Yonsei Medical Journal
Vol. 41, No. 4, pp. 468−476, 2000

A Bone Replaceable Artificial Bone Substitute: Morphological and Physiochemical Characterizations

Jong-Chul Park, Dong-Wook Han, and Hwal Suh

Abstract

A composite material consisting of carbonate apatite (CAp) and type I atelocollagen (AtCol) (88/12 in wt/wt%) was designed for use as an artificial bone substitute. CAp was synthesized at 58°C by a solution-precipitation method and then heated at either 980°C or 1,200°C. In this study, type I AtCol was purified from bovine tail skins. A CAp-AtCol mixture was prepared by centirfugation and condensed into composite rods or disks. The scanning electron-microscopic (SEM) characterization indicated that the CAp synthesized at 58°C displayed a crystallinity similar to that of natural bone and had a high porosity (mean pore size: about 3−10 μm in diameter). SEM also revealed that the CAp heated at 980°C was more porous than that sintered at 1,200°C, and the 1,200°C-heated particles were more uniformly encapsulated by the AtCol fibers than the 980°C-heated ones. A Fourier transformed-infrared spectroscopic analysis showed that the bands characteristic of carbonate ions were clearly observed in the 58°C-synthesized CAp. To enhance the intramolecular cross-linking between the collagen molecules, CAp-AtCol composites were irradiated by ultraviolet (UV) ray (wave length 254 nm) for 4 hours or vacuum-dried at 150°C for 2 hours. Compared to the non cross-linked composites, the UV-irradiated or dehydrothermally cross-linked composites showed significantly ($p<0.05$) low collagen degradation and swelling ratio. Preliminary mechanical data demonstrated that the compressive strengths of the CAp-AtCol composites were higher than the values reported for bone.

Key Words: Bone substitute, carbonate apatite, type I atelocollagen, cross-linking, compressive strength

INTRODUCTION

Ceramic materials such as hydroxyapatite (HAp, $[Ca_{10}(PO_4)_6(OH)_2]$), carbonate apatite (CAp, $[Ca_{10}(PO_4)_6CO_3]$), or -tricalcium phosphate (β-TCP) are currently available for orthopedic applications involving grafting or defect filling to restore bone tissue.[1-5] The results of a large number of investigations confirm the acceptable biocompatibility of HAp[6,7] and verify that implants of this material are osteoconductive. Its biocompatibility makes HAp attractive for use in medical applications[1,2] involving both orthopedics and dentistry. An implanted HAp surface is chemically and directly integrated into the native bone, but a bony substitution is precluded due to the high density and brittleness of the HAp.[7] In contrast to HAp, β-TCP is a material foreign to living tissue, and thus is likely to be phagocytosed by macrophages, evoking a local inflammatory response.[7,8]

CAp, one of the primary inorganic components, that composes some 60−70% of bone, has been shown to be an effective biomaterial for use as a bone filler.[9-11] However, the characteristics of low crystallinity and high solubility have been a barrier to the use of CAp in clinical practice. High temperature crystal fusion of calcium phosphate, or sintering,[12-14] results in more suitable dense, porous, or particulate forms of apatites. Because of insufficient bending strengths, dense sintered apatites have seldom been used as bone implant materials.[15] Porous apatites are more easily carved than dense ones, and the pores presumably provide a mechanical interlock by the regenerated new bone, leading to a firmer initial fixation of the implants.[16] For periodontal osseous lesion repair and alveolar ridge augmentation, particulate forms are preferred, primarily because they are easier to handle. Since the presence of carbonate in apatite lattice is known to increase chemical reactivity

Received January 24, 2000
Accepted April 15, 2000
Department of Medical Engineering, Yonsei University College of Medicine, Seoul, Korea.
This study was supported by the Ministry of Health and Welfare of the Republic of Korea (Grant No. HMP-97-B-2-0015).
Address reprint request to Dr. H. Suh, Department of Medical Engineering, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel: 82-2-361-5406, Fax: 82-2-363-9923, E-mail: hwal@yumc.yonsei.ac.kr

in weak acids and would probably contribute to the ease of resorption in bony tissue,[17] CAp would have a more favorable biological response than HAp as a bone substitute material.

Collagen, a structural protein of bone, has been used clinically as an effective biomaterial for numerous biomedical applications.[18,19] Cells can live in contact with collagen fiber because it is an extracellular matrix and has cell adhesive properties. However, collagen imparts a highly sensitive immunity by the telopeptide chains that exist at both extremities of the molecule. However, a telopeptide-free collagen, a so called atelocollagen (AtCol), is known to be non-immunogenic.[11,19,20] Additionally, atelopeptide type I collagen was previously shown to be an effective carrier[21,22] for bone morphogenetic proteins which produce ectopic osteoinduction.

This study was intended to develop a composite material consisting of pre-heated CAp and type I AtCol extracted from bovine tail skins and to characterize its morphological, physiochemical, and biomechanical properties.

MATERIALS AND METHODS

Unless otherwise specified, all reagents used for CAp synthesis, collagen extraction, and apatite-collagen composite fabrication were purchased from Sigma (St. Louis, Mo., USA).

