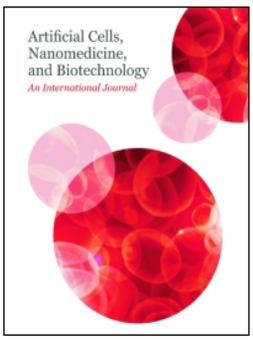

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Artificial Cells, Blood Substitutes, and Biotechnology

ISSN: 1073-1199 (Print) 1532-4184 (Online) Journal homepage: http://www.tandfonline.com/loi/ianb19

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To cite this article: Qi-Qing Zhang, Lei Ren, Chun Wang, Ling-Rong Liu, Xue-Jun Wen, Yu-Hua Liu & Xing-Dong Zhang (1996) Porous Hydroxyapatite Reinforced with Collagen Protein, Artificial Cells, Blood Substitutes, and Biotechnology, 24:6, 693-702, DOI: 10.3109/10731199609118892

To link to this article: http://dx.doi.org/10.3109/10731199609118892

Published online: 11 Jul 2009.

ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH., 24(6), 693-702 (1996)

POROUS HYDROXYAPATITE REINFORCED WITH COLLAGEN PROTEIN

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KEYWORDS: Hydroxyapatite, collagen, composite material

ABSTRACT: Porous hydroxyapatite(HAP) with certain porosity and pore size was prepared, and incorporated with bovine collagen protein. The composition and structure of the HAP was confirmed by X-Ray Diffraction(XRD) and ICP. Scanning Electron Microscopy(SEM), mechanical tests and in vitro degradation were performed. Collagen protein with low antigenicity was obtained from bovine tendon by enzyme digestion, and was then forced to fill in the HAP matrix to form composites. Scanning Electron Microscopy(SEM), Mechanical tests and in vitro degradation were performed. The test results show that first, HAP thus made has specific pore size and directions; second, mechanical properties of the composites have been markedly improved; third, the in vitro degradation rate of the composite is almost the same as and mainly controlled by the degradation rate of collagen.

INTRODUCTION

With the advancement of modern medical science, traditional artificial bone materials can no longer fulfill the needs for the prosthesis and replacement of human hard tissues. In the case of injuries caused by excessive sports and traffic accidents, hard tissue reconstruction and replacement are becoming increasingly complicated and ages of these patients may vary. For many years, the choice of traditional artificial bone materials have been restricted to a few kinds like metal alloys, synthetic polymers, aluminum oxide ceramics, carbides, etc. The properties of these materials are not totally satisfying when being applied to complex operations of hard tissue reparations. Therefore, biomedical materials whose physical-chemical and biological properties similar to those of human tissues and organs are highly desirable and bear the common interests of both laboratory researchers and clinical doctors.

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From the view of material science, human bone tissues can be regarded as natural composite materials composed of organic part -- collagen fiber and inorganic part -- calcium phosphate(1). Thus, research work is underway aiming to develop a kind of artificial bone material which has similar constituents, structures and properties to those of natural bones. In recent years, hydroxyapatite ceramic material is the focus of artificial bone research all over the world. Hydroxyapatite(HAP) is expected to be an ideal substitution of human bone tissues because of its good biocompatibility and bioactivity, that is, HAP can form firm osseous combination with natural bones(2-4). However, because of its brittleness and weakness in physiological environment and unsatisfactory growth rate of bone tissues into the HAP pores, the porous HAP material has only been used as bone filler and its application has been greatly limited(5-9).

Collagen is the principal component of mammal connective tissues. It is a kind of protein and can be extracted from bovine tendon or calf skin by enzyme or acid(10,11). Collagen protein is fibrous and is formed by three α-polypeptide chains entangling together through hydrogen bonds. Strong light polarization reveals the helix structure of protocollagen. Stress-strain curve indicates the existence of plastic phase and elastico-viscous phase and thus resulting good tenacity and strength. It is recognized that collagen also contributes to the growth of cells(7). The α-chains and certain peptide degradation residues have the ability to affiliate tissue cells(12). When applied to human, collagen is biocompatible(18), nontoxic and has no anaphylaxis, pyrogen and carcinogenicity(12). The degradation products can be excreted out of the body through urine(12). Because of these advantages, collagen is being regarded as an important biomaterial(13-17). But collagen has very poor stiffness and can not be used alone for hard tissue packing and substitution.

