JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 13 (2002) 993–997

The cross-linkage effect of hydroxyapatite/collagen nanocomposites on a self-organization phenomenon

M. C. CHANG[1,3], T. IKOMA[2,3], M. KIKUCHI[2,3], J. TANAKA[2,3]
[1]*Kunsan National University, Kunsan 573-701, Korea*
[2]*NIRIM, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*
[3]*CREST, Japan Science and Technology Corporation, Japan*

Hydroxyapatite(HAp)/collagen nanocomposites were prepared by a coprecipitation method controlling the degree of cross-linkage between collagen molecules using glutaraldehyde. The precipitates filtered were dried in a freeze drier or naturally dried in the air at 25 °C. The naturally dried cakes had open channels of 5–15 μm in diameters, which were three-dimensionally and regularly developed over the whole samples, and showed a pretty good mechanical strength. The channels that were formed at spaces among the HAp/collagen particles, cross-linked one another, which had been filled up with water before its evaporation. The ordering state of the open channels depended on the degree of cross-linkage with glutaraldehyde; the optimal self-organized state was found when 30 molecules of glutaraldehyde were added per collagen molecule, though an excess amount of glutaraldehyde suppressed the appearance of the ordered state. From SEM and FT-IR measurements, it was indicated that the self-organization in the HAp/collagen nanocompsites continuously occurred during the drying process together with the removal of water and the increase of the density.

Introduction

In order to develop noble bone substitutes, hydroxyapatite (HAp) has been intensively investigated from the viewpoints of biocompatibility, bioactivity, biodegradation and osteoconductivity similar to bone [1–4]. The composites of HAp and some polymers, e.g. PMMA, polylactide, chondroitin sulfates, chitosan and collagen, could improve the above properties [5–13]. Among those materials, especially the composite of HAp and collagen had a quite similar microscopic structure to bone [8]. Bone is an extracellular matrix in which HAp nanocrystals align their *c*-axes along collagen fibers. Such nanostructure of HAp embedded in collagen could have been reproduced through a self-organization process in a biomimetic coprecipitation method [8]. The self-organization mechanism of HAp/collagen composites has been studied by some authors [14, 15].

In the present paper, we tried to develop a porous structure of HAp/collagen nanocomposite by controlling the degree of cross-linkage with glutaraldehyde. The ordered porous structure obtained was analyzed as functions of cross-linkage and drying condition in relation to the self-organization.

Experimental procedure

The starting materials used were $CaCO_3$ (Alkaline analysis grade, Wako, Japan), H_3PO_4 (AP grade, Wako, Japan) and glutaraldehyde (25% aqueous solution, AP grade, Wako, Japan). Collagen (Nitta Gelatin, Japan: MW = 300 000) was extracted from porcine dermis; as its telopeptide was removed by treating with an enzyme, the collagen molecule was 300 nm in length with a triply helical chain. The collagen was diluted in a phosphoric water solvent of pH 2.23 to be 20 mM.

Pure $Ca(OH)_2$ was obtained through the hydration of CaO calcined at 1150 °C for 3 h [10]. HAp/collagen composite was prepared by a simultaneous titration method under constant pH described by Kikuchi *et al.* [8]. A $Ca(OH)_2$ aqueous suspension of 99.7 mM and a H_3PO_4 aqueous solution of 59.7 mM with collagen of 5 g were gradually added into a reaction vessel through tube pumps while controlling pH 8.4. The temperature of the reaction vessel was set at 38 °C and the weight ratio of HAp/collagen was fixed at 80/20. After the coprecipitation process, the slurry obtained was aged at 38 °C for 12 h and pH was gradually lowered to 7.0. Then an aqueous solution of glutaraldehyde (0.2%) was

slowly dropped into the slurry solution at 38 °C; the total amount of glutaraldehyde was regulated to be 30, 90, 300 and 600 molecules per collagen molecule. The samples obtained were hereafter denoted by HCG30, HCG90, HCG300 and HCG600, respectively; the pure collagen and the HAp/collagen composite without cross-linkage were abbreviated as COL and HAp/COL, respectively.