Synthesis of carbonate apatite (CAp)

CAp was synthesized by the previously reported method.[11] A volume of 0.058 M ammonium carbonate solution was mixed with an equal volume of 0.12M ammonium phosphate solution. Using a peristaltic pump (Micro Tube Pump; EYELA, Tokyo, Japan), this mixed solution and 0.2 M calcium acetate solution were added to 1.3 M ammonium acetate solution, which was pre-heated to 58°C within a mantle heater, at a rate of 280 ml/hr and adjusted to pH 7.4±0.1 by adding 5 N NaOH solution. The resultant precipitate was centrifuged at 3,200×g for 30 minutes and washed with distilled water for 24 hours. The washed precipitate was vacuum-dried at 58±2°C. The powder obtained from the vacuum-drying was placed into a platinum crucible in an electric furnace (JELRUS, Model 16000 and 7115, Hicksville, NY, USA) that was programmed as follows; dry time : 2 min, low temperature : 400°C, high temperature : 980°C or 1,200°C, heat rate : 60°C/min, hold time no vacuum : 2 min (at 980°C or 1,200°C), cool time : 20 min. The resulting products were the 980°C-heated CAp and the 1,200°C-sintered CAp powders, respectively.

Extraction of type I atelocollagen

As previously described by Suh et al.,[19] type I AtCol was extracted from bovine tail skins. After the purification of type I AtCol by differential salt precipitation, the collagen precipitate was lyophilized at −40°C and kept at 4°C.

Preparation of apatite-collagen composite

The lyophilized type I AtCol was dissolved in 0.001 N HCl solution at 4°C for 1 day and buffered to pH 7.4 by adding 0.05 N NaOH solution. The CAp powders heated at 980°C or 1,200°C were poured into the 0.1 wt% collagen solution and stirred at 300 rpm for 1 hours with an electronic stirrer equipped with a Teflon blade. The CAp to AtCol ratio was held at 88 to 12 in weight. After controlling the pH to 7.4 by adding 0.05 N HCl solution, the mixture was centrifuged at 3,000×g for 30 minutes at 4°C and the resulting precipitate was referred to as a CAp-AtCol composite. The composite was condensed in a polytetrafluoroethylene mold to produce disks (diameter 15 mm×thickness 2.5 mm and diameter 10 mm×thickness 5 mm) or rods (diameter 5 mm×height 10 mm) (Fig. 1). To increase the collagen fibrillar cross-links, the obtained specimens were irradiated by ultraviolet (UV) ray (wave length 254 nm) at 4°C for 4 hours (UV-irradiation method[7,8]) or vacuum-dried at 150°C for 2 hours (dehydrothermal (DHT) method[22]). The specimens were placed in a self-designed UV chamber (DBO231S, Daeil, Seoul, Korea) and irradiated by eight surrounding 10 W UV bulbs. The distance between the light sources and the specimen was 5 inches. As a result, four groups of CAp-AtCol composites were prepared; 980- or 1,200-UV groups (composites composed of 980°C- or 1,200°C-heated CAp and AtCol and cross-linked by UV-irradiation method), and 980- or 1,200-DHT groups (cross-linked by DHT method).

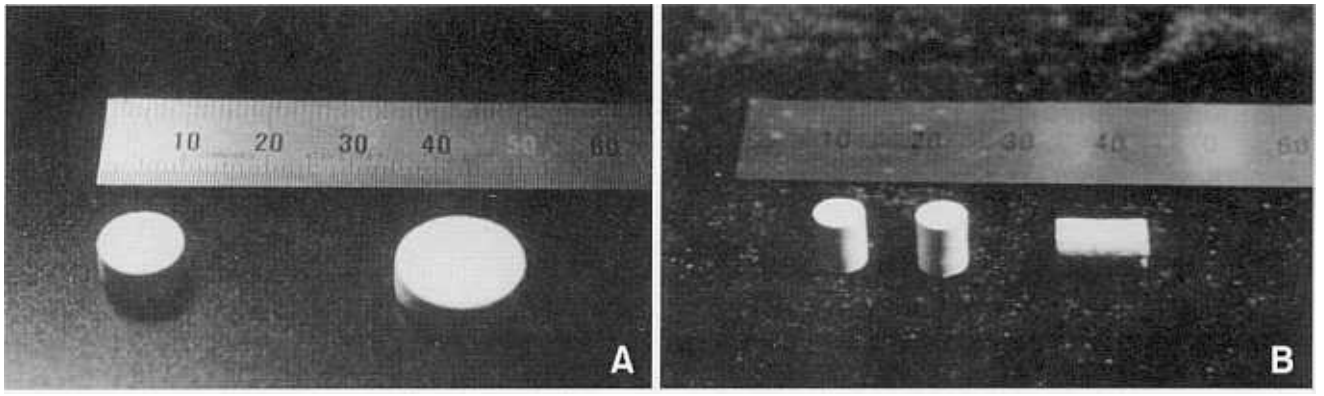

Fig. 1. The CAp-AtCol composite disks (A) and rods (B).