In this article, after fully considering the properties of HAP ceramics and collagen protein, we experimented with porous HAP and collagen composites to form new artificial bone materials. It is expected that during the early stage of implantation, the porous HAP could act as the framework for the in-growth of bone tissues and the collagen could act as a reinforcement so as to enhance the strength and tenacity of the composites. At the same time, the growth rate of the new bone tissues into the HAP pores could be matched by the degradation rate of collagen so that eventually, the new bone combined with HAP would become permanent and naturally bioactive.

METHODS

1.Preparation of porous HAP

Raw HAP were ground and meshed to form fine powder. Certain amount of hydrogen peroxide (aqueous solution) and paper pulp were added and mixed thoroughly. The slurry was then desiccated at 90°C in a rectangular container and slowly heated to 1280 °C and remain there for 3 hours. After being slowly cooled to room temperature, porous HAP ceramics with specific porosity, porous size and directions was thus formed.

2.Preparation of collagen materials

Fresh bovine tendon was washed, frozen and sliced to 1-2mm thick. Proteinase was used for 16-hour-digestion at 37 °C. Then the enzyme was inactivated and the tendon was washed by distilled water and dissolved in acid solution. The about 10% collagen solution was centrifuged and stored at 5°C.

3.Preparation of HAP-collagen composites

Collagen solution with various concentrations was centrifuged with porous HAP. Collagen was thus pressed to fill in the porous HAP at a single direction as deep as possible. The HAP ceramics were dried in vacuum desiccator at 35 °C and cross-linked by 1.4%(V/V) formaldehyde for 1 hour at room temperature. Then the HAP-collagen composites were removed from the cross-link agent, washed to neutral with distilled water and ambiently dried in vacuum desiccator for further use.

4.Reconfirmation of HAP composition and structure

A D/MAX-RA X-ray diffractor was used to obtain XRD diagrams of HAP samples in the form of powder, and the result was compared with standard diagram of ASTW(9-432). Elemental analysis was conducted on a Jarrall-Ash 9000+2000 ICP apparatus to ensure that the HAP sample has sufficient purity.

5. Porous microstructure investigation

A Cambridge-S200 Scanning Electron Microscope was used to characterize the microstructure of the HAP-collagen composite. Porosity and pore size were calculated.

6.Mechanical tests

Mechanical parameters like compressive strength, bending strength and deformation ratio of the composite samples fabricated with different collagen concentrations were obtained using WD-10 Electronic material testing apparatus.

7.In vitro degradation

95mg HAP-collagen composites (containing 1mg collagen) and 1mg pure collagen protein were loaded into test tubes and 2ml Stock solution and 0.1ml Stock-collagenase solution were added respectively. The tubes were vibrated at the speed of 100r.p.m. at 37°C. Concentration changes of the major degradation product - hydroxyproline -- were recorded by UV-265 ultra-violet spectrophotometer at different time spans.

RESULTS

1.Sample preparations

Currently there are three methods employed in the fabrication of porous bioceramics -- using separate bubble-generating agents other than reactive ingredients, or using other porous materials as a framework, or achieving bubble generation during synthetic and/or sintering processes by properly choosing reactive agents. Among these, the first method is found to be the most effective. Hydroxyperoxide is the most commonly selected bubble-forming reagent, but in many cases, it is rather difficult to assert some control on the size and shape of the pores in the ceramic matrices. Therefore, we experimented by adding in paper pulp during the formation of the HAP matrix, and were successfully

able to produce samples with specific pore size and shape by altering ratios of the involved paper pulp. We prepared three types of HAP samples with various porosity and pore size as shown in Table 1. The formed micro pores could be interconnected since the paper pulp has a higher vaporization temperature. Special effort had also been paid to grant a certain extent of direction to the arrayed pores.

