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Cross-linkage of hydroxyapatite/gelatin nanocomposite using imide-based zero-length cross-linker

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Abstract Hydroxyapatite [HAp]/Gelatin [GEL] nanocomposite was prepared at 37 and 48 °C through coprecipitation process. The HAp/GEL nanocomposite slurries were cross-linked by imide-based zero-length cross-linking agent such as *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The chemical bond formation and microstructure in HAp/GEL nanocomposite was investigated as a function of cross-linking agents and temperature. The single addition of EDC into the composite slurries resulted in a tougher microstructure in both samples prepared at 37 and 48 °C. However, in the case of the simultaneous addition of EDC and NHS the sample prepared at 48 °C showed a coarse microstructure. These results were consistent with the fact that the chemical reactivity of NHS is degraded at 48 °C whereas the reactivity of EDC increases up to 80 °C.

Introduction

For several years hydroxyapatite [HAp]/gelatin [GEL] nanocomposite [1–4] using biomimetic [5–6] coprecipitation has been pursued as a biological bone substitute [7], which is biocompatible, biodegradable and as a scaffold site for regeneration of new bone. In the development of biomimetic HAp/GEL nanocomposite [2, 3] using the commercial GEL materials there have been several difficulties due to the complexity of the reaction mechanism and typical low toughness. Currently, the accumulated data have showed that a single morphology of needle-shaped apatite particles was induced either through the dynamic processing under high-speed stirring or the introduction of fluoride source. For the increase of final toughness, the cross-linkage for the nanocomposite slurries was performed by using glutaraldehyde [GA] [2].

Cross-linking of collagen-based materials is an effective method to optimize the mechanical properties. The cross-linkage using GA [8–10] has been extensively used because of its strong activity, but it has difficulties due to cytotoxicity and the problematic control of the very cross-linking reaction. So in order to improve the toughness of the compact body of HAp/GEL composite with much reduced little cytotoxicity, alternative cross-linking methods were investigated. In commonly available collagen-coated vascular grafts, following the in vitro and in vivo degradation of the cross-linked polymer (especially the glutaraldehyde-crosslinked) evoke cytotoxic reactions due to the release of the relevant aldehyde. Crosslinking of collagen using the water soluble *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) may result in a material that has shown to be non-cytotoxic in vitro and biocompatible in vivo [11].

We chose the reagents, which activate carboxylic acid groups of glutamic or aspartic acid residues to react with amine groups of another chain providing cross-links with the formation of amide bonds. In particular, the nonzero-length cross-linkage agents such as EDC or NHS were investigated for the purpose of cross-linking the HAp/

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GEL nanocomposite slurries. Normally EDC can be used without the introduction of other chemicals, but in the present work NHS is used with EDC in order to trigger the cross-linking reaction of gelatin molecules. Water soluble carboimide caromed (WSC) has well been extensively used to trigger the cross-linking of gelatin-based bio-glue [11–14] and NHS-esters [15] was used to yield stable products upon reaction with primary or secondary amines. In this research we planned two experimental groups. One group is using EDC only and the other using NHS with EDC.

Experimental process

Preparation of samples

The preparation process of HAp/GEL nanocomposite is reported in detail elsewhere [1]. HAp/GEL nanocomposite slurries were precipitated at pH 8 by the controlled supply of the aqueous solution of $Ca(OH)_2$ and the mixture solution of GEL and H_3PO_4, respectively. The input amount of GEL content was set as 3 g for all samples, and the reaction temperatures were 37 and 48 °C, code named HG3-37 and HG3-48, respectively. After the coprecipitation process the aging was continued for 24 h at 37 °C for all samples. The pH value was decreased from pH 8 during the coprecipitation reaction and reached close to 7 at the end of the aging. The cross-linking agent was added for the aged slurries and kept for 20 min, and the reacted slurries were immediately collected using a vacuum filter and washed with double-distilled water. The formed cake using a vacuum glass filter was dried at ambient temperature for the characterization. As cross-linking agent we used imide-based zero-order reagents such as a water soluble carbodiimide and succinimide, specifically 0.6 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Aldrich, USA) and 0.9 g *N*-hydroxysuccinimide(NHS)-esters (Aldrich, USA), respectively. The resulted slurries were highly expanded and filtering-off was slow using a vacuum filter. We performed two methodologies: one-way is to add EDC (0.6 g) singly and another is to add EDC (0.6 g) and NHS (0.9 g) simultaneously. This resulted in four samples with different cross linking and temperature characteristics, code-named HG3-37E, HG3-37EN, HG3-48E and HG3-48EN, respectively, with the letters E and N indicating EDC and NHS, respectively.