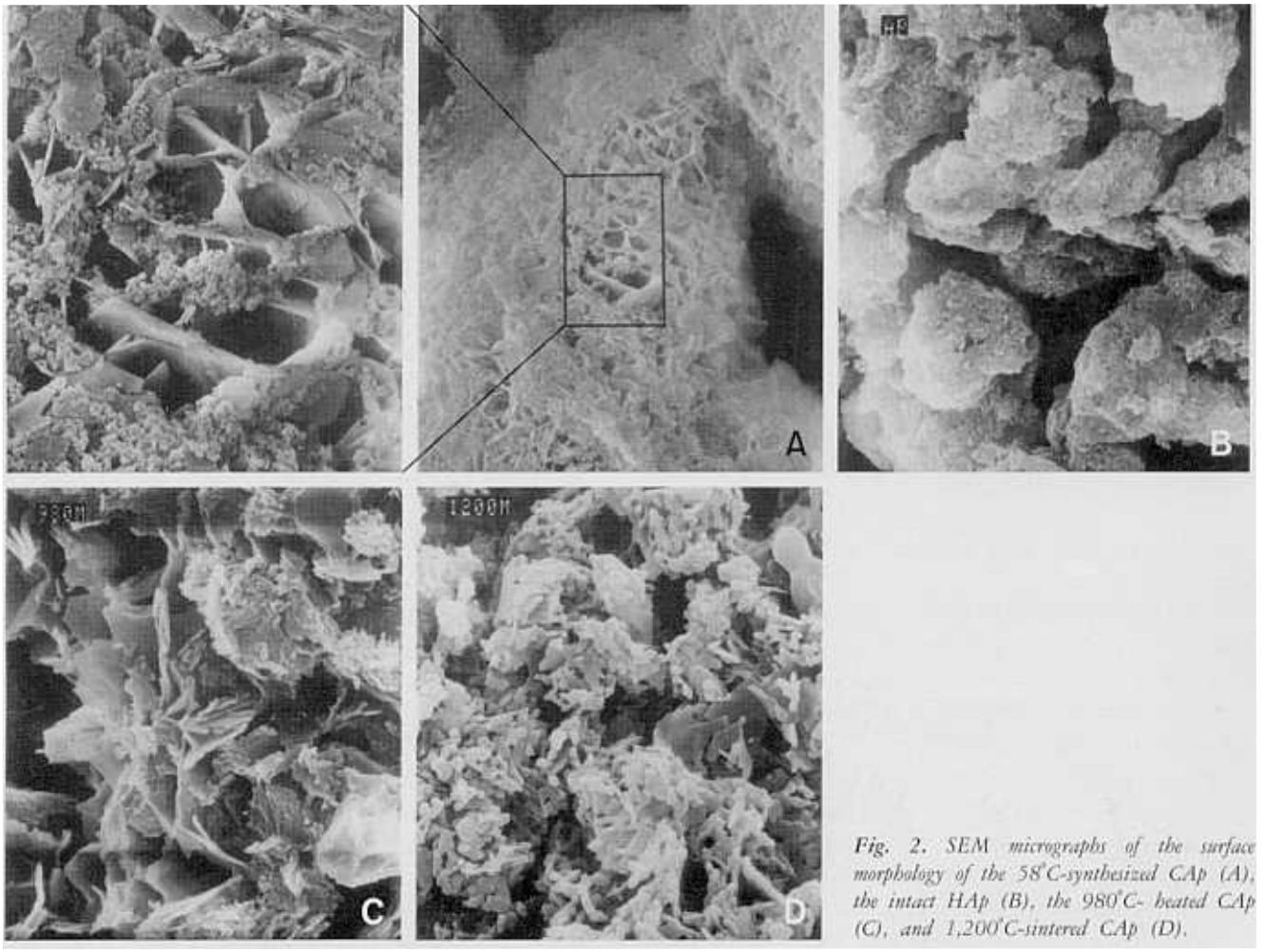

Fig. 2. SEM micrographs of the surface morphology of the 58°C-synthesized CAp (A), the intact HAp (B), the 980°C- heated CAp (C), and 1,200°C-sintered CAp (D).

Scanning electron microscopy (SEM) observation

The surface morphologies of the 58°C-synthesized CAp powder, the 980°C- or 1,200°C-heated CAp powder, and the fabricated apatite-collagen composite were examined with a field emission scanning microscope (Hitachi S-800, Tokyo, Japan). The specimens were mounted and sputter-coated with gold using an

ion coater, and then observed at an accelerating voltage of 20 kV. For purposes of comparison, intact HAp (Sigma) was also observed.

Infrared (IR) spectroscopy analysis

For the 58°C-synthesized CAp powder and the 980°C- or 1,200°C-heated CAp powder, the infrared (IR) spectra of the specimens were obtained between 4,000 and 500 cm^{-1} using a Shimazu FTIR-4200 spectrophotometer. Intact HAp was also analyzed for comparison.

