Cross-linkage of hydroxyapatite/gelatin nanocomposite using EGDE

MYUNG CHUL CHANG
Kunsan National University, Kunsan 573-701, Korea; CREST, Japan Science and Technology Agency
E-mail: mcchang@kunsan.ac.kr

T. IKOMA, J. TANAKA
Biomaterials Center of NIMS, Ibaraki 305-0044, Japan; CREST, Japan Science and Technology Agency

Calcified tissue, such as bone and teeth, is a biologically and chemically bonded composite of inorganic apatite nanocrystals embedded in an organic matrix of collagen [COL] and noncollagenous proteins [1, 2], where the anionic and cationic side chains of the latter bind readily to the calcium and phosphate on the surface of apatite mineral. Bone collagen with a higher hydroxylysine content, has a good chemical affinity for hydroxyapatite [HAp] because of the covalent bonding interaction between Ca^{2+} in HAp and carboxyl group in hydroxylysine [2]. Recently, Chang and his co-workers have developed imitation bone components using the biomimetic process [3, 4], which is based on the idea that biologic systems store and process information at the molecular level. The imitation bone materials been prepared by the coprecipitation reaction of apatite nanocrystals in soluble COL [5–7] or gelatin [GEL] [8, 9]. Double-diffusion process [10, 11] has been used for decades as a conventional process to obtain the apatite phase, but we have developed the coprecipitation process using highly active $Ca(OH)_2$ [5] as a Ca^{2+} ion source. Especially the commercial sources of GEL materials show good water solubility, and well-defined physical and chemical properties [12, 13]. In the development of HAp/GEL composites the cross-linkage is an important technology to give a higher toughness for the compact body of HAp/GEL nanocomposites and glutaraldehyde (GA) has been used because of its strong cross-linking reactivity [9]. However, normally the cross-linking reaction by glutaraldehyde is completed within several minutes, so it was hard to control the degree of cross-linkage in order to consistently obtain the compact body from the precipitates. Moreover, unreacted or partially reacted GA molecules have a cyto-toxicity problem, causing inflammation in the human body. In order to develop the biocompatible biomaterials with higher toughness, the selection of the proper cross-linkage agent is a prerequisite step. In this research we utilized EGDE (Ethylene Glycol Diglycidyl Ether) as a cross-linkage agent [14], which slowly reacts with GEL molecules of HAp/GEL composite slurries.

The preparation details of HAp/GEL nanocomposites were well described by Chang [8]. The precursors used here were $CaCO_3$ (Alkaline analysis grade, Wako, Japan), H_3PO_4 (AP grade, Wako, Japan), and Gelatin (Nitta Gelatin, Osaka, Japan). Pure $Ca(OH)_2$ powders were obtained through the calcination of $CaCO_3$ powders and the slaking process. The amount of $Ca(OH)_2$ and H_3PO_4 was calculated to make 10 g of HAp and the amount of GEL was 4 g. Before the coprecipitation reaction, the $Ca(OH)_2$ powders were vigorously stirred in deionized [DI] water at room temperature for 12 hrs and the weighed GEL powders were dissolved in the mixture of DI water and phosphoric acid at 37 °C for 12 hrs. During the entire coprecipitation process the temperature and pH of the reaction solution was maintained at 8.0 and 37 °C, respectively. After the reaction, the obtained slurries were aged at 37 °C for 24 hrs. Aqueous solution of EGDE (5 mM) was gradually added to the reaction vessel under vigorous stirring and the slurry samples were picked up after 0, 2, 9 and 24 hrs, respectively. The sample names were coded as HG4-E0, HG4-E2, HG4-E9 and HG4-E24, respectively. HAP-37 is a pure hydroxyapatite sample prepared at 37 °C without GEL matrix.

The filtered cakes were naturally dried at 37 °C in the incubator and phase formation was confirmed using X-ray diffraction (AFC-5R, Rigaku, Japan) on the crushed powders. The dry body microstructures were characterized by scanning electron microscopy (SEM, JSM-5600, Jeol, Japan). A chemical interaction between HAp crystals and the GEL matrix was estimated using the diffuse reflectance FT-IR (Spectrum 2000, Perkin-Elmer, UK). Following initial analysis of the raw spectra to determine the precise constituents, the spectral band positions were analyzed by using GRAMS AI (7.0) (Thermo Galactic, Salem, USA).

