

Modification of Hydroxyapatite-gelatin Nanocomposite using Side Group Reaction of $\text{Ca}^{2+}\text{-RCOO}^-$

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ABSTRACT

In the preparation of a hydroxyapatite [HAp]/gelatin [GEL] nanocomposite, the GEL matrix in aqueous solution of H_3PO_4 was modified by the introduction of aspartic acid [Asp], asparagine [Asn], and glycine [Gly]. The addition of Asp, Asn and Gly greatly affected the slurry formation of HAp/GEL nanocomposite and the resulting dry body showed variations in toughness with the addition of the different amino acids. The introduction of Asn into HAp/GEL nanocomposite was effective for producing the organic-inorganic interaction between HAp and GEL, and caused the increase of toughness. The formation reaction of the modified HAp/GEL nanocomposites was investigated by using XRD and FT-IR. The organic-organic interaction between the GEL matrix and the additives of Asp, Asn and Gly was confirmed from FT-IR analysis, and the organic-inorganic interaction between HAp nanocrystallites and the modified GEL matrix was also discussed, using FT-IR spectra patterns. Nanocrystallites of HAp were covalently bound with the GEL macromolecules and differently influenced by the modification species of Asp, Asn, and Gly.

Key words : Hydroxyapatite-gelatin nanocomposite, Aspartic acid, Asparagine, Glycine, Organic-inorganic interaction

1. Introduction

One is the extracellular matrix mainly composed of Hydroxyapatite [HAp: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] nanocrystals and COL fibers, in which the HAp nanocrystals align their c-axes with the COL fibers.¹⁻⁶⁾ As one of the artificial bone substitutes, the HAp-embedded gelatin [GEL] nanostructure⁷⁻⁹⁾ has been reproduced using a biomimetic¹⁰⁻¹³⁾ coprecipitation reaction of HAp nanocrystals in the soluble GEL matrix.^{14,15)} The properties of biological bone tissue¹⁶⁾ are essentially based on the organic-inorganic interaction between calcium phosphate [CaP] and the protein matrix, such as each type of COL, glucosamine or other proteins. In this report the GEL matrix was modified with Aspartic acid [Asp], Asparagine [Asn] and Glycine [Gly] to investigate the influence on the organic-inorganic interaction in the HAp/GEL nanocomposite.^{8,17)} The incorporation of Asp, Asn, and/or Gly can be applied to introduce anionic residues into the gelatin gels as side chain amides. The objectives of this research were to prepare dense dry bodies with a good toughness through the modification of HAp/GEL nanocomposite.

2. Materials and Methods

The technical background of sample preparation was based on multiple experiments since 2000^{7,8,17)}. The addition

of side groups into an HAp/GEL nanocomposite was tried several times since 2004 and the mechanical properties^{21,22)} of sample bodies have been checked. It was not easy to obtain stable values of advanced mechanical properties because of the complicated process of making a sample body similar to real bone. At first the precipitation process has to be precisely controlled to make a stable HAp/GEL nanocomposite, and the dry process has to be stabilized to obtain the test samples for the evaluation of mechanical properties. The side group chemicals of Asp, Asn, and Gly were added into the HAp/GEL nanocomposite slurries in order to make a multi-composite of HAp/GEL nanocomposite having bone-like architecture.

2.1. Preparation of HAp/GEL Nanocomposite

The preparation process of an HAp/GEL nanocomposite was explained by Chang et.al.⁷⁾ The precursors used here were CaCO_3 (Alkaline analysis grade, Wako, Japan), H_3PO_4 (AP grade, Wako, Japan), GEL (Unflavored, Natural Food Inc., Canada), Glycine (Gly, 99%, Aldrich), Asparagine (Asn, 99%, Aldrich), and Aspartic acid (Asp, 99%, Aldrich). Pure $\text{Ca}(\text{OH})_2$ was obtained through the hydration of CaO . The slurry for HAp/GEL composite was prepared by the simultaneous titration method using peristaltic pumps, water bath and the pH controller (Burkert 8280H, Germany). The amount of $\text{Ca}(\text{OH})_2$ and H_3PO_4 was calculated to make 10 g of HAp. A homogeneous suspension of $\text{Ca}(\text{OH})_2$ in DI water was prepared at 37°C after 12 h stirring in the beaker. The homogeneous suspension of $\text{Ca}(\text{OH})_2$ of 2 L, and H_3PO_4 aqueous solution with GEL of 1.5 L, were simultaneously added to the reaction vessel through peristaltic pumps.