Collagen degradation test

In order to investigate the collagen degradation, four groups of CAp-AtCol composite disks (diameter 15 mm × height 2.5 mm) were used. The non cross-linked specimens were regarded as the controls. All specimens were immersed in 10 mM $CaCl_2$ solution containing 20 units/ml collagenase IA (Sigma) and incubated at 37°C for 12 and 24 hours with shaking

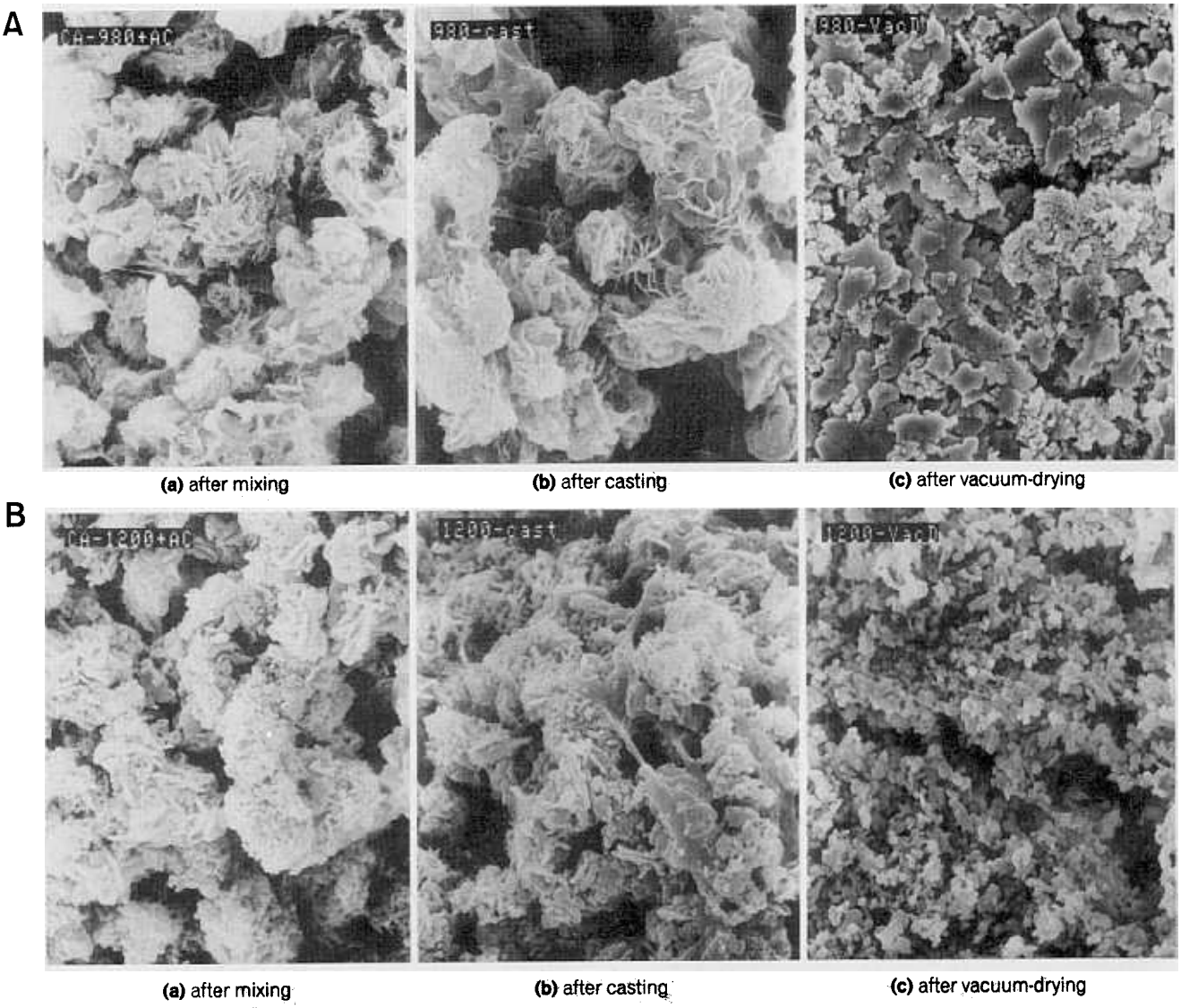

Fig. 3. SEM micrographs of the surface morphology of the composite with 980°C-heated CAp and AtCol (A) and the composite with 1,200°C-sintered CAp and AtCol (B) according to each process of composite preparation ((a): after mixing, (b): after casting, (c): after vacuum-drying).

at 200 rpm. After incubation, the amount of collagen digested by collagenase treatment was indirectly determined by Bradford assay.[23] Each specimen solution was mixed with Bradford reagents (Bio-Rad, Protein Assay Reagent, Hercules, CA., USA) at a ratio of 4 : 1 in volume and transferred into a 96-well plate. The absorbance of each well was measured at 610 nm by an automatic microplate (ELISA) reader (Spectra Max 340, Molecular Device Inc., Sunnyvale, CA., USA). The results of the test were examined statistically, in comparison with the control, using t-test.

Swelling (water uptake) test

In order to examine the water uptake, each specimen was put into a grilled basket after weighing and immersed in distilled water, followed by incubation at 37°C for 1, 2, 4, and 24 hours. The specimens were air-dried for 10 minutes, and then the increased weight due to the absorption of water was measured. The swelling ratio (mg/mg) was expressed as the increased weight against the original weight of the specimen. The results of the test were investigated statistically, compared with the control, using t-test.