Before vacuum filtering a part of slurry was sampled for TEM observation and microstructures were characterized by transmission electron microscopy TEM (TEM, JEOL JEM1210, Japan). Phase formation was identified using X-Ray Diffraction (XRD D-5005, Siemens, Germany) on the crushed powders. For the dry body, microstructures were characterized using scanning electron microscopy (SEM, JSM-6700F, JEOL, Japan). A chemical interaction between HAp crystals, GEL macromolecules and cross-linking agent was estimated using the diffuse reflectance FT-IR (Magna 750, Nicolet, USA). Diffuse reflectance of IR was measured for powder specimens diluted ratio 1:10 with KBr powder of spectroscopic grade (Spectratech) by one tenth: background noise was corrected with pure KBr data. The measuring resolution was 4 cm^{-1} and iteration was performed for 256 times in the range of 650–4,000 cm^{-1}. The sample compartment of the FT-IR spectrophotometer was purged with dry air. 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, MacScience, Japan) was carried out on the dried samples to evaluate the bond formation between HAp crystal and GEL molecules. The measurements were done between 25 and 1,200 °C at a heating rate of 10 °C/min. All experiments were carried out in Platinum pans in air atmosphere, and Al_2O_3 powders (10 mg) were used as a reference.

Chemistry of gelatin cross-linking

Cross-linkers are chemical reagents used to conjugate molecules together by a covalent bond. Several atoms usually separate the two molecules, forming the 'spacer arm'. Carbodiimides react with carboxyls to form an intermediate (o-acylisourea) that can stabilize with reaction with amines, forming a peptidic bond, without spacer length. In this chemistry, the cross-linking reagent is not incorporated into the final product. This is ''zero-length'' cross-linking. EDC is soluble up to 100 mg/mL in water. The reaction take place in an acidic buffer (pH 4.7–5.5), but coupling can actually be accomplished in a buffer system up to pH 7.4. The intermediate undergoes hydrolysis in aqueous solutions, thus a stabilization is usually necessary for further coupling to amines. A classic way to do it consists to add *N*-hydroxysuccinimideal form. Easy removal of excess reagent and corresponding urea after coupling may be achieved by washing with dilute acid or water. In general, the amino acid content of gelatin is glycine, 25.5%; proline, 18%; hydroxyproline, 14.1%; glutamic acid 11.4%; lysine, 4.1%; and so on [16, 17].

In our experiment the average pH value is set up to 8.0 during the coprecipitation, but the actual pH is changed up to 7.0 with the aging process. The cross-linking is per-

formed for the aged HAp/GEL nanocomposite slurries having pH = ~7. So two step process using EDC with NHS is effectively working for the chemical modification of GEL molecules with HAp nanoparticles.