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2.2. Modification of HAp/GEL Nanocomposite with Asp, Asn and Gly

A part of the GEL powders was stirred at 37°C for an hour in the H_3PO_4 aqueous solution of 1.5 L. The dry sample was named GEL1 and a reference sample named GEL0 indicates the pure GEL powders. As mentioned in Section 2.1 the amount of $\text{Ca}(\text{OH})_2$ and H_3PO_4 was calculated to make 10 g of HAp for the preparation of the HAp/GEL nanocomposite.⁷⁾ For the modification of HAp/GEL nanocomposite, 0.5 gram of Asp, Asn, or Gly was dissolved in the H_3PO_4 aqueous solution of GEL. The samples were coded HG3Asp, HG2Asn, HG3Gly, and etc., respectively. HG3 and HG2 indicate the samples prepared by using 3 g and 2 g of GEL, respectively. HAp is the sample prepared without GEL matrix. After the coprecipitation reaction, the total volume became 4L through the adjustment. During the coprecipitation process the temperature and pH of the reaction solution in vessel were set and maintained at 37°C and 8.0, respectively. After the reaction, the obtained slurry was aged at 37°C for 24 h and the precipitated HAp/GEL slurry was passed through a glass filter. The final cakes were naturally dried at 25°C in the air and a chemical interaction between HAp crystals and functional groups of GEL was estimated using the diffuse reflectance FT-IR (Magna 750R, Nicolet, USA) on the crushed powders. 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). The powders were also used for characterization by XRD (Siemens 500).

2.3. Mechanical Testing

Compression strength was evaluated for the samples of HG3Asp, HG2Asn and HG3Gly. Results from the compression tests for HAp/GEL nanocomposite have been reported in detail elsewhere.^{21,22)} The tests were performed on three replicate samples from each material group. The mechanical test samples were cut from a fully dried block using a cylindrical saw. The ends of each cylindrical sample were sanded down to adjust the sample height using 400- and 600-grit sandpaper; the height was at least twice the diameter to meet the standards set by ASTM C39 for compression testing. The samples were then placed between the compression plates on the MTS Synergie 100 machine (MTS System Corp., Eden Prairie, MN). A piece of damp paper towel was placed between the compression plate and the sample to prevent the cylinder from slipping. The machine was set to compress the sample at the rate of 0.5 mm/min. The force as a function of the amount of extension was recorded, and the data was captured on the Testworks 4 software (MTS System Corp.). Analysis of variance was used to compare mechanical properties for various compositions of HAp/GEL nanocomposite samples. The effect of gelatin and additive content on fracture toughness was measured by examining eight different batches of material.

A short rod chevron-notch method based on ASTM E1304-89 was used for fracture toughness tests. The chevron notch located in the central plane of the rod formed an isosceles triangular shape to initiate the crack tip.²²⁾ The angle between the two equal

sides of the triangle was 53.13, and the height of the triangle was 4 mm. The purpose of initiating a crack in the sample is to provide a standard area where the crack propagates. The sample height and diameter were 7.6 and 4 mm, respectively. The samples ($n = 3$ for each batch) were then loaded onto a MTS 810 Material Test System (MTS System Corp.) and tested in dry conditions. A noncontact capacitance transducer (model PX305HB; Lion Precision, Minneapolis, MN) was set up to measure crack opening displacements.

The force and displacement at breakage were recorded and used to calculate the fracture toughness using the equation

$$K_{IC} = Y \frac{F}{D\sqrt{W}} \quad (1)$$

where Y is stress intensity factor coefficient, here equal to 29.39, F represents the maximum load at breakage, and D and W are the sample diameter and length, respectively.