Biomechanical property: Compressive strength

For the 980-UV and 1,200-UV groups of the CAp-AtCol composite disks (diameter 10 mm × thickness 5 mm), a universal mechanical testing machine (Instron model 8511, Instron Co., Canton,

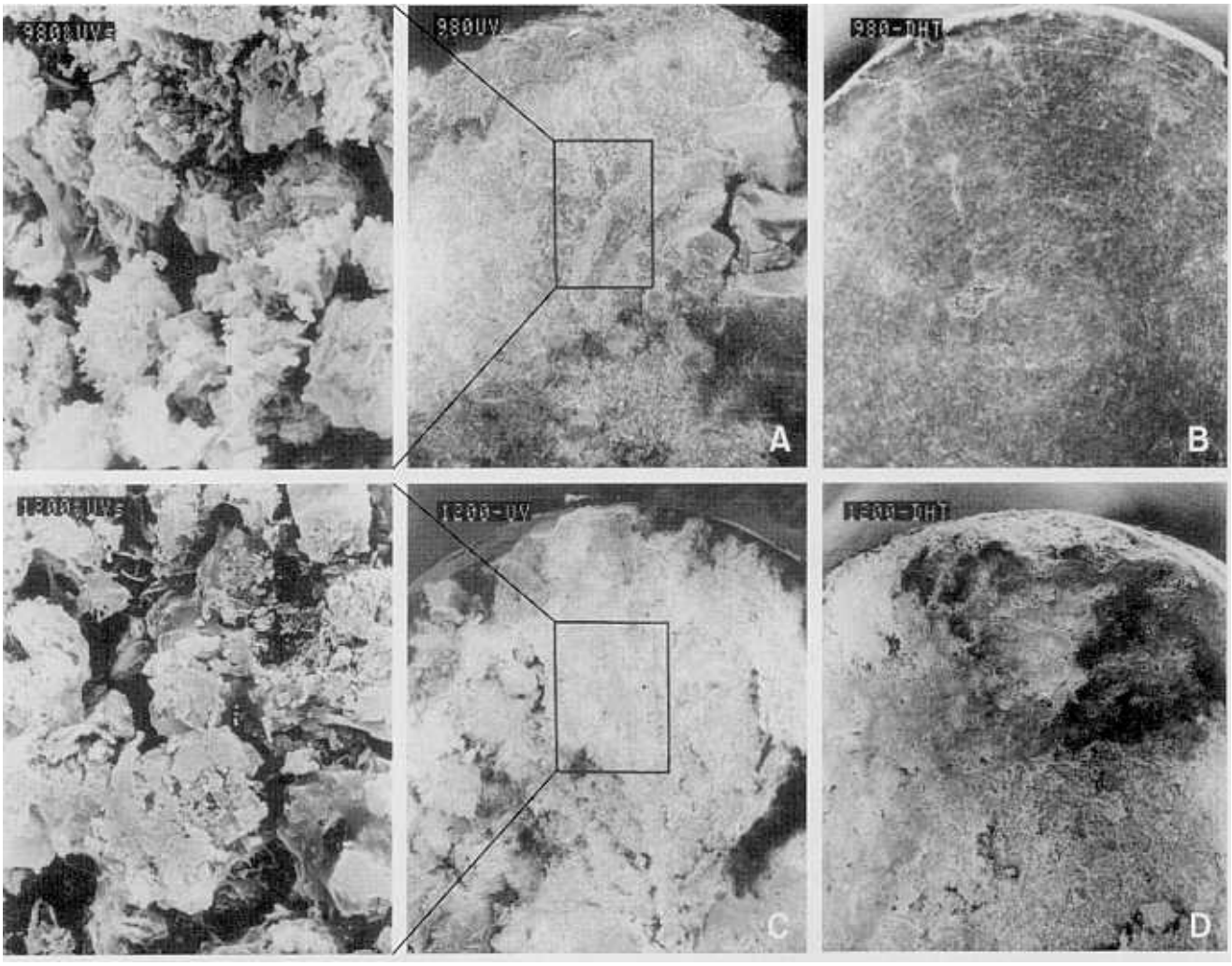

Fig. 4. The microstructures of the four groups of composites cross-linked by UV-irradiation or DHT method ((A): 980-UV, (B): 980-DHT, (C): 1,200-UV, (D): 1,200-DHT).

Mass., USA) equipped with a 2 ton load cell, and operating at a cross-head speed of 0.005 mm/sec and a sampling rate of 1 Hz was used to measure the compressive strengths.

RESULTS

Characterizations

For the purpose of comparison, the microstructural development in CAp and HAp is illustrated in Fig. 2. The SEM micrograph of the CAp synthesized at 58°C (Fig. 2A) showed that the CAp crystallites were highly porous and their pore size was about 3−10 μm in diameter, while the intact HAp (Fig. 2B) was apparently shown to be dense rather than porous. Compared to the CAp synthesized at 58°C, the porosity of the CAp heated at 980°C (Fig. 2C) was substantially maintained, and it seemed more porous than that sintered at 1,200°C (Fig. 2D). The surface morphology of the CAp-AtCol composites according to each process of composite preparation (after mixing, after casting, and after vacuum-drying) (Fig. 3) demonstrated that the type I AtCol molecules were randomly distributed between the CAp particles. After vacuum-drying, the 980C-heated CAp particles were roughly encapsulated by AtCol fibers (Fig. 3A(c)), while the 1,200°C-sintereded CAp demonstrated a uniform encapsulation by the AtCol (Fig. 3B(c)). As shown in Fig. 4A-D, the microstructures of the composites physically cross-linked by UV-irradiation or DHT method indicated the importance of both the cross-linking and the retention of the fibrillar collagen in the composite formation.

