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Fluoride incorporation in hydroxyapatite/gelatin nanocomposite

Myung Chul Chang

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Abstract The incorporation of fluoride ion into hydroxyapatite (HAp)/gelatin (GEL) nanocomposite was investigated. The F^- ion incorporation into OH^- site of HAp phase was an energetically active process, which could be confirmed from the spray solution reaction. The precursors of Ca^{2+} in water and phosphates in aqueous gelatin were mixed in the humidified air chamber by air spray, and then the precipitates were aged in a reactor. The F^- ion precursor was dissolved in the starting solution of $Ca(OH)_2$ in water, and the resulted $Ca(OH, F)_2$ complex droplets induced the formation of stable fluoroapatite (F, OH)Ap. The reaction kinetics could be assumed from TEM morphology with ED, XRD and FT-IR analysis.

1 Introduction

Calcified tissue, such as bone and teeth is considered as a biologically and chemically bonded composite between apatite nanocrystals and type-I collagen (COL) [1–3]. Type-I COL is a kind of template for the heterogeneous nucleation of apatitic phase, and the space available for apatitic crystal growth in COL matrix is a thin groove confined in their growth in two but not three dimensions [4]. In general apatites can incorporate numerous impurities of F, Cl, CO_3, Zn, Mg and so on [2, 5]. Recently, Chang and his workers [6–12] have developed an imitation bone using biomimetic process, and the biomimetically imitation bone has been prepared at 35–48°C by a coprecipitation reaction of HAp nanocrystals in the soluble collagen [6–9] or denatured collagen [10–12]. Especially the denatured collagen, GEL precursor is not homogeneous, while in COL solution individual macromolecules are homogeneously dispersed [13]. The GEL contains a variety of macromolecule morphologies, and the morphology variety [11] was strongly involved in the formation reaction of octacalcium phosphate (OCP) and/or HAp phases during the mineralization using the coprecipitation process [10]. The formation mechanism of apatitic phase in the GEL macromolecules can be understood on a basis of heterogeneous nucleation and epitaxial growth, which is generally accepted in the formation of biological bone [14, 15].

We report the effect of fluoride incorporation into apatitic phase in HAp/GEL nanocomposite, which can be observed in TEM morphology development [5, 16, 17]. Fluorine ions (F^-) from the fluoride source are potentially incorporated into hydroxyl (OH^-) site of HAp, and therefore contribute to the intensive development of apatite crystallites. We have tried how to show the effect of the crystallization energy difference by the introduction of F^- into (OH)Ap lattice. So we designed another precipitation reaction process, which is based on the supply of ion sources by using air-spray system (Fig. 1).

2 Experiments

2.1 Coprecipitation process

The starting materials were $CaCO_3$ (Alkaline analysis grade, Aldrich, USA), H_3PO_4 (AP grade, Aldrich, USA),

M. C. Chang
MDRCBB, School of Dentistry, University of Minnesota,
16-280 Moos Tower, 515 Delaware S. E., Minneapolis,
MN 55455-0329, USA

M. C. Chang (✉)
School of Material Science and Chemical Engineering,
Kunsan National University, Kunsan 573-701, Korea
e-mail: chang083@umn.edu; mcchang@kunsan.ac.kr

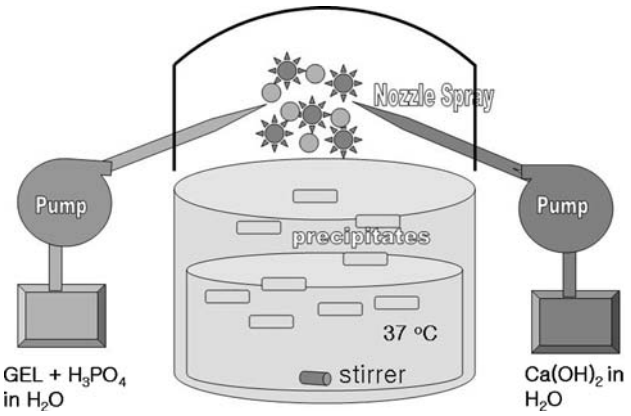

Fig. 1 Schematic design for the spray precipitation reaction. The precursor solution was injected by peristaltic pump and sprayed by using air pressure nozzle. The reaction to keep pH = 8 was controlled by the electronic system

and Gelatin (Unflavored, Natural Food Inc., Canada). The process has been reported in detail elsewhere [10]. Pure $Ca(OH)_2$ was obtained through the hydration of CaO, calcined at 1,150°C for 3 h. The slurry of the HAp-GEL composite was prepared by the simultaneous titration method using peristaltic pumps, a stirrer, a water bath, and a pH controller (Bukert 8280H, Germany). The amount of $Ca(OH)_2$ and H_3PO_4 was calculated to make 10 g of HAp. Homogeneous suspensions of $Ca(OH)_2$ dispersed in 2 l of H_2O and an aqueous H_3PO_4 solution with 5 g of GEL were gradually added to the reaction vessel using the peristaltic pumps. Before the coprecipitation process the GEL powders were stirred with H_3PO_4 in DI water for the phosphorylation of GEL molecules. After the coprecipitation reaction, the total volume became 4 l with pH adjustment. During the coprecipitation process the temperature and pH of the reaction solution in the vessel were set and maintained at 37°C and 8.0, respectively. The obtained slurry was aged at 37°C for 24 h. A part of slurry was used for TEM observation, and microstructures were characterized by transmission electron microscopy TEM (JEM-1210, JEOL, Japan). The samples, prepared by the coprecipitation process are coded as HAp/GEL, while HAP indicates the hydroxyapatite (HAp) sample prepared without using GEL.

2.2 Spray process

As shown in Fig. 1 each solution of phosphate in aqueous GEL and $Ca(OH)_2$ in water was sprayed by using an air-compressor nozzle. The sprayed droplets in the mist were reacted in the open air environment on the upper space of the reactor, and then precipitated in water vessel under stirring. The upper space of the main reactor was covered with a half-sphere shell to keep the mist of the sprayed solution. The spray precipitation process was controlled to maintain pH = 8.0 in the main reactor, and the slurries of dropped precipitates were aged at 37°C for 24 h. Five mM of ammonium fluoride (Aldrich, USA) solution was mixed with the water suspension of $Ca(OH)_2$ for an hour before the precipitation process. The sample names were coded as spray-HAp/GEL, spray-FAp/GEL and so on, as the spray process is applied.

3 Results and discussion

3.1 TEM and XRD

TEM morphology (Fig. 2) shows the directional crystal growth in the fluorinated sample, and we can confirm the nearly single crystalline phase from ED patterns. Most of phases in the fluorinated nanocomposite showed