Results and discussion

XRD diffraction

From XRD (Fig 1) the HAp/GEL composite samples prepared at 48 °C (HG3-48E, HG3-48EN) show the stronger peak development compared to the samples prepared at 37 °C (HG3-37E, HG3-37EN). The reaction temperature of 48 °C is a critical temperature for keeping the molecular behavior of GEL, leading the degradation [17], and so the organic-inorganic interaction between GEL and apatite is seriously deteriorated [18]. During the coprecipitation process the precipitation reaction is instantaneously occurred between Ca^{2+} and PO_4^{3-} on GEL molecules. The phosphoric GEL molecules in liquid states are dropped into the main reactor and the calcium–phosphates are precipitated on the GEL molecules, which are supposed to be severely deformed or degraded above 48 °C. Before the precipitation reaction the acidic reagent of GEL with H_3PO_4 in DI water was kept in water bath at 37 °C for 10 h. The precipitation reaction of apatitic phase is very fast and supposed to be occurred on the nearly normal GEL molecular structure before the severe degradation. After the completeness of dynamic coprecipitation the entire slurries were aged at 37 °C for 10 h in the same heat bath. Consequently, the further degradation of GEL molecules may be progressed with aging time. Actually we could observe a highly swelled slurries status for a while after the coprecipitation process was completed. However, the swelled slurries were slowly deposited on the bottom of the reactor after several hours aging.

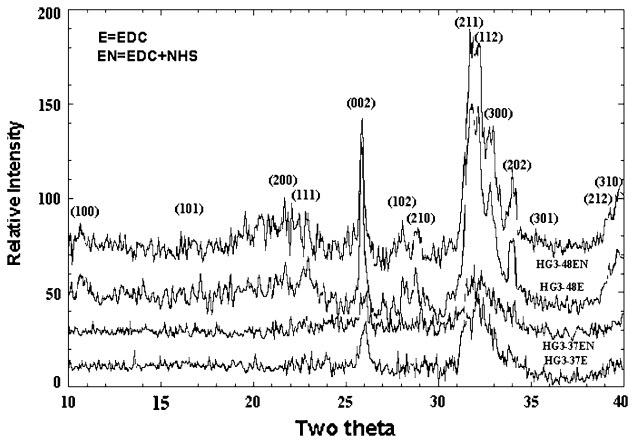

Fig. 1 XRD pattern for HG3-37E and HG3-48E, respectively. The HG3-48 samples prepared at 48 °C showed the very strong crystal development, but HG3-37 samples prepared at 37 °C showed the mere peak development indicating the immature fine crystallites

Thermal analysis

Thermal analysis (Fig. 2) shows interesting results for two ways of cross-linking. As shown in Fig. 2A DT spectra for HG3-E37 and HG3-EN37, several exothermic peaks were distinctly developed between 250 and 520 °C. Normally we observe two distinct peaks (T1, T2) and one more shoulder (T3) in exothermic spectra of HAp/GEL nanocomposite [1, 2]. T1 and T2 correspond to the thermal degradation and pyrolyzation of organic molecules,