The IR spectra of both the intact and heated CAp are demonstrated in Fig. 5. For comparison, the IR spectrum of intact HAp is also included in Fig. 5. The broad, large bands in the range of 1,300−1,000 cm^{-1} and a band at −570 cm^{-1} that were specific to phosphate (PO_4^{3-}) groups were in all the specimens. The OH bands at 3,570 and 630 cm^{-1} were evident in the HAp but obscured in the CAp. In particular, a prominent single band in the range of 1,750−1,600 cm^{-1} that was characteristic of a water molecule was distinctly observed only in the intact HAp. The doublet-like bands in the range of 1,600 −1,400 cm^{-1} and a small band at −870 cm^{-1} that were characteristic for carbonate (CO_3^{2-}) ions were clearly observed in the intact CAp.[12] It was revealed that the width of the CO_3 band at −870 cm^{-1} decreased gradually as the heating temperature increased 980°C to 1,200°C. It also appeared that the CO_3 bands in the range of 1,600−1,400 cm^{-1} were slightly widened because the CO_3 groups were affected by the H_2O molecules.

Collagen degradation

The relative collagen degradation of non cross-linked and UV/DHT-cross-linked apatite-collagen composites is compared in Fig. 6. Even after 12 hours of collagenase treatment, the collagen degradation of the composites cross-linked by UV-irradiation or

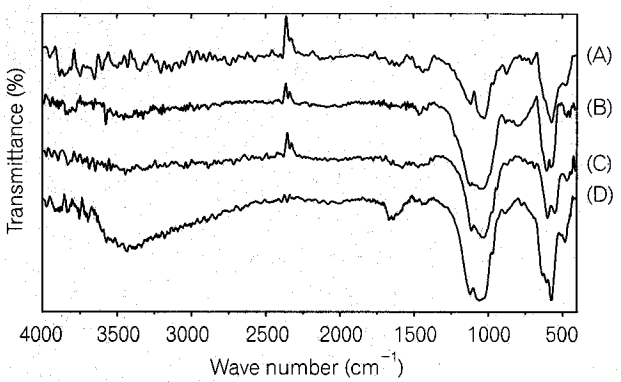

Fig. 5. FT-IR spectra of the 58°C-synthesized CAp (A), the 980°C-heated CAp (B), 1,200°C-sintered CAp (C), and the intact HAp (D).

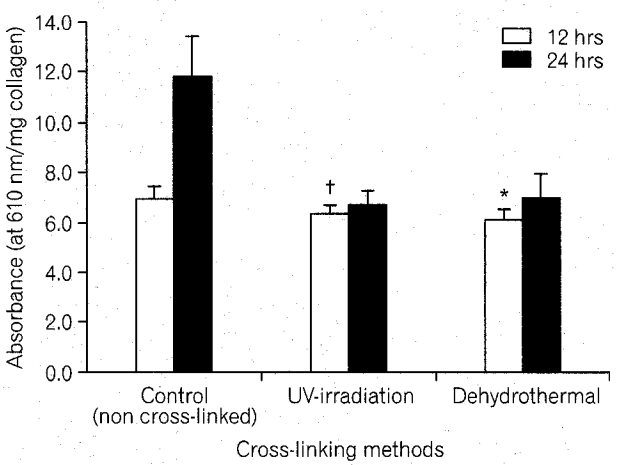

*Fig. 6. Comparison of the collagen degradation of apatite-collagen composites according to cross-linking methods. *$p < 0.05$ and †$p < 0.005$ vs. control ($n=6$).*

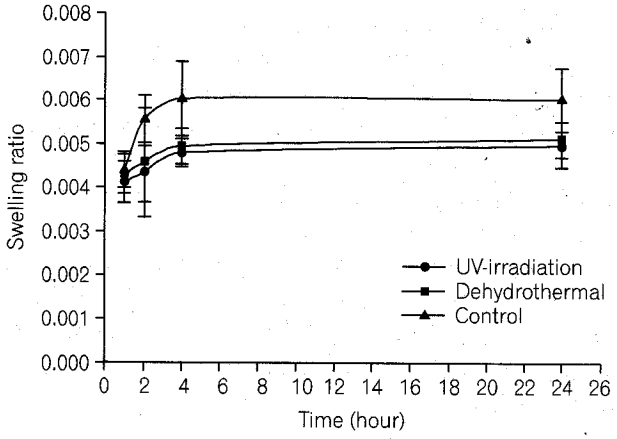

Fig. 7. Comparison of the swelling ratio of apatite-collagen composites according to cross-linking methods.

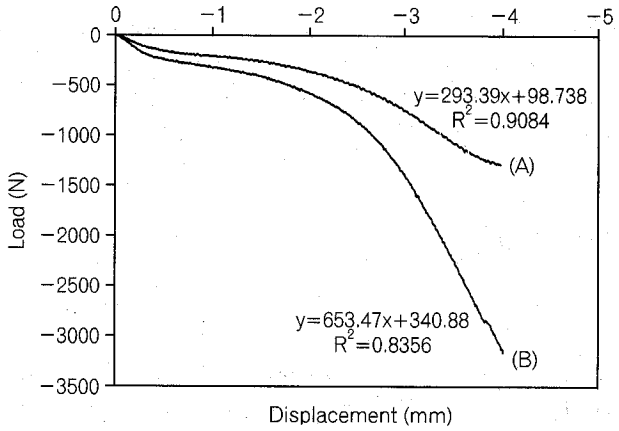

Fig. 8. The compressive strengths of the 980-UV groups (A) and the 1,200-UV groups (B) of the CAp-AtCol composite disks (diameter 10×5 mm thickness).