Thermal analysis (TG-DTA TG8120, Rigaku, Japan) was carried out on the dried samples to evaluate the decomposition of organics. The measurements were done between 25 and 1200 °C at a heating rate of 10 °C/min. All experiments were carried out in platinum pans at atmospheric temperature, and Al_2O_3 powders (10 mg) were used as a reference. 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 and the typical samples size was $5 \times 3 \times 20$ mm^3.

The HAp/GEL composite slurries obtained were highly swollen by the cross-linkage, and the highly swollen status in the solution was not significantly changed even 24 hr after the addition of EGDE. The appearance of the vacuum filtered cakes was like a lump of milky gel. Fig. 1 shows a comparison of XRD

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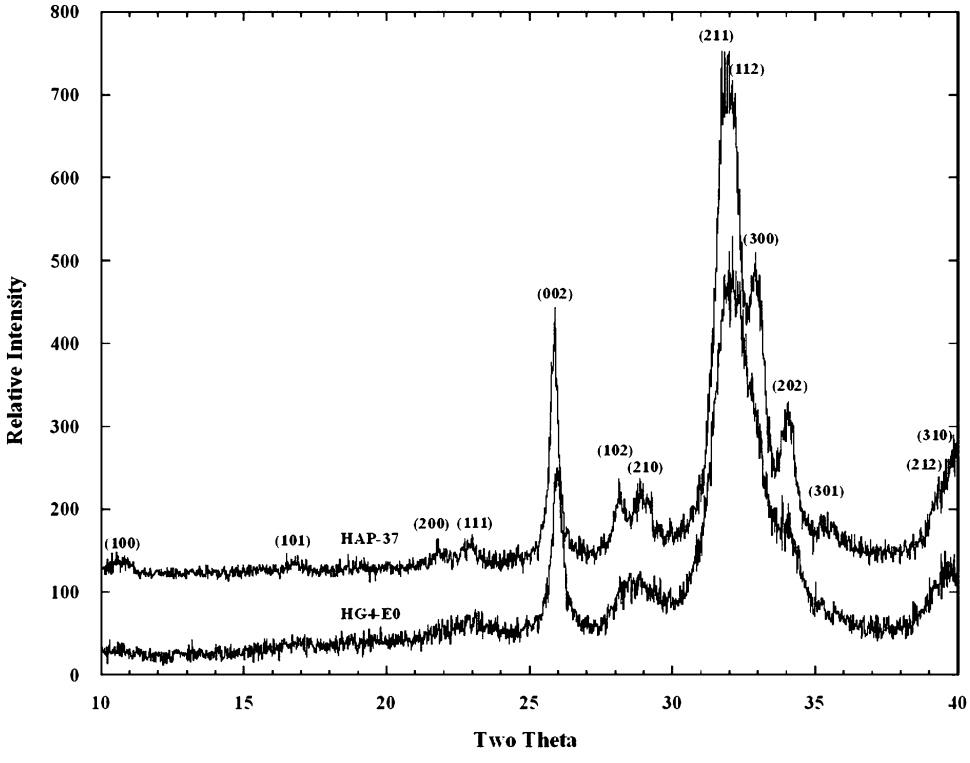

Figure 1 XRD patterns for HAP-37 and HG4-E0. HG4-E0 shows the weak peak development because of the heterogeneous nucleation on the GEL molecules.