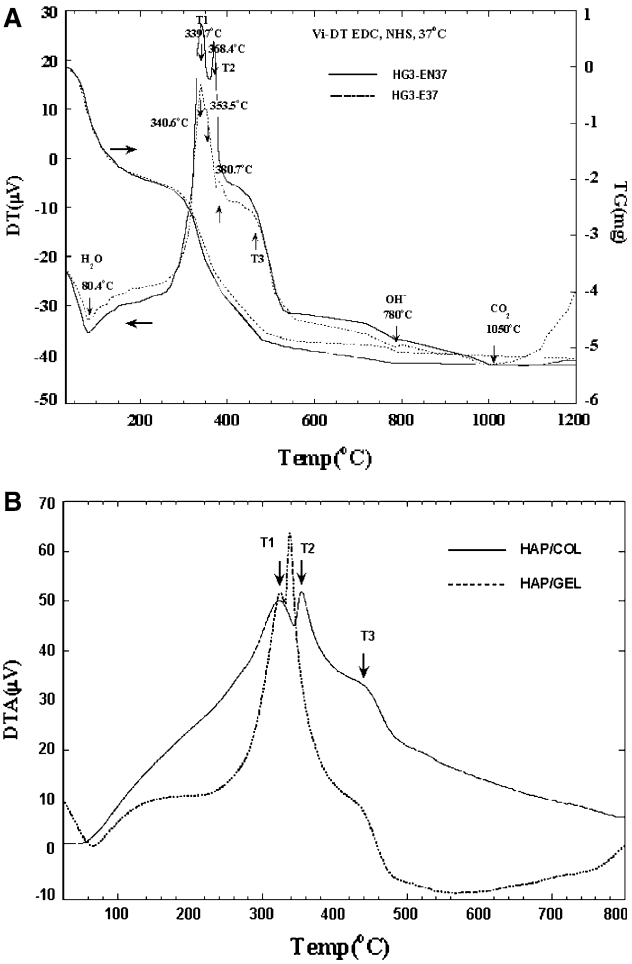

Fig. 2 (**A**) DT/TG analysis for the HG3-37E and HG3-37EN samples prepared at 37 °C. The single introducing of EDC in HG3-37E induced additional peak besides typical three exothermic peaks (T1, T2, T3) and the appearance of additional peak may be attributed to the uncompleted intermediate reaction. The big endothermic peak at 80 °C is coming from the highly swelled slurries structure by the cross-linkage agent. (**B**) DTA spectra comparison between HAp/COL [19] and HAp/GEL [1] nano-composite samples

respectively, and T3 is associated with the final decomposition of the residual organics. In gelatin macromolecules [16] T1 peak corresponds to the disorganization of helical structure in denatured collagen fibrils and renatured collagen fibrils, and T2 peak reflects the disintegration of each chain of fibrils. The disorganization of helical structure is the unique property of collagen fibril [17]. T3 peak indicating the combustion of organics is located at ~510 °C and most of residual organics is disappeared at ~575 °C. The introduction of EDC agent definitely induced the additional peak in the exothermic zone, but the introduction of EDC + NHS agent did not induce the additional peak. That is, it is assumed that the single addition of EDC left the intermediate reaction compounds in the sample products and this compound is strongly related with the helical structure of the GEL molecule. As mentioned above, T1 and T2 are related with the thermal degradation, mostly helical structure, pyrolyzation and mostly fibril structure of GEL. The new peak exists between T1 and T2, and so it is considered that a new compound is connected with both helices of the GEL molecules and the fibril structure. In general compared with HAP/COL system [19] HAP/GEL system [1] has smaller T1 peak and bigger T2 peak as shown in Fig. 2B. The characteristic behavior of T3 is almost similar because it is due to the final decomposition of protein to gas. So the spectral behavior of T1 and T2 peaks has a structurally important meaning. T1 is mainly related with the primary or secondary structure on the triple helices and T2 indicates the tertiary structure on the fibrils or bundles. So HAP/COL has stronger T1 peak and, on the other hands, HAP/GEL has a relatively stronger T2 peak as shown in Fig. 2B. The HAP/COL system has a very big exothermic energy compared to that of HAP/GEL system because of the internal structure energy. From Fig. 2A it is evident that EDC in HG3-E37 introduces some kinds of intermediate reaction compounds in the final HAP/GEL sample, and the use of NHS with EDC eliminated the intermediate compounds as shown in HG3-EN37. Moreover, NHS greatly contributed to the development of T1 and T2 peaks, intensifying the internal structure of helices, fibrils and bundles of GEL molecules. As commonly stated in the literature [11–13] the co-use of EDC and NHS greatly improved the molecular structure of HAP/GEL composite, but the increase of reaction temperature from 37 to 48 °C resulted in the controversy as shown in FT-IR analysis and SEM microstructure.

Because of the surface water in the composite, HAP/GEL has a bigger broad exothermic H_2O peak under 100 °C. Endothermic peaks by the hydrated water appeared at ~60 °C. The water molecules are partially associated with the organic components combined with the individual apatite crystals. Another endothermic peaks were located at