DHT method showed significant ($p<0.005$ or $p<0.05$) differences from that of the non cross-linked controls. As the time of enzyme treatment increased up to 24 hours, these differences were more significantly distinctive. In terms of the cross-linking methods, there was no significant difference between the UV-irradiation method and the DHT method.

Swelling ratio

The swelling ratios of the apatite-collagen composites were compared according to the cross-linking methods (Fig. 7). It would seem that the swelling ratios of the composites cross-linked by UV-irradiation or DHT method were slightly increased at 4 hours, while those of the non cross-linked controls were sharply increased. The swelling ratios of the cross-linked composites were significantly lower than the controls ($p<0.05$, $n=4$). After 4 to 24 hours, the water uptake of all the specimens was consistently maintained.

Measurement of compressive strength

The mechanical properties for the 980-UV and 1,200-UV groups of composite disks (diameter 10 mm × thickness 5 mm) was investigated respectively. As a result of this study, it was shown that the compressive strength of the 1,200-UV composite disk was approximately 2 times higher than that of the 980-UV group (Fig. 8). It was also found that the compressive strength of both groups was in the higher range of those reported for bone.

DISCUSSION

The calcium phosphate family of ceramics such as HAp, β-TCP, and CAp has been shown to be an effective biomaterial as a bone substitute material. However, synthesizing these calcium phosphates as powders limits their clinical use.[24,25] For practical applications, dense, porous, or particulate forms have been prepared by subjecting the compacted apatite powders to a high temperature fusion process called sintering. Based on the knowledge of the importance of the carbonate presence in the apatite lattice, CAp may be superior to HAp or β-TCP among these ceramics. In this study, a highly porous CAp with a chemical composition and crystallinity similar to those of bone was successfully synthesized, and its surface morphology was characterized by SEM observation (Fig. 2). The existence of carbonate ions, known to increase the chemical reactivity in weak acids and contribute to easy resorption in bony tissue, were also clearly observed in the synthesized CAp under IR spectroscopic analysis (Fig. 5).

As natural polymers, collagens are the main component of the organic substrate in bone, and are structural proteins that participate in the assembly of various kinds of macromolecules in the extracellular matrix. Collagen, specifically type I, is an attractive molecule for manufacturing biomaterials due to its favorable biological properties.[18,19,25] It has the ability

to support cell adhesion and plays a crucial role in cell proliferation and tissue remodeling. Collagen can also serve as a matrix in which the particles of CAp or HAp are anchored. In particular, composites composed of apatite and collagen show great promise as biomaterials, since bone is a natural composite primarily composed of these two phases.[25] However, in contrast to bone, a firm bonding between these phases cannot be established in composites.

In this study, the heated CAp powders were mixed with the bovine collagen solution, the antigenicity of which had been removed by enzymatic treatment, and formed into apatite-collagen pellets. The 980°C-heated CAp particles had a coarse surface morphology with a rough encapsulation by AtCol, but the 1,200°C-sintered CAp particles were uniformly encapsulated. These different features would be related to the pores in the particles. The extremely high viscosity of the AtCol molecules was interrupted to be infiltrated into the pores of 980°C-heated CAp particles, which were larger than the particles sintered at 1,200°C, and this resulted in an indentation-rich surface. However, the pore-less 1,200°C-sintered particles could be encapsulated by the AtCol molecular fibers in a compact and uniform appearance. To overcome the weak bonding between apatite and collagen, the fabricated apatite-collagen composites were physically cross-linked by UV-irradiation or DHT method. Fibrillar collagen must remain structurally stable for a few weeks in order to act as a scaffold for cell adhesion and proliferation. To protect against proteolytic digestion *in vivo*, conventional collagen-based biomaterials are usually cross-linked using chemicals such as glutaraldehyde or hexamethylene diisothiocyanate.[22] However, unreacted chemicals used for the cross-linking gradually leach from the collagen implant, increasing the duration and intensity of the inflammatory response and cytotoxicity, thereby delaying cellular infiltration of the collagen. Both the collagen degradation test and swelling test further support the importance of collagen cross-linking in the composite formation.

While a very broad range of values have been reported for the mechanical properties of bone and other tissues,[26,27] mechanical property data for synthetic CAp-AtCol composites could not be found in the literature. The yield strengths of wet human bone under flexural loading in the transverse direction have been measured to range from 7−120 MPa by Rice et al.[28] Curry and Brear[29] reported Young's modulus for animal bones to range from 2−50 GPa. The compressive strengths of the CAp-AtCol composites fabricated in this study were in the higher range of those reported for bone.

In conclusion, the present study shows that a composite composed of pre-heated CAp and non-immunogenic AtCol possesses excellent physiochemical and biomechanical properties when cross-linked without chemicals and the CAp-AtCol composite can be expected to be a compatible substitute of bone grafts.