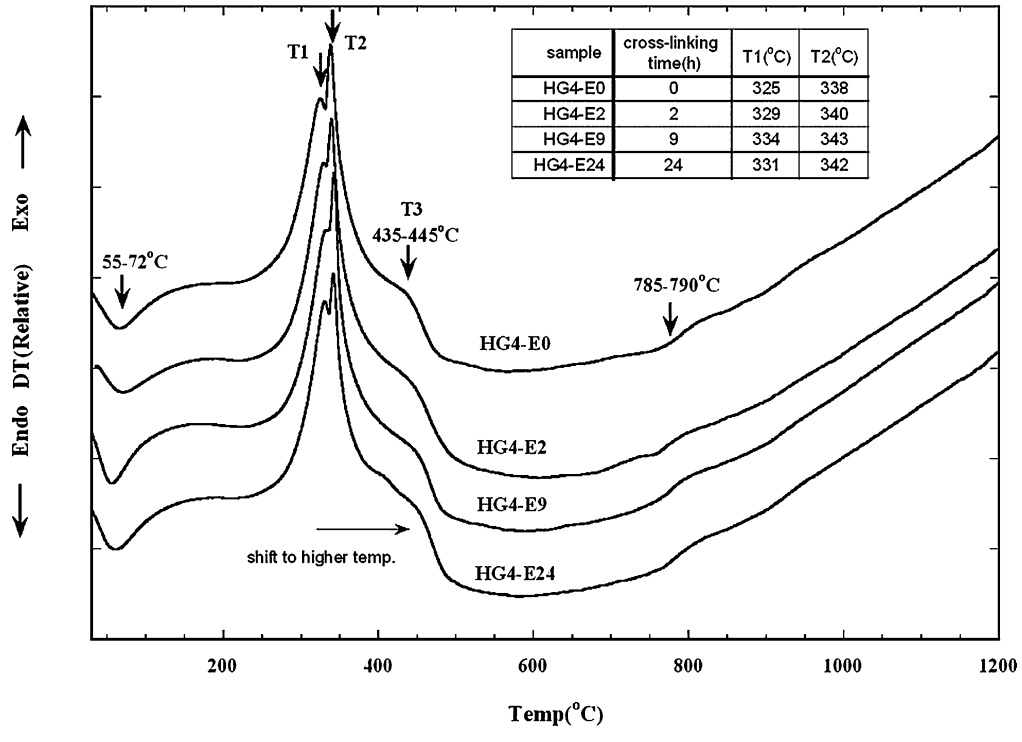

Figure 2 DT analysis for the samples of HG4-E0, HG-E2, HG4-E9 and HG4-E24. The peak temperatures of T1 and T2 increase with the increase of cross-linking time, as shown in HG4-E2 and HG4-E9.

patterns between HAP-37 and HG4-E0, which was prepared at the same temperature of 37 °C. The HAP-37 sample revealed characteristic peaks in the XRD pattern of ASTM 9-432. On the other hand the HG4-E0 sample showed broad weak peaks, indicating the poorly crystalline HAp phase. Normally the existence of GEL matrix during the precipitation of apatite phase induces the immature crystallites because of the heterogeneous nucleation on the GEL molecules [8]. TEM morphologies have shown the development of very tiny needle-shaped apatite crystallites with the existence of amorphous calcium phosphates [9].

From DT data (Fig. 2) the samples showed double exothermic peaks at T1 (325–331 °C) and T2 (338–342 °C), and a distinct exothermic shoulder at T3 (435–445 °C). T1 and T2 correspond to the thermal