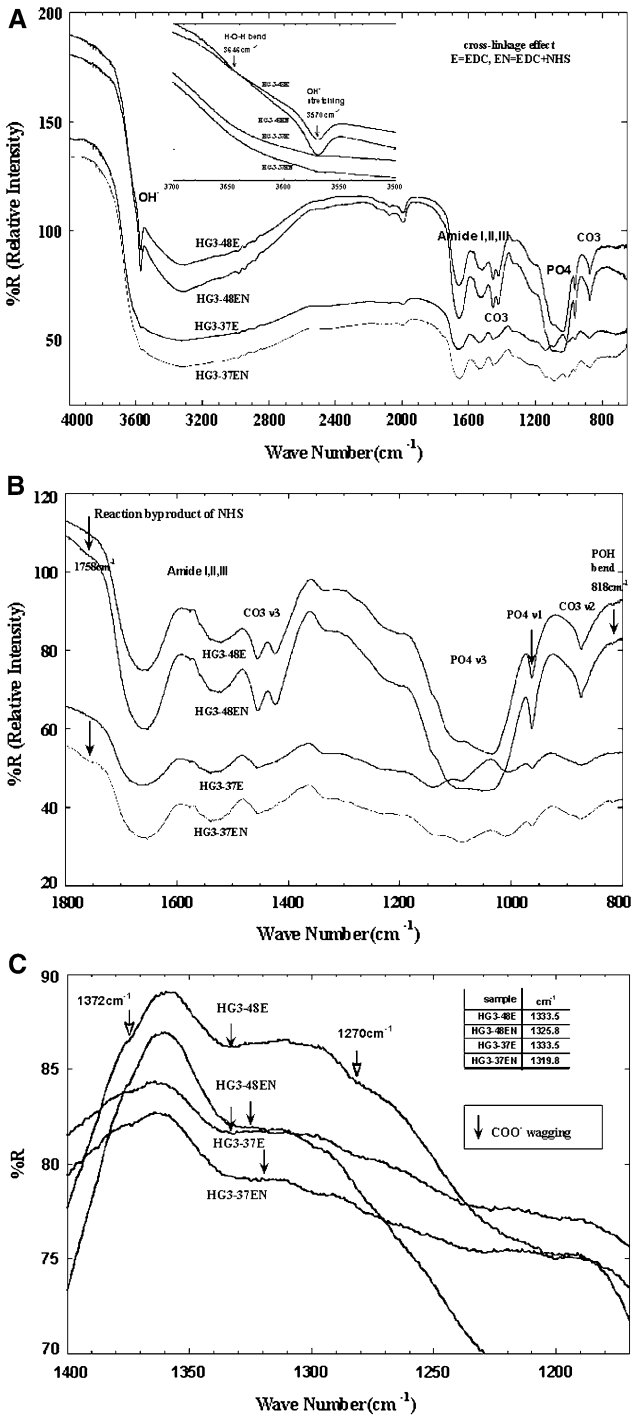

Fig. 3 (**A**) FT-IR spectra for HG3-37E, HG3-37EN, HG3-48E and HG3-48EN, respectively. The organic coordination of HAp with GEL matrix is confirmed from amide I, II, III bands and PO_4 bands with the reaction temperature. (**B**) We can observe the typical bands such as C=O stretching at 1,700–1,600 cm^{-1} for amide I, N–H bending at 1,550–1,500 cm^{-1} for amide II, and N–H deformation at 1,300–1,200 cm^{-1} for amide III band. As HAp related bands, there are OH^- stretching (3,570 cm^{-1}), and phosphate contours: $v_1$962 cm^{-1} and v_3 1,089 cm^{-1}. It is noted that a new band at 1,758 cm^{-1} is appeared by the NHS agent. (**C**) The spectral change of $-COO^-$ wagging bands is showing the modification of ε-amine group in GEL molecule

just below 800 °C, corresponding to the release of lattice OH^- along the c-axis. At 1,190 °C we can observe another endothermic peak, showing the CO_2 releasing in the carbonated apatite. From Fig. 2A we can know that the cross-linkage reaction by EDC induced complicate polymeric structure. There are endothermic peaks at ~80 °C for the physically adsorbed water, just below 800 °C for the OH lattice and 1,050 °C for the CO_2 releasing, respectively.