3. Results and Discussion

3.1. X-ray diffraction (XRD)

In Fig. 1 XRD patterns of HG2Asn, HAp and HG3Gly are

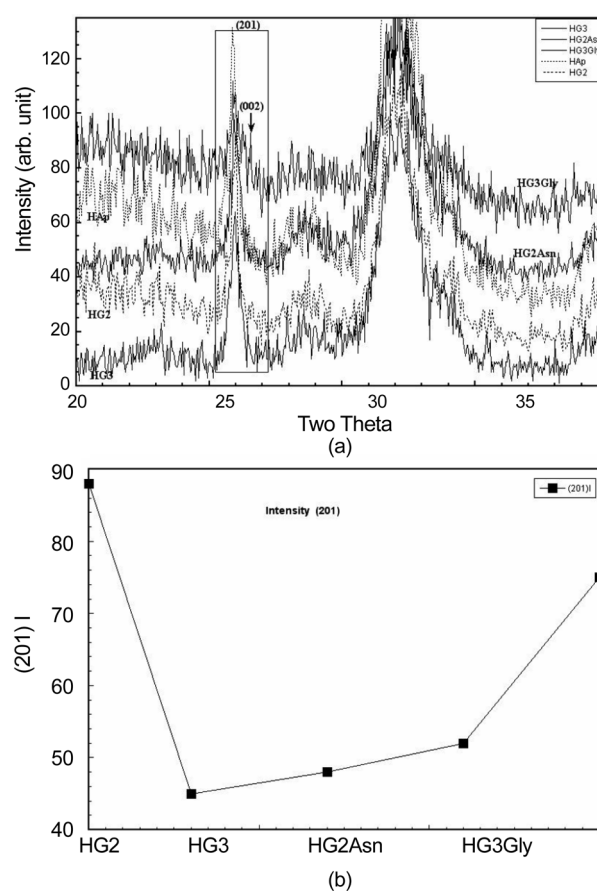


Fig. 1. (a) XRD pattern for HG3Gly, HG2Asn, HG3, HG2 and HAp. It shows the mineralization of the HAp phase in the combined matrix of GEL with Gly and Asn. (b) The intensity of (201) with the addition of side groups of Gly and Asn, and the amount of GEL in the HAp/GEL nanocomposite.

essentially similar to those of HG3. The precipitated product of HG2Asn is the composite of HAp and modified GEL matrix, in which 2 g of GEL with 0.5 g of Asn was dissolved in the aqueous solution of phosphoric acid. Fig. 1(b) shows the (201) intensity with the samples. The corresponding (201) peak intensity was reduced with the addition of Asn or Gly, indicating the decrease of crystal development.

It may be said that the addition of Asn or Gly contributed to enhancing the organic-inorganic interaction between HAp and the GEL through the modification of GEL macromolecules. Normally the HAp nucleation is caused by the interaction between Ca^{2+} as a free ion and R-COO^- as a phosphorylated group in the GEL matrix. In the modified GEL matrix there will be greater amounts of the side chain's functional groups, which may be coordinated with free Ca^{2+} ions. Therefore the chance of nucleation will be increased. The crystal growth is based on the growing of the CaP nuclei at the nucleation sites. If each of the added side groups is attached on the same side group in GEL there will be more chances for the growth of CaP nuclei. The nucleation is based on two factors. One is the organic-organic interaction between GEL and the added side groups. Another one is the organic-inorganic interaction between HAp and the modified GEL macromolecules. This will be discussed through the FT-IR analysis.

3.2. FT-IR analysis

Fig. 2 shows FT-IR for Asp, Asn, Gly, GEL0 and GEL1. In GEL the amide A band is located in the range of 3300 and 3600 cm^{-1} . In Fig. 2(a) GEL1 shows the red-shifted spectra pattern¹⁷⁾ of the amide A band and the typical PO₄ bands between 900 and 1200 cm^{-1} . Gly and Asp show the OH⁻ spectral bands at 3315 and 3097 cm^{-1} , respectively. It is noted that the reported FT-IR spectra of Asn²⁴⁾ show the complicated bands at 2860, 2930, 3115, and 3395 cm^{-1} in the range of 2800 and 3600 cm^{-1} . In Fig. 2(b) the 1,339 cm^{-1} band in GEL0 corresponds to the wagging vibration mode in the proline side chains of GEL molecules. The proline band in GEL1 was red-shifted through the formation of phosphorylated groups in GEL macromolecules.