The HAp/collagen slurry was filtered using a glass filter and gently washed five times with ion-exchanged water. The precipitates obtained were dried in a freeze dryer (Advantage, VirTis, USA) at −30 °C under vacuum, or were naturally dried in the air at 25 °C. The samples dried were dipped in ion-exchanged water or in simulated body fluid [12] in order to check dissolution and water absorption.

The microstructures of the composites were observed by a scanning electron microscope (SEM; TOPCON, Japan). A chemical interaction between HAp nanocrystal surfaces and functional groups of collagen was evaluated using a diffuse reflection FT-IR (Spectrum 2000, Perkin-Elmer, UK). Thermal analyses (TG8120, Rigaku, Japan) were performed for dried samples between 25 °C and 800 °C at a heating rate of 10 °C/min to evaluate the cross-linkage; all measurements were carried out in open Pt pans in the air using a Al_2O_3 powder of 10 mg as a reference. Thermo-gravimetric and differential thermal analysis (TG-DTA) data were analyzed with an associated computer (software: ThermoPlus-2, Ragaku, Japan). FT-IR and TG-DTA data were measured for the collagen and HAp/collagen freeze-dried, and for the HCG30, HCG90, HCG300 and HCG600 naturally dried.

Three-point bending strength and Young's modulus were measured by a universal testing machine (AGS-H, Shimadzu, Japan) at a cross-head of 500 μm/min with a span of 15 mm for the samples of $5 \times 3 \times 20\,mm^3$ in size.

Results and discussion

Fig. 1 shows the SEM photographs of the samples prepared. Fig. 1(a) shows a well-known porous network structure of collagen. Fig. 1(c) is the microstructure of a freeze-dried HCG30, and Figs. 1(b) and (d)–(f) those of naturally dried samples. Peculiar pore structures could be found for the cross-linked samples; though pores were randomly distributed in the freeze-dried sample (c), a channel structure with a spacing of about 100 μm were found parallel to a glass filter plane in the naturally dried samples (d, e). As many open pores were found in the plane (b) which was perpendicular to the planes (d, e), it was considered that the open pores formed columnar channels in matrices. In a higher magnified structure (f), many small pores were observed in the channels; therefore, the open channels probably formed a 3D network.

Glutaraldehyde ($OHCCH_2CH_2CH_2CHO$) has two functional groups (–CHO) to be able to link with $\varepsilon-NH_2$ groups of lysine or hydroxylysine residues in collagen. It is known that all available free amine groups react with glutaraldehyde to form Schiff's bases within 5 min [16–18]. A collagen molecule of type I has approximately 3000 ε-amine groups. When glutaraldehyde was added into the composite slurry during the preparation process, the size of precipitated particles was found to grow. According to Kikuchi *et al*. [8], such precipitated particles formed the assembly in which many HAp nanocrystals of about 50 nm in size were aligned around collagen molecules of 300 nm in length, forming bundles. Therefore, it is considered that the cross-linkage is formed between the bundles of HAp/collagen composites. The color of the HAp/collagen composite was essentially white, and the color changed from pale yellow to slightly dark yellow with the increase in the cross-linkage.

In the reaction process between glutaraldehyde and bundles, a part of glutaraldehyde molecules could not probably react at their both ends due to the hindering effect of HAp nanocrystals and/or hydrated water around collagen molecules. When the samples were dried after filtering, gel-like bundles lost the hydrated water bound around HAp nanocrystals or collagen molecules; as a result, an average spacing between the bundles shrank and the free glutaraldehyde molecules could gradually react to enable further cross-linkage and long-range polymeric networks. Especially, in the case of naturally dried samples, bundles were well packed and continuously polymerized during the drying process resulting in the formation of the rod-shaped spaces. From Fig. 1(c), the size of the polymerized bundles was estimated to be about 30 μm and that of the rod-shaped spaces distributed between 5 and 15 μm.

The chemical interaction in the HAp/collagen system was probed by the red shift of the FT-IR peak observed at $1340\,cm^{-1}$ which was ascribed to an antisymmetric stretching mode of $–COO^-$ in collagen [8]. As shown in Fig. 2 the wave number shifted to a lower value with the cross-linkage; i.e. $1339\,cm^{-1}$ for the pure collagen, $1334\,cm^{-1}$ for the HAp/collagen composite without cross-linkage and $1331\,cm^{-1}$ for the HCG30 and HCG90. When the cross-linkage was further high (HCG300, HCG600), however, the chemical shift oppositely decreased, corresponding to the reduction of structural ordering observed by SEM. The self-organization mechanism was therefore enhanced by the cross-linkage below 90 molecules of glutaraldehyde per collagen molecule.