FT-IR analysis

FT-IR spectra in Fig. 3 show the good chemical bond formation between GEL and apatite, and the structure modification of HAp/GEL nanocomposite by using the cross-linkage agent. We could confirm the organic-inorganic bonding [18–20] between HAp phase and GEL matrix from the strong amide bands; amide I in the range 1,700–1,600 cm^{-1} and amide II band in the range 1,600–1,460 cm^{-1}. The high PO_4 bands ($v1$, $v3$; 1,200–900 cm^{-1}) indicate the existence of apatite phase. The further crystal growth with aging at 48 °C as shown in Fig. 3A was occurred as confirmed from the peaks of OH^- stretching band at 3,570 cm^{-1}, HOH bending mode at 3,646 cm^{-1} and PO_4 v_3 band at 1,150–950 cm^{-1}.

Normally PO_4 v_3 domain in Fig. 3B is known to indicate the mineralization, stoichiometry, symmetry of tetrahedral PO_4, and the existence of HPO_4^{2-} [18–20]. On the other hands PO_4 v_1 band at 962 cm^{-1} effectively indicates the crystallinity of the apatite phase. The PO_4 v_3 domain well represents the organic coordination of apatite crystallites with the GEL matrix and so the spectral feature is deformed by the organic coordination of mineral phases [19, 21]. Actually the XRD pattern doesn't show the effect of the organic coordination of apatite, but the FT-IR spectral feature of PO_4 v_3 domain was effectively reflecting the chemical coordination of the organic apatite. The samples at 37 °C show the higher amount of organic coordination with apatite phase. From Fig. 3B we can observe a peculiar band for samples of HG37EN and HG48EN at 1,758 cm^{-1}, which may be led by the reaction byproduct of NHS. These chemical agents are supposed that no chemical groups are left in the cross-linkage structure of protein. So it is assumed that a part of functional groups from NHS is left in the composite. The reaction byproducts by NHS were existed in the GEL molecules for both samples prepared at 37 °C and 48 °C, respectively. Additionally it seems that the modification of POH bending mode at 818 cm^{-1} is not affected by cross-linkage, but mainly caused by the reaction temperature. The POH bending mode is related with the organic–inorganic coordination of the interface bonding between GEL and HAp [18].

Figure 3C shows the change of the wagging band in ε-amine groups according to the cross-linkage reaction. The spectral behavior of this wagging band in HAP/GEL nanocomposite is intimately related with the organic-inorganic bond formation in the interface between apatitic phase and GEL molecule [1, 2, 14]. The band frequency was changed between 1,319.8 and 1,333.5 cm^{-1}. Here it is noted that the band frequency in HG3-37E and HG3-48E is not moved, but the EN samples shows the considerable frequency change. That is, the chemical modification of GEL molecular structure was appeared by the cross-linking effect of NHS agent in HG3-37EN and HG3-47EN. The chemical modification of GEL molecules by cross-linkage agent affected the lattice interaction between organic molecules and apatite interfaces. The –COO groups in GEL molecules make a covalent bond with Ca^{2+} sites in the interfacial surface of HAp. The chemical bond between Ca^{2+} and –COO will be modified with the amount of EDC + NSH and the reaction temperature.

TEM and SEM morphology

TEM morphology (Fig. 4) for HG3-48E shows needle-type crystals with the strong directionality. Because of the cross-

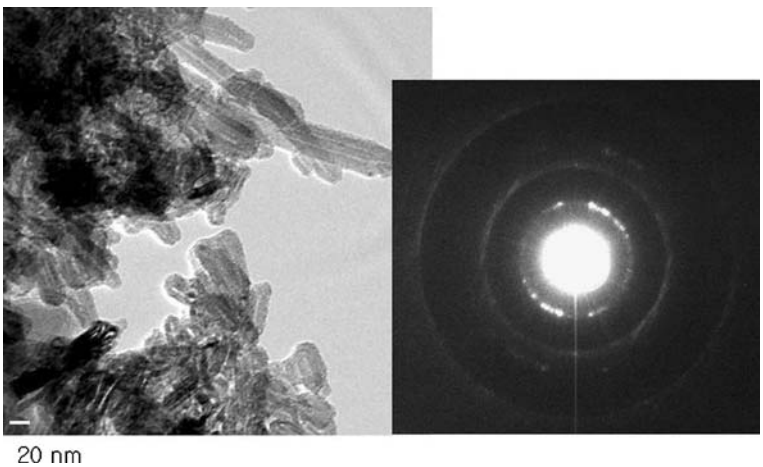

Fig. 4 TEM morphology for HG3-48E, indicating the needle-shaped morphology. We could observe the needle-like particles over the entire range