In the preparation of modified HAp/GEL nanocomposite the GEL powders were stirred at 37°C with the powders of Asp, Asn, or Gly in the aqueous solution of H_3PO_4 . The added side group elements might be attached to the corresponding side group of GEL macromolecules and so the Ca^{2+} -ROO⁻ interaction might be affected during the precipitation reaction of calcium phosphate in the mixed GEL solution. Fig. 3 shows FT-IR for the modified HAp/GEL nanocomposite using Asp, Asn, or Gly. In Fig. 3(a) HG3 and HG2 represent the typical FT-IR spectra similar to HAp/GEL nanocomposites.^{7,8)} The spectral intensity of the OH⁻ band at 3569 cm^{-1} in HAp decreases with the increase of GEL amount in HAp/GEL nanocomposites.⁷⁾ The organic-inorganic interaction between HAp phase and the modified GEL macromolecules could be confirmed from the individual spectra patterns of OH⁻, amide and PO₄ bands.

In Fig. 3(b) amide I, II, III bands were critically developed in the modified HAp/GEL nanocomposites. The 1335 cm^{-1} band

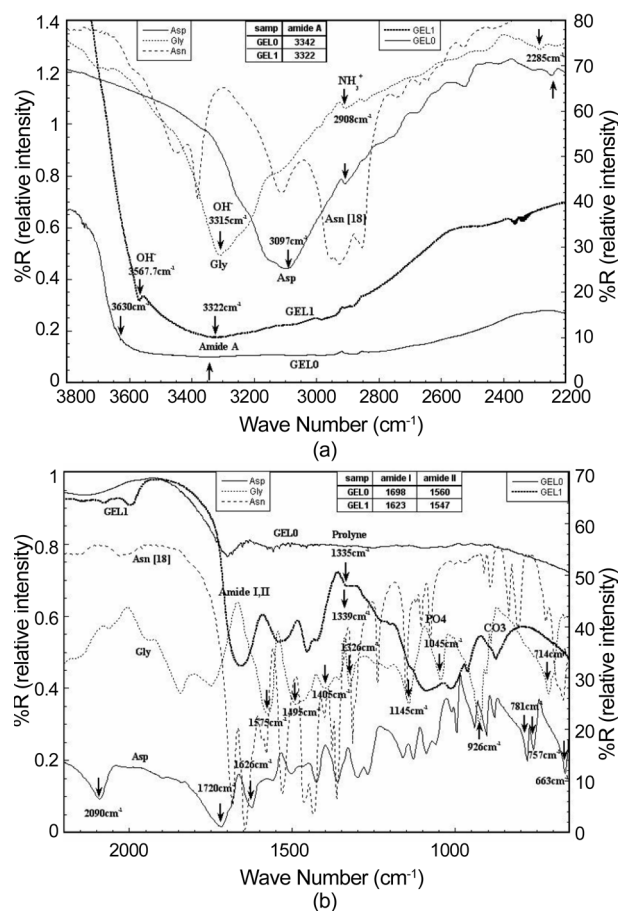


Fig. 2. FT-IR spectra for Asn,¹⁸⁾ Gly, Asp, GEL0 and GEL1. For high resolution amide and PO₄ spectra the detailed spectra analysis was performed for (a) amide spectra between 3800 and 2200 cm^{-1} and (b) PO₄ spectra between 2200 and 650 cm^{-1} .

in HG3 is a spectra pattern indicating the resonance of a Ca^{2+} -COO complex, which is caused by the wagging vibration of proline side chain through covalent bond formation with the HAp phase.⁷⁾ The 1339 cm^{-1} band of GEL is red-shifted in HAp/GEL nanocomposites. The lower chemical shift of 1339 cm^{-1} band is caused by the smaller crystallites of HAp bound with GEL macromolecules, suggesting the lower energy wagging of the proline side chain. The proline band peak of HG2Asn at 1328 cm^{-1} is indicating a greater chemical shift, compared to that of HG2. In HG2Asn there might be a higher degree of organic-organic interaction between the GEL matrix and Asn molecules. There will be larger amounts of carboxyl groups to interact with Ca^{2+} during the coprecipitation, which has resulted from the added side groups. The PO₄ spectra patterns in HG2Asn were more deeply developed and the PO₄ ν_1 band showed the critically stronger pattern. In the PO₄ ν_3 bands the 1091 cm^{-1} asymmetry PO₄ band was more critically developed than those of the 1010 cm^{-1} P-O stretch band. When we compare the PO₄ band spectra among HG2, HG2Asn, and HG3 the spectra pattern of HG2Asn was greatly organized and approached that of HG3. Especially, the asymmetry of