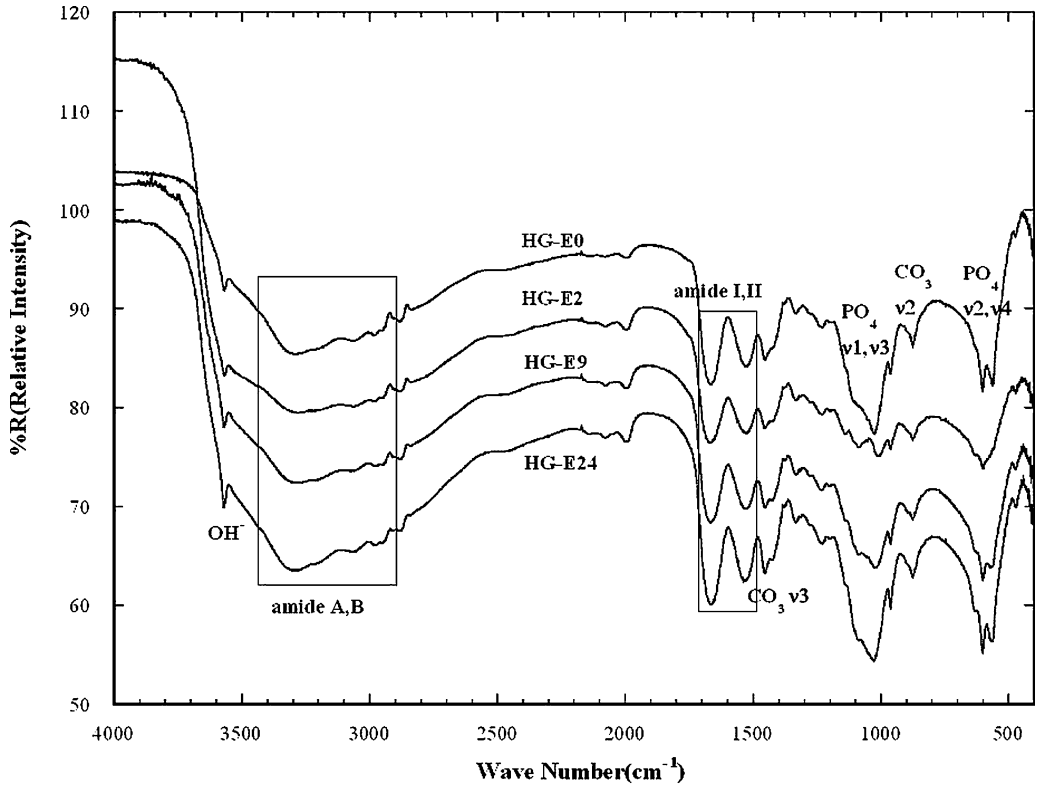

Figure 3 FT-IR spectra for the samples of HG4-E0, HG-E2, HG4-E9 and HG4-E24. PO4 bands show a higher degree of organic-inorganic interaction in HG-E2, and the crystal development with the aging time in HG4-E9 and HG4-E24.

degradation and pyrolyzation of GEL molecules respectively, and T3 is associated with the final thermal degradation of the residual organics. Endothermic peaks by the hydrated water appeared between 55 and 72 °C. The water molecules are partially associated with the organic component combined with the individual apatite crystals. Another endothermic peak was located at just below 800 °C, corresponding to the release of carbon dioxide from the carbonated apatite. It is interesting that the peak temperatures of T1 and T2 increased with the cross-linking time as shown in HG4-E2 and HG4-E9, and then the temperature slowly decreased in HG4-E24. The cross-linkage by EGDE contributed to the stability of GEL molecules at the interface between GEL and HAp crystallites. On the other hand, the crystal development progressed with the cross-linkage time and more amount of mineralization was attained. That is, the stability of GEL molecules at the interface of HAp phase decreased a little with the crystal development as shown in the HG4-E24 sample.

FT-IR spectra (Fig. 3) show the organic-inorganic bonding between HAp phase and GEL matrix from the strong amide bands: amide I in the range 1700–1600 cm^{-1} and amide II band in the range 1600–1460 cm^{-1}. The high PO_4 bands ($\nu 1$, $\nu 3$; 1200–900 cm^{-1}) and the low PO_4 bands ($\nu 2$, $\nu 4$; 700–450 cm^{-1}) indicate the existence of an apatite phase. Most of the cross-linkage reaction on GEL was completed within two hours after the addition of EGDE, and the long time treatment (9–24 hrs) was not found useful anymore for the improvement of the cross-linkage. Instead, further crystal growth occurred as confirmed from the peak of OH^- band at 3570 cm^{-1} and PO_4 ν_3 band at the range of 1150–950 cm^{-1}. Normally PO_4 ν_3 domain is known to indicate the mineralization, stoichiometry, symmetry of tetrahedral PO_4, and the existence of HPO_4^{2-} [15]. On the other hand, PO_4 ν_1 band at 962 cm^{-1} effectively indicates the crystallinity of the apatite phase. The resolution of PO_4 ν_3 domain was found to be getting worse in the HG4-E2 sample compared to that of HG4-E0 and the incomplete resolution was caused by the cross-linkage reaction of GEL molecules. As shown in HG4-E9 and HG4-E24 the resolution of PO_4 ν_3 domain was getting better with the increase of treatment time, indicating the completeness of the cross-linkage reaction and further development of the apatite crystals.

In the preparation of HAp/GEL composites the precipitated apatite phases are embedded in GEL matrices through the covalent interaction between Ca^{2+} ions of apatite and $R\text{-}COO^-$ ions of GEL molecules. The apatite crytals are matured through the aging process and many of the chemical bridges among GEL molecules are formed by the cross-linkage reaction, resulting in complicated reaction intermediates in HG4-E2. The PO_4 ν_3 domain well represents the organic coordination of apatite crystallites with the GEL matrices and the spectral feature is deformed by the organic coordination of mineral phases [15]. Actually the XRD pattern does not show the effect of the organic coordination of apatite, but the FT-IR spectral feature of PO_4 ν_3 domain was effectively reflecting the chemical coordination of the organic apatite.