2.HAP composition and structure

The XRD diagram of HAP sample is given in Figure 1(A), and compared with standard XRD diagram(ASTW9-432) in Figure 1(B). The obvious disparity between major diffraction peaks of the two diagrams indicates that the porous sample is virtually composed of HAP. Solution concentrations of the present elements are listed in Table 2. It can be seen that Ca and P are taking the dominant role, while the sum of all the other constituents is less than 0.2% of the total concentration.

3.SEM observations

Figure 2 shows the SEM photo of the HAP structure, and Figure 3 shows the SEM image of the HAP-collagen HAP-collagen composite sample. The SEM results show that collagen filled into the HAP ceramics in the form of solutions shrinks along and is loosely attached to the pore walls after desiccation. Examinations of the structure of vertical layers indicate that collagen materials have been imbibed into the pores and stretch along the pore directions. But the pores are not fully imbued with collagen because of large viscosity of the collagen solutions.

The calculated values of pore size are given in Table 3. The average porosity is 62.2%. Actually, the porous matrix is filled with macro pores of 300-1500um as well as micro pores of 0.5-20um. The pores has a certain direction and internally connected. According to Klawitter and Hulbert(19), the minimum pore size is 100um for bone ingrowth into ceramic structures. The average pore size of the sample here is large enough to allow ample ingrowth of tissues and cells.

4.Mechanical properties

The results of mechanical tests show that compressive strength increases after the HAP was filled with collagen and is larger when the direction of stress is parallel to the pores (see Figure 4). Bending strength and deformation ratio also increase to various extent. Bending strength is larger when stress is vertical to the pores whereas deformation ratio is larger when stress is perpendicular (see Figures 5 and 6). We have not found a distinct explanation of the irregular sudden rises and falls of the mechanical parameters. We suspect that this could be explained by the non-homogeneous distribution of collagen protein within the HAP matrix caused by the composition procedure, which, at the meantime, being modified and improved so as to avoid this situation.

5.In vitro biological degradation test

The result of in vitro degradation test is given in Figure 7 in which the solution concentrations of hydroxyproline were monitored by an UV spectrophotometer at different time intervals. The degradation curve indicates that degradation rates of the composite and collagen are largely the same. Degradation rate of