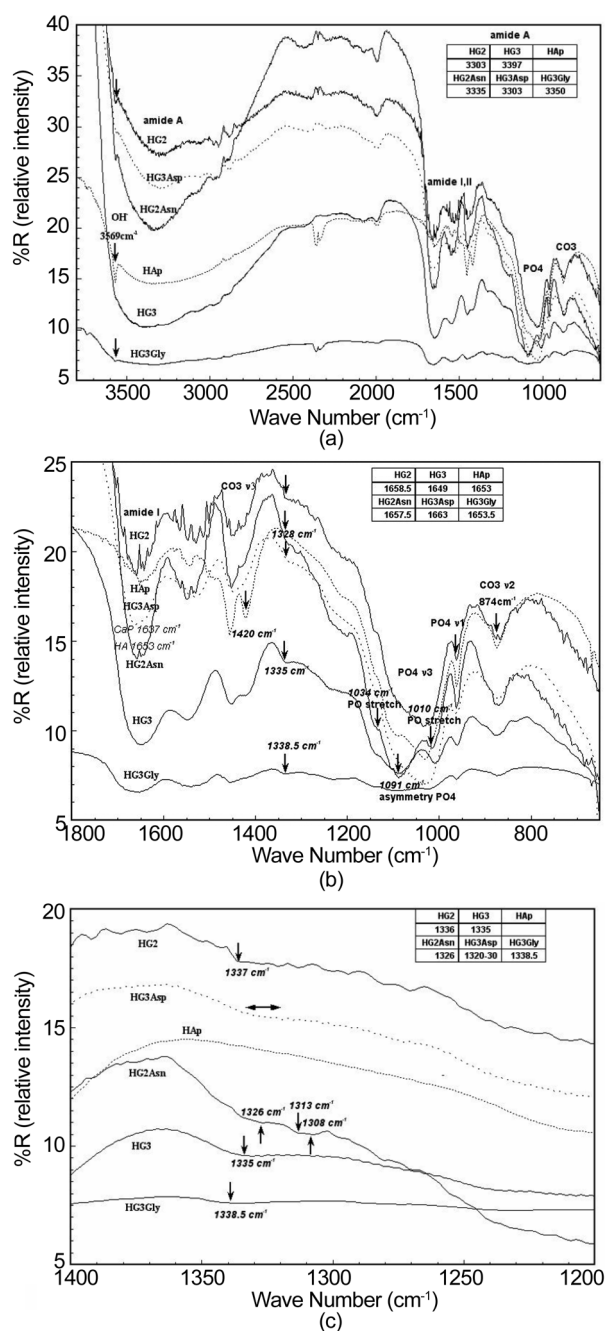


Fig. 3. (a) FT-IR spectra for HG3Gly, HG3Asp, HG2Asn, HG3, HG2 and HAp. For high resolution amide and PO4 spectra the detailed spectra analysis was performed for amide spectra and PO4 spectra (b) between 1800 and 650 cm^{-1} . (c) High resolution FT-IR spectra between 1400 and 1200 cm^{-1} for $\text{Ca}^{2+}\text{-COO}^-$ coordination bond.

the PO4 band at 1091 cm^{-1} was strongly developed, indicating the formation of crystalline HAp in which the band is closer to the position of octacalcium phosphate as observed by Fowler et al.^{24,25} than to the position of hydroxyapatite. Among Asp, Gly and Asn the effect of Asn was most effective for modifying the HAp/GEL nanocomposite. Among HG2, HG3 and HAp,

HG3 shows the broad band in the phosphate spectral region, such as the PO4 v3 and PO4 v1 bands, but the HG2 spectra pattern is close to that of HAp.

It is interesting that the Asn in HG2Asn induced the increase of total spectral intensity in both the amide regions and phosphate regions. There may be two effects affecting the property of the modified HAp/GEL nanocomposite system. One effect is on the organic-organic interaction between GEL and Asn. Another one is on the organic-inorganic interaction between the HAp and the modified matrix. On the contrary, in HG3Gly the total spectral pattern was broadened through the addition of Gly. The addition of Gly greatly induced the organic-organic interaction between GEL macromolecules and Gly, which can be analyzed from the broadened spectra of the amide band and the PO4 band. As can be seen in Fig. 3(c), the wagging band of the prolyne peak locates at 1338.5 cm^{-1} , close to that of Collagen. In HG3Asp the spectral pattern was very close to that of HG2 even though the matrix slurry was based on HG3. The amide band spectra patterns were weak, compared to those of HG3. On the contrary PO4 band spectral patterns were considerably developed, indicating the stronger mineralization. In HG3Asp the original matrix of GEL was based on HG3, but the spectral pattern was close to that of HG2. It means that Asp induced the enhancement of the mineralization reaction rather than that of the organization reaction between GEL and Asp. In HG2Asn the spectral patterns of amide and PO4 bands were simultaneously increased. Actually the obtained slurries of HG2Asn were pretty stiff and a tough dry body was obtained.