REFERENCES

1. Ducheyne P, Lemmons JE. Bioceramics: Material characteristics versus in vivo behavior. Ann NY Acad Sci 1985; 523:219-36.
2. Heimke G. Advanced ceramics for biomedical applications. Angrew Chem Int Ed Engl 1989;28:111-6.
3. Flahiff CM, Blackwell AS, Hollis JM, Feldman DS. Analysis of a biodegradable composite for bone healing. J Biomed Mater Res 1996;32:419-24.
4. Higashi S, Yamamuro T, Nakamura T, Ikada Y, Hyon SH, Jamshidi K. Polymer-hydroxyapatite composites for biodegradable bone fillers. Biomaterials 1986;7:183-7.
5. Zhang QQ, Ren L, Wang C, Liu LR, Wen XJ, Liu YH, et al. Porous hydroxyapatite reinforced with collagen protein. Artif Cells Blood Substit Immobil Biotechnol 1996;24:693-702.
6. Bagambisa FB, Joos U, Schilli W. Mechanisms and structure of the bond between bone and hydroxyapatite ceramics. J Biomed Mater Res 1993;27:1047-55.
7. TenHuisen KS, Martin RI, Klimkiewicz M, Brown PW. Formation and properties of a synthetic bone composite: Hydroxyapatite-collagen. J Biomed Mater Res 1995;29: 803-10.
8. Miyamoto Y, Ishikawa K, Takechi M, Toh T, Yuasa T, Nagayama M, et al. Basic properties of calcium phosphate cement containing atelocollagen in its liquid or powder phases. Biomaterials 1998;19:707-15.
9. Okazaki M, Ohmae H, Hino T. Insolubilization of apatite-collagen composites by UV irradiation. Biomaterials 1989; 10:564-8.
10. Okazaki M, Ohmae H, Takahashi J, Kimura H, Sakuda M. Insolubilized properties of UV irradiated CO_3 apatite-collagen composites. Biomaterials 1990;11:568-72.
11. Suh H, Lee CK. Biodegradable ceramic-collagen composite implanted in rabbit tibiae. ASAIO J 1995;41:M652-M6.
12. Rao WR, Boem RF. A study of sintered apatites. J Dent Res 1974;53:1351-4.
13. Ellies LG, Nelson DG, Featherstone JD. Crystallographic structure and surface morphology of sintered carbonated apatites. J Biomed Mater Res 1988;22:541-53.

14. Tatsumi J, Kurihara N, Kanai A, Takahashi T, Ikeda K. Clinical application of sintered bone (II). Effects of collagen coating on implant materials in vitro. Nippon Shishubyo Gakkai Kaishi 1989;31:200-12.
15. deWith G, van Dijk HJA, Hattu N, Pris D. Preparation, microstructure and mechanical properties of dense polycrystalline hydroxyapatite. J Mater Sci 1981;16:1952-8.
16. De Groot K. Bioceramics consisting of calcium phosphate salts. Biomaterials 1980;1:47-50.
17. Doi Y, Koda T, Wakamatsu N, Goto T, Kamemizu H, Moriwaki Y, et al. Influence of carbonate on sintering of apatites. J Dent Res 1993;72:1279-84.
18. Suh H. Treatment of collagen for tissue regenerative scaffold. Biomed Eng Appl Basis Comm 1999;11:167-73.
19. Suh H, Suh SW, Min BG. Anti-infection treatment of a transcutaneous device by a collagen-rifampicine composite. ASAIO J 1994;40:M406-M11.
20. Li S, Zheng Z, Liu Q, de Wijn JR, de Groot K. Collagen/apatite coating on 3-dimensional carbon/carbon composite. J Biomed Mater Res 1998;40:520-9.
21. Kusumoto K, Bessho K, Fujimura K, Konishi Y, Ogawa Y, Iizuka T. Self-regenerating bone implant: ectopic osteoinduction following intramuscular implantation of a combination of rhBMP-2, atelopeptide Type I collagen and porous hydroxyapatite. J Craniomaxillofac Surg 1997; 24:360-5.
22. Omura S, Mizuki N, Kawabe R, Ota S, Kobayashi S, Fujita K. A carrier for clinical use of recombinant human bone morphogenetic protein-2: dehydrothermally cross-linked composite of fibrillar and denatured atelocollagen sponge. Int J Oral Maxillofac Surg 1998;27:129-34.
23. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
24. Doi Y, Shibutani T, Moriwaki Y, Kajimoto T, Iwayama Y. Sintered carbonate apatites as bioresorbable bone substitutes. J Biomed Mater Res 1998;39:603-10.
25. Doi Y, Horiguchi T, Moriwaki Y, Kitago H, Kajimoto T, Iwayama Y. Formation of apatite-collagen complexes. J Biomed Mater Res 1996;31:43-9.
26. Suh H, Hwang YS, Lee JE, Kim KT, Park J-C, Park KD, et al. Type I atelocollagen grafting on polyurethane tube and it's mechanical property. Biomater Res 1998;2:158-62.
27. Suh H, Park J-C, Kim KT, Lee WK, Cho BK. Mechanical properties of the UV irradiated porcine valves. Biomater Res 1998;2:95-9.
28. Rice JC, Cowin SC, Bowan JA. On the strength dependence of the elasticity and strength of cancellous bone on apparent density. J Biomech 1988;21:155-68.
29. Curry JD, Brear K. Hardness, Young's modulus and yield stress in mammalian mineralized tissue. J Mater Sci Mater Med 1990;1:14-20.