Table 1. Porosity and pore size of three divisions of samples

Sample	Porosity(%)	Pore size (um)
1	74	875
2	72	633
3	68	426

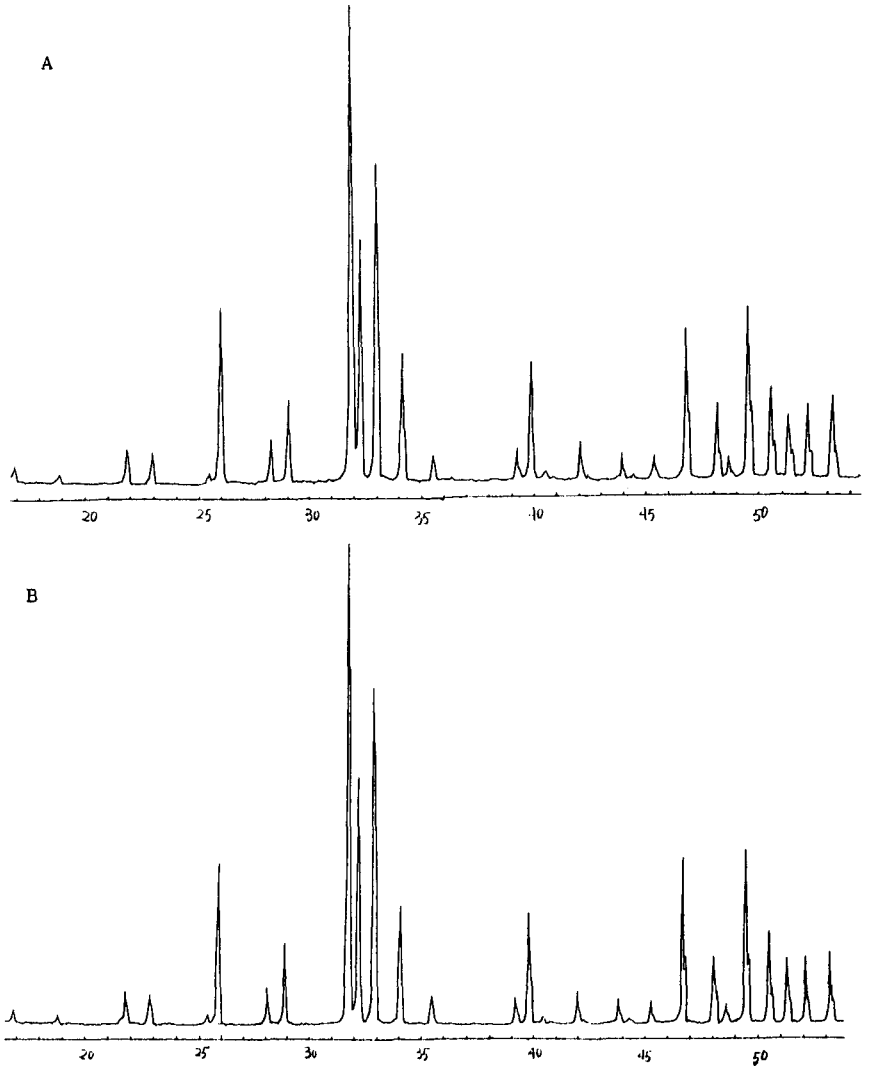

Figure 1. XRD diagrams of (A) HAP sample and (B) HAP standard sample

Table 2. Results of elemental analysis

Element	Concentration(ug/ml)	Percentage in solution(%)
Ca	185.41	37
P	88.302	17.67
Cu	0.0039	0.0007
Fe	0.9170	0.0917
K	0.4056	0.0406
Na	0.9687	0.0968
Mn	0.0068	0.0068
Al	0.3704	0.0037
Si	< 0.000	< 0.000

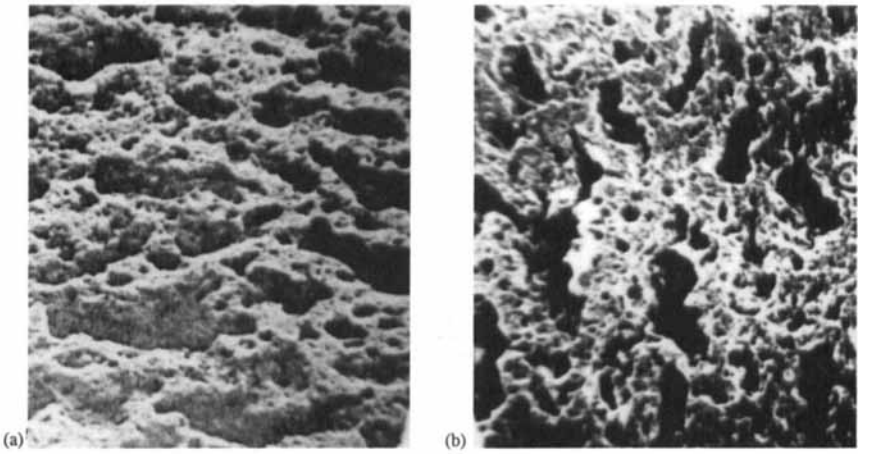

Figure 2. SEM photos of the HAP sample. (A) area cut vertical to the pore direction, and (B) area cut parallel to the pore direction

the composite is mainly determined by the rate of collagen protein inside the HAP matrix. The collagen protein almost entirely disappears after about 120 hours when the concentration of hydroxyproline reaches its peak.