The SEM micrograph shows the fracture surface for the dry sample. We can observe the agglomeration of small aggregates, which are composed of the apatite

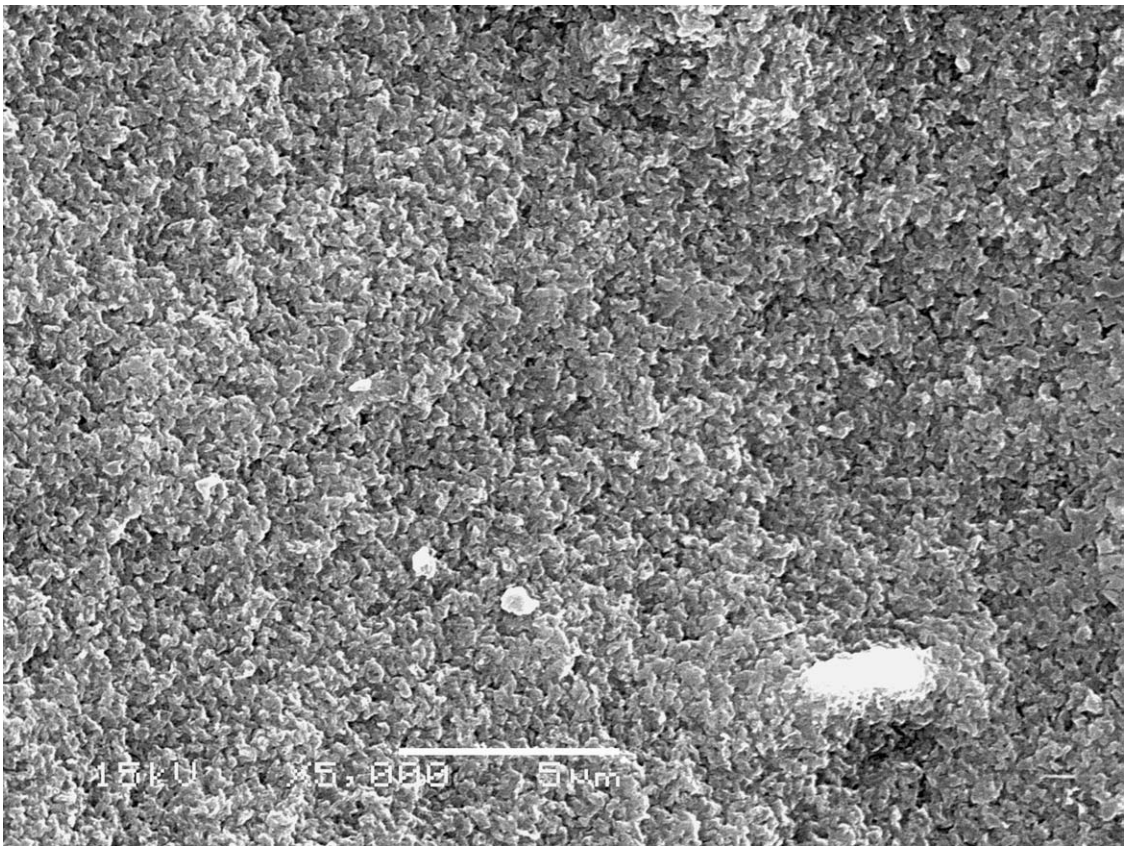

Figure 4 SEM micrograph for the HG4-E9 sample. The scale bar indicates 5 μm. The small grains ($\sim$0.5 μm size) correspond to the aggregates of precipitated particles embedded in the GEL matrices.

particles embedded in GEL matrices. We prepared two types of compact samples. In one method the slurries were cross-linked for 9 hrs by EGDE and then vacuum filtered and dried at 37 °C for several days in the incubator. In the other method the vacuum filtered slurries were consolidated for two days in the steel mold under 50 MPa using the uniaxial press. The estimated 3 points bending strength values were 20 and 25 MPa for the uniaxially pressed sample and the dry sample, respectively. By using the precipitated slurries and EGDE we could easily handle the sample processing in order to obtain the tough body.

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