Fig. 3 shows thermal analysis data for a lower temperature region; the temperature T_d in Fig. 3(b) was evaluated from the DTA curves as shown in Fig. 3(a), i.e. the temperature for the dehydration process. The dehydration temperatures increased with the increase in cross-linkage (Fig. 3(b)): here, the abscissa is the number of glutaraldehyde molecules per collagen molecule. This result suggests that the cross-linked network structure caused the removal of the hydrated water from the gels. As the temperature T_d was nearly saturated between 90 and 300 molecules, the optimal number of glutaraldehyde molecules was limited below 300.

The hydrated water in the dried samples were also estimated from thermo-gravimetric weight loss (Fig. 4(a)). From Fig. 4(b) the water content sharply increased even for the formation of small amount of cross-linkage (HCG30), and then the content moderately increased with the cross-linkage. As seen from weight loss at

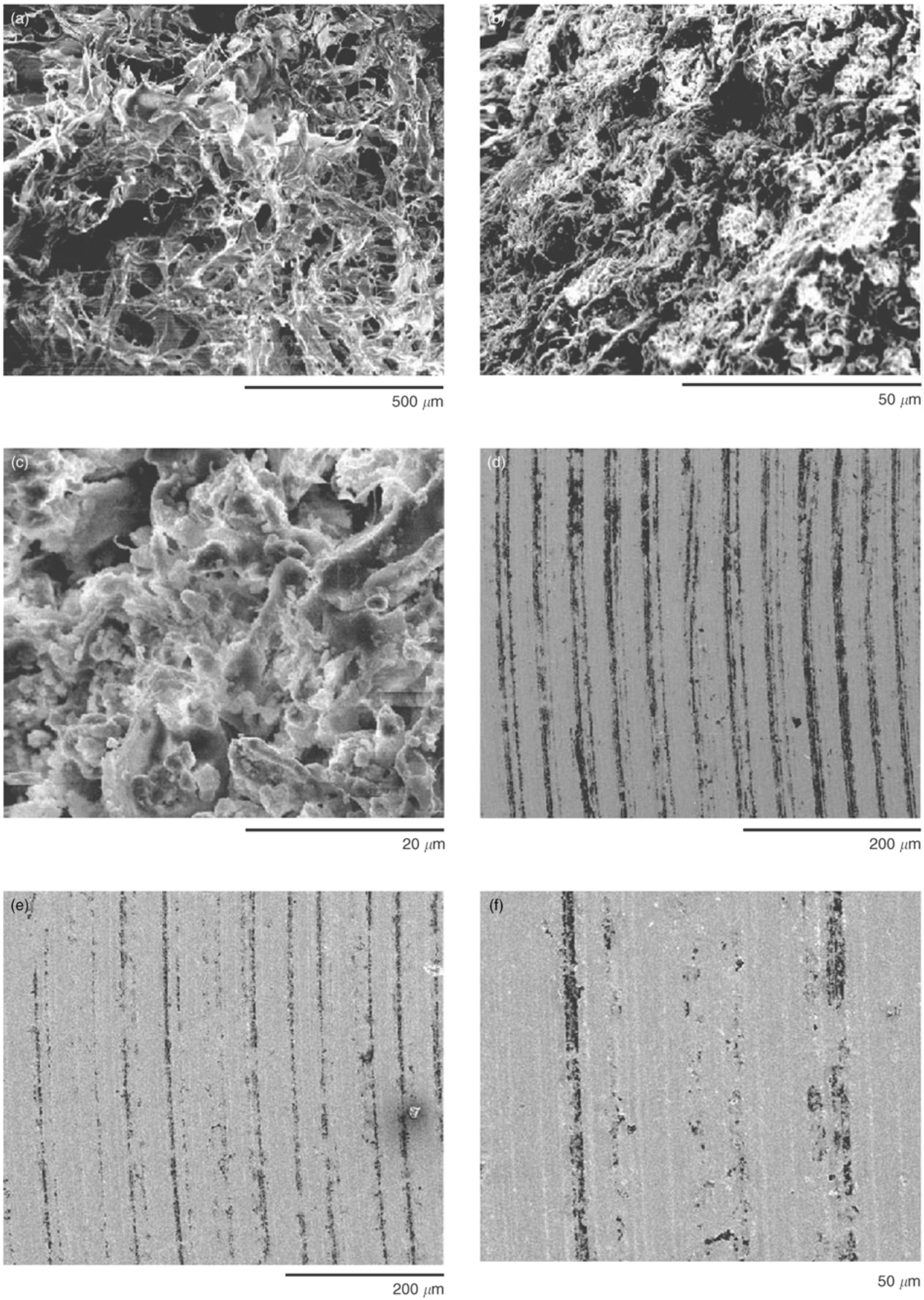


Figure 1 SEM micrographs for COL (a), HCG30 (b), HCG30 (c, d) and HCG300 (e, f). (a) and (c) indicate the freeze dried samples, while (b), (d), (e) and (f) indicate the naturally dried samples. Fig. 1(b) is a perpendicular section of Fig. 1(d).

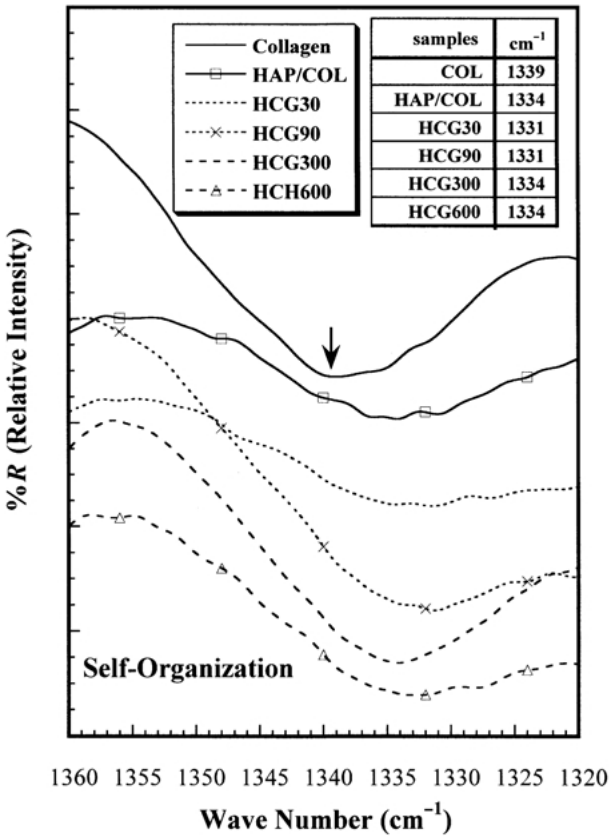


Figure 2 FT-IR spectra for COL, HAP-COL, HCG30, HCG90, HCG300 and HCG600. COL and HAP-COL are the freeze dried samples, and HCG30, HCG90, HCG300 and HCG600 are the naturally dried samples.