DISCUSSIONS AND CONCLUSION

The vital weakness of porous HAP ceramics is its brittleness. When the inner stress exceeds the critical point, unavoidable collapse of the material is often abrupt and disastrous. We have used a kind of fibrous protein -- collagen to form composites with HAP. In the case of the new composites, when the HAP matrix

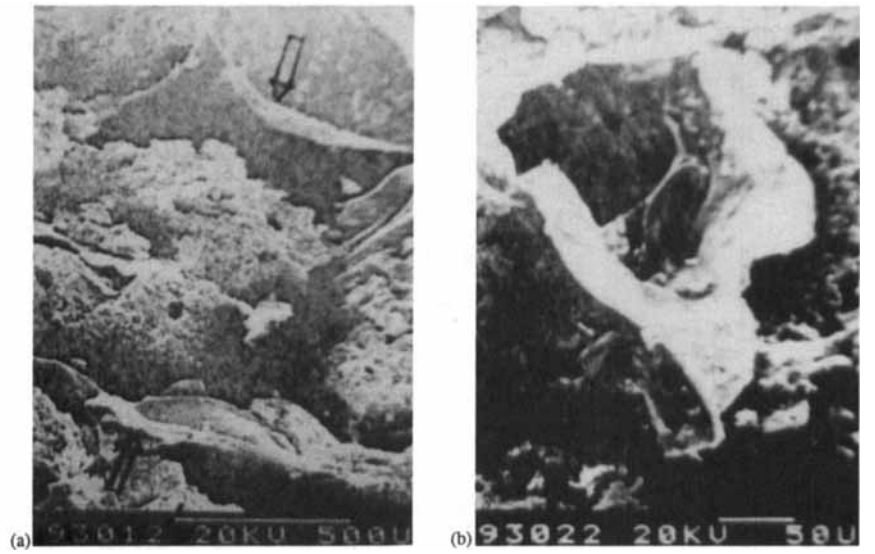

Figure 3. SEM photos of the HAP-collagen Composite. (A) shows the combination of HAP and collagen fibers (the arrows are pointing to the places where HAP and collagen combine), and (B) is a more detailed view of the HAP-collagen interface.

Table 3. Pore size (um)

Maximum	Minimum	Average
1500	313	675

is pressed, the collagen fiber inside could partially afford the stress and retard the broadening of rifts, thus enhancing the strength and tenacity of the material. This is distinctively demonstrated by the mechanical tests. However, it is extremely important to properly control the HAP-collagen ratio. The strength of the material increases with the concentration of collagen only within a certain range of ratios. Since the viscosity of the collagen solutions becomes too large when the collagen concentration reaches a certain value, it is discovered that it is rather difficult to fill more collagen into the HAP pores. Then, the strength of the composite may decrease with increasing collagen concentration.

The in vitro degradation test suggest that the collagen protein does not change its nature after composites are formed.

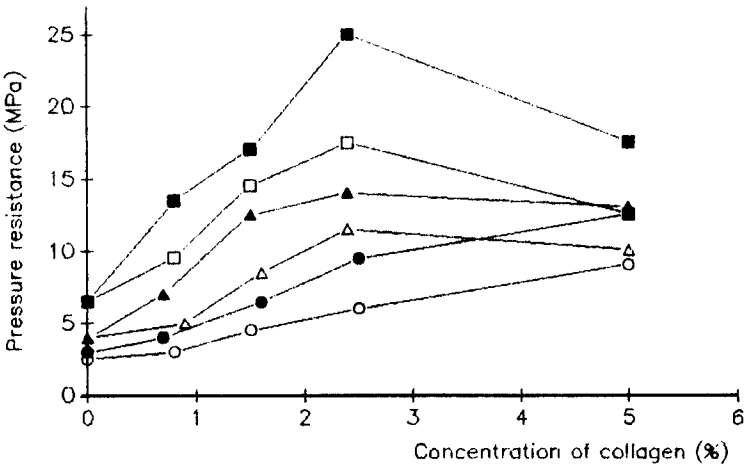

Figure 4. Compressive strength versus collagen concentration curves of the composites.
o—o, △—△, □—□ -- No.1,2,3 HAP-collagen composites, directions of stress vertical to the pores

●—●, ▲—▲, ■—■ -- No.1,2,3 HAP-collagen composites, directions of stress parallel to the pores

