peak of chitosan at 2θ=20° was observed. In Figure. 3b, two main diffraction peaks of HAp of chitosan/ HAp composite scaffold at 2θ=26°and 32° can be found.[1] The characteristic peaks of DCPD of chitosan/DCPD composite scaffold at 2θ= 20.94, 29.25, 30.50, 34.15 and 34.42 can be found in Figure. 3c[5] The XRD patterns of the chitosan/calcium phosphate composite scaffolds shown in Figures. 3b and 3c are characterized by specific diffraction peaks arising from pure chitosan scaffolds. However, the specific board peak for chitosan (2θ=20°) disappeared in the composite scaffolds, which may be the result of the interaction of chitosan and calcium phosphate which are compatible.

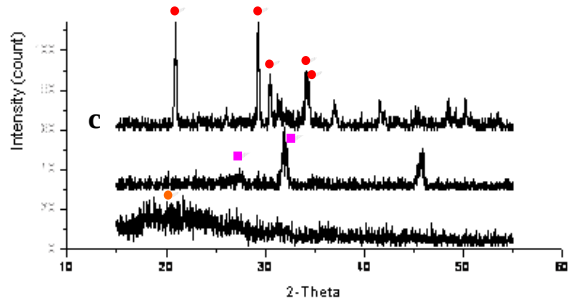

Figure 3. XRD patterns of (a) chitosan scaffold, (b) chitosan /HAp composite scaffold, (c) chitosan/DCPD Composite scaffold

Compressive Modulus

Compression testing was performed on the chitosan pure and chitosan/calcium phosphate composite scaffold with DMA. It was found that the chitosan/ calcium phosphate composite scaffolds were having a better compressive modulus than chitosan pure scaffolds Table 3.

Table 3. Compressive modulus of the chitosan pure and chitosan/calcium phosphate composite scaffold

Solution	***Compressive modulus of the scaffold (N/m^2)***		
	Chitosan scaffold	***chitosan/HAp composite scaffold***	***chitosan/DCPD composite scaffold***
Non solvent	7.20×10^2	2.00×10^3	6.40×10^3
Sterilization solvent (70% v/v EtOH)	7.00×10^1	2.60×10^2	7.80×10^3
Cell culture media (DMEM)	4.20	2.40	7.60×10^1

Conclusions

In this study, chitosan/HAp and chitosan/ DCPD composite scaffolds was successfully prepared by a simple hybridization through membrane diffusion process followed by effective freeze-drying without using any cross-linking agent. Moreover, it should be noted that the as prepared composite scaffolds are ideal scaffolds with good and complicated three-dimensional irregular porous structure and a better compressive modulus than chitosan scaffolds to be used for tissue engineering material. As for the biological properties, the related research including *in vitro* cell culture experiment is on-going and the corresponding result will be further reported.

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