3.3. Mechanical Properties

All the samples were crushed from one end of the cylinder. They showed the following strengths: 120 ± 10.0 MPa for HG3, approx. 70 ± 14.0 MPa for HG2, approx. 100 ± 10.0 MPa for HG3Gly and HG3Asp, and 120 ± 10.0 MPa for HG3Asn. The HG3Asn nanocomposite showed a good dry strength. For the HAp/GEL nanocomposite formed by using GEL macromolecules modified with side group additives, the Asn addition resulted in better mechanical strength (approx. 120 MPa) and higher effective crack resistance in the water immersion test. The modified GEL was well mineralized by HAp, but the increase in toughness was different from that of HAp/GEL nanocomposite.

The toughness values for HG3, HG2, HG3Gly, and HG3Asp were $0.6 \text{ MPa m}^{1/2}$, $0.5 \text{ MPa m}^{1/2}$, $0.6 \text{ MPa m}^{1/2}$, and $0.7 \text{ MPa m}^{1/2}$, respectively. The fracture toughness values (KIC) increased with the addition of Asn, resulting in a fracture toughness of $1.1 \text{ MPa m}^{1/2}$, equivalent to that of pure hydroxyapatite. The fracture toughness values are substantially smaller than those observed for natural bone, which has KIC values around $2.5 \text{ MPa m}^{1/2}$. Natural bone has the toughening microstructures such as a Haversian system or lamellar structure.³ The addition of Asn may help to make the artificial bone samples of HAp/GEL nanocomposite with toughening microstructures. The increase of organic-inorganic interaction by Asn addition may induce the toughening microstructure.

3.4. The effect of side group addition into HAP/GEL nanocomposite

The addition of Asn, Gly and Asp into HAP/GEL nanocomposites could be analyzed by using XRD and FT-IR. One effect of side-group additives is on the organic-inorganic interaction between HAP and the GEL matrix. Another effect is on the organic-organic interaction between the GEL macromolecules matrix and the additives. In GEL the band position of amide A is sensitive to the GEL conformation⁷⁾ and the most significant change of GEL is the blue-shift (25-30 cm^{-1}) of the amide A band in the native collagen spectrum.^{15,26)} The high amide A frequency indicates the existence of some level of collagen [COL] structure. The organic coordination of HAP with the GEL matrix is confirmed from amide bands and PO₄ bands. The organic-inorganic interaction between HAP phase and the modified GEL matrix could be confirmed from the existence of the strong amide bands of amide I and II. As another evidence for the organic-inorganic interaction in Fig. 3(c), the spectral feature of the $\text{Ca}^{2+}\text{-COO}^-$ bond⁷⁾ at 1,335 cm^{-1} was smeared with the addition of side groups in the GEL. From the 1,335 cm^{-1} band spectra in Fig. 3(a) indicating the existence of an $\text{Ca}^{2+}\text{-COO}^-$ complex between HAP and the GEL molecules, most of the free Ca^{2+} was consumed during the coprecipitation process. In preparing the modified HAP/GEL nanocomposite, for the samples using some side-groups, such as Asn and Gly, the obtained slurries were sticky, and the toughness of the dry body varied with the species of added side groups.

4. Conclusion

An HAP/GEL nanocomposite was modified with Gly, Asp, or Asn, and the introduction of Asn into the HAP/GEL nanocomposite was effective for achieving the organic-inorganic interaction between HAP and GEL. Dense dry bodies with fracture toughness values of $K_{\text{IC}} = 1.1 \text{ MPa m}^{1/2}$ were obtained by using Asn. Through FT-IR analysis we could confirm the organic-inorganic interaction between HAP and the organic matrix of modified GEL, and the organic-organic interaction between GEL and side group additives. It is believed that a better toughness value could be attained by the adjustment of two factors on interactions in a real bone, which has KIC values around 2.5 $\text{MPa m}^{1/2}$.

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