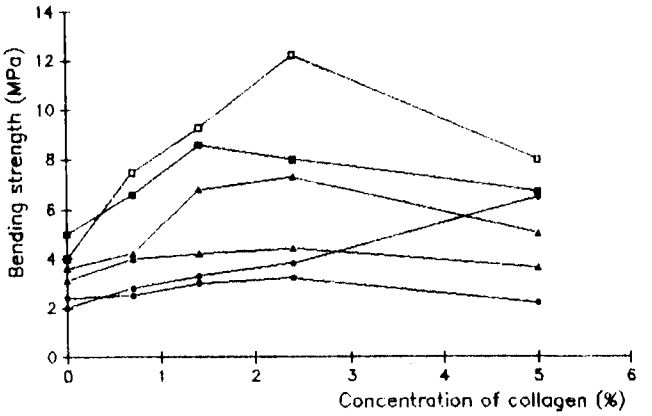

Figure 5. Bending strength versus collagen concentration curves of the composites.
o—o, △—△, □—□ -- No.1,2,3 HAP-collagen composites, directions of stress vertical to the pores

●—●, ▲—▲, ■—■ -- No.1,2,3 HAP-collagen composites, directions of stress parallel to the pores

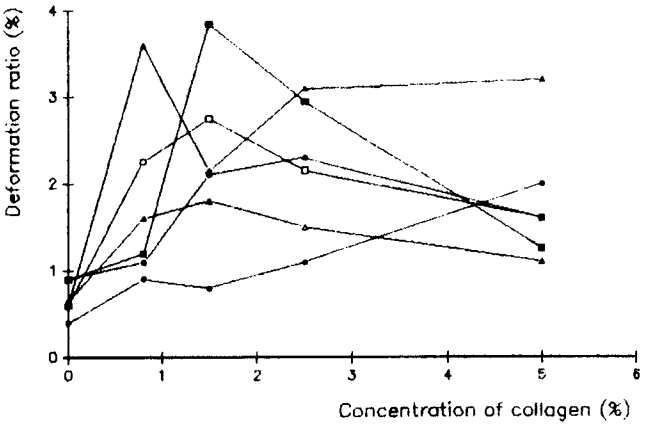

Figure 6. Deformation ratio versus collagen concentration of the composites.
o—o, △—△, □—□ -- No.1,2,3 HAP-collagen composites, directions of stress vertical to the pores

●—●, ▲—▲, ■—■ -- No.1,2,3 HAP-collagen composites, directions of stress parallel to the pores

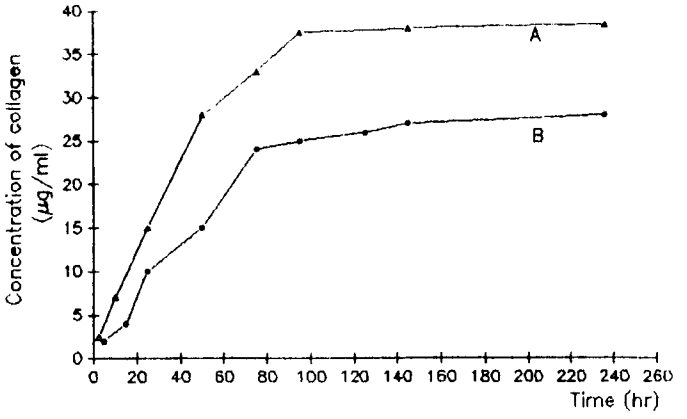

Figure 7. In vitro degradation curves of the composite and collagen. A—collagen, B—composite.

o—o, △—△, □—□ -- No.1,2,3 HAP-collagen composites, directions of stress vertical to the pores

●—●, ▲—▲, ■—■ -- No.1,2,3 HAP-collagen composites, directions of stress parallel to the pores

So it is possible to control the degradation rate of HAP-composites by controlling the cross-linking conditions and degradation rate of the collagen. Previous in vivo study of collagen protein reports that pure collagen starts to degrade at about 15 days after implantation. This roughly matches the growth rate of natural hard tissues. It is thus predicted that after the HAP-collagen composite is implanted, the collagen component will degrade concomitantly with the growth of the bones. So at the early stage of implantation, it can reinforce the material matrix, induce the growth of tissues and gradually disappear during the course of tissue regeneration. Then the collagen will be completely replaced and new bone which consists of natural bone tissues and HAP ceramics will be ultimately formed.

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