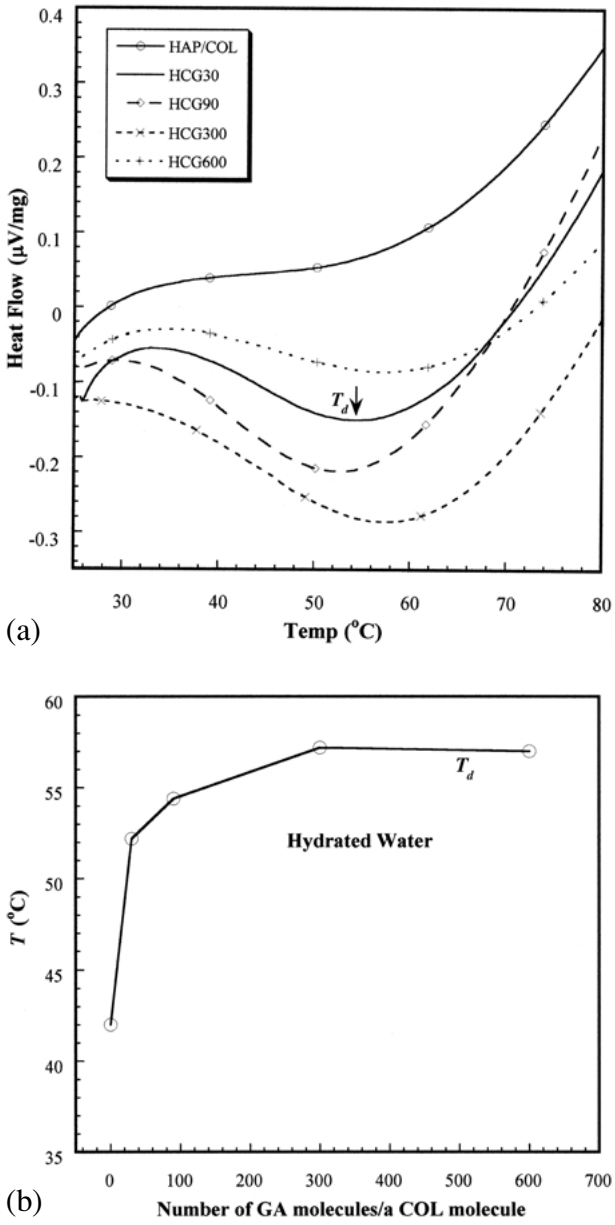


Figure 3 (a) DTA curves for HAP-COL, HCG30, HCG90, HCG300 and HCG900. (b) T_d as a function of number of GA(glutaraldehyde) molecules per COL molecule. The dehydration temperature (T_d) for hydrated water was determined from (a).

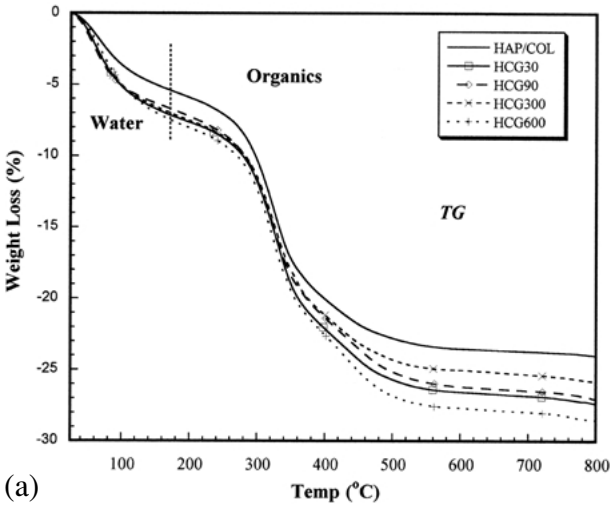
higher temperatures, the collagen content relatively decreased with the cross-linkage, resulting from the increase of the water content. The cross-linked network structure can increase the storage ability of the hydrated water.

These results suggest that the higher-order structure of HAp/collagen nanocomposites was stabilized by the introduction of a small amount of cross-linkage. From mechanical strength measurements, the sample HCG30 had better flexibility like rubber before drying, and good toughness after drying; the bending strength and Young's modulus were 7.7 MPa and 0.12 GPa for the freeze dried sample, and 16.1 MPa and 0.58 GPa for the naturally dried sample, respectively. The strength of the naturally dried sample is smaller in comparison to the HAp/collagen composite solidified by cold isostatic pressure (bending strength: 50 Mpa, Young's modulus: 3 GPa) [6], but sufficiently higher than a pure HAp porous materials.

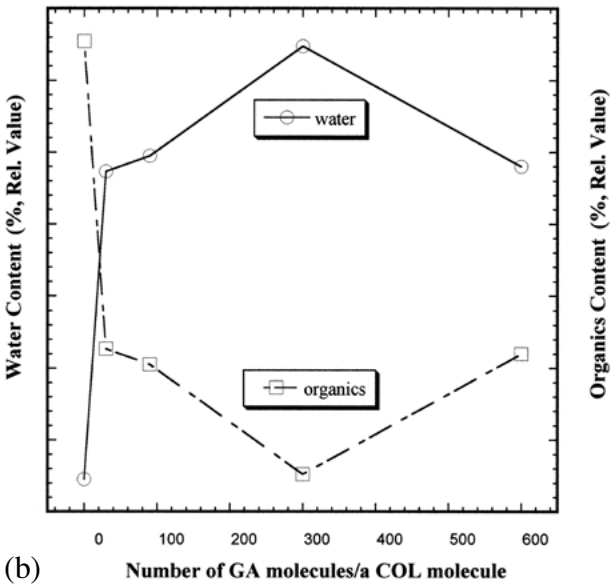
When the cross-linked samples were dipped into water and simulated body fluid, they were expanded to the original wet cake state by absorbing water; however, they maintained their shape and no dissolution took place.