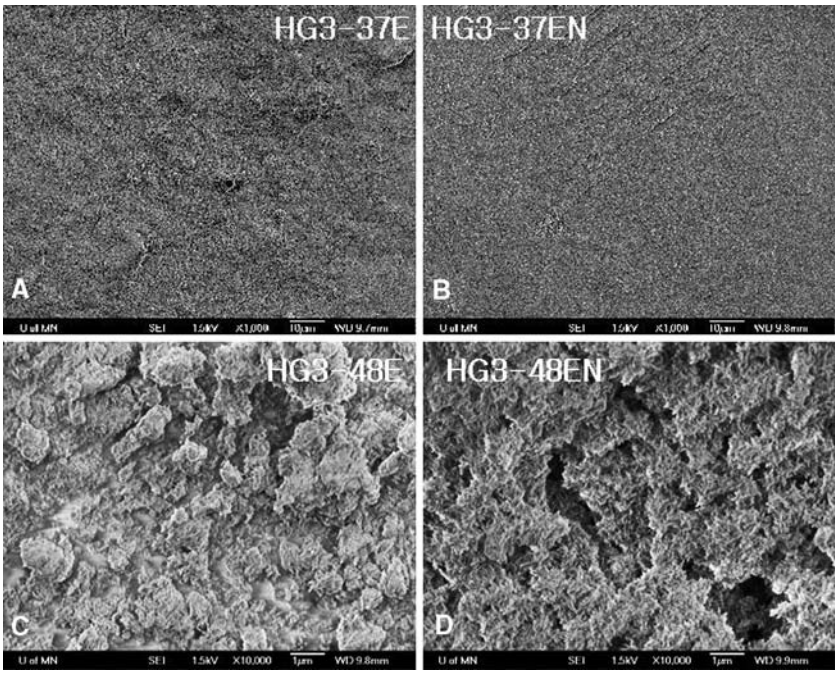

Fig. 5 SEM morphology for HG3-37E (**A**), HG3-37EN (**B**), HG3-48E (**C**) and HG3-48EN (**D**), respectively. Co-use of EDC and NHS produced dense microstructure in the 37 °C reaction (**B**), but the single use of EDC produced dense body in the 48 °C reaction

linkage by using EDC, there are very strong coagulation between apatite crystals and GEL molecules. In HG3-48E we can observe a very dense region, which looks like it was coagulated by EDC agent, but the same kinds of dense region is not observed in HG3-48EN. HG3-37EN shows a very dense microstructure, but HG3-37E shows a coarse structure.

SEM morphology (Fig. 5) for HG3-48E and HG3-37EN shows relatively dense microstructure, but HG3-48E shows coarse microstructure. So it can be said that EDC agent is better working at a higher temperature of 48 °C, but NHS is better working at a lower temperature of 37 °C. It seems that EDC agent is very active at 48 °C, but NHS activity is abruptly degraded at this temperature. Normally the EDC agent becomes more active with the temperature increase from the room temperature to 80 °C [11, 12]. On the other hands the appropriate reaction temperature of NHS is said to be room temperature.

Conclusion

The chemical bond between Ca^{2+} and –COO in HAp/GEL nanocomposite would be modified with the amount of EDC and/or NSH, and the reaction temperature. At higher temperature of 48 °C the single addition of EDC into HG3 slurries was effective to make a dense microstructure, but at 37 °C the simultaneous addition of EDC and NSH was a better way to have a dense body of HG3 nanocomposite. The role of zero-length cross-linking agent for HAp/GEL nanocomposite was well confirmed from FT-IR, DTA and SEM microstructure.

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