Conclusions

A polymeric network structure was constructed by introducing cross-linkage into HAp/collagen nanocomposites using glutaraldehyde. The polymerization took place among bundles of hydroxyapatite nanocrystals and collagen molecules, resulting in the formation of porous channel structure with pores of 5–15 μm in size. From thermal analyses, it was shown that the optimal amount of glutaraldehyde was around 30 molecules per collagen molecule. HAp/collagen composite cross-linked by 30 glutaraldehyde molecules had a good bending strength of 16.1 MPa.



(a)



(b)

Figure 4 (a) TG curves for HAP-COL, HCG30, HCG90, HCG300 and HCG600. (b) Contents of the water and the organics as a function of number of GA molecules per COL molecule, which were determined from (a).

References

1. J. S. HANKER and B. L. GIAMMARA, *Science* **242** (1988) 885.
2. W. R. WALSH, M. OHNO and N. GUZELSU, *J. Mater. Sci.: Mater. Med.* **5** (1994) 72.
3. H. OHASHI, M. THERIN, A. MEUNIER and P. CHRISTEL, *ibid.* **5** (1994) 237.
4. L. GINESTE L, M. GINESTE, X. RANZ, A. ELLEFTERION, A. GUILHEM, N. ROUQUET and P. FRAYSSINET, *J. Biomed. Mater. Res.* **48** (1999) 224.
5. P. WEISS, M. LAPKOWSKI, R. Z. LEGEROS, J. M. BOULER, A. JEAN and C. DACULSI, *J. Mater. Sci.: Mater. Med.* **8** (1997) 621.
6. S. DEB, M. BRADEN and W. BONFIELD, *ibid.* **8** (1997) 829.
7. C. I. VALLO, P. E. MONTEMARTINI, M. A. FANOVICH, J. M. LOPEZ and T. R. CUADRAO, *J. Biomed. Mater. Res.* **48** (1999) 150.
8. M. KIKUCHI, Y. SUETSUGU, J. TANAKA, S. ITO S, S. ICHINOSE, K. SHINIYAMA, Y. HIRAOKA, Y. MANDAI and S. NAKATANI, *Bioceramics* **12** (1999) 393.
9. S. B. CHO, M. KIKUCHI, Y. SUETSUGU and J. TANAKA, *Key Eng. Mater.* **132–136** (1997) 802.
10. M. KIKUCHI and J. TANAKA, *J. Jap. Soc. Pow. Pow. Met.* **45** (1998) 36.
11. M. MURATA and J. TANAKA, *Bioceramics* **12** (1999) 429.
12. C. DU, F. Z. CUI, D. ZHU and K. D. GROOT, *J. Biomed. Mater. Res.* **44** (1999) 407.
13. M. ITO, Y. HIDAKA, M. NAKAJIMA, H. YAGASAKI and A. H. KAFRAWY, *ibid.* **45** (1999) 204.
14. K. SATO, Y. KUMAGAI and J. TANAKA, *Bioceramics* **12** (1999) 137.
15. S. H. RHEE and J. TANAKA, *ibid.* **20** (1999) 2155.
16. L. H. H. OLDE DAMINK, P. J. DIJKSTRA, M. J. A. VAN LUYN, P. B. VAN WACHEM, P. NIEUWENHUIS and J. FEIJEN, *J. Mater. Sci.: Mater. Med.* **8** (1997) 621.
17. J. M. RUIJGROK, J. R. D. WIJN and M. E. BOON, *ibid.* **5** (1994) 80.
18. F. CASAGRANDA, J. A. WERKMEISTER and J. A. M. RAMSHAW, *ibid.* **5** (1994) 332.

Received 7 September 2000
and accepted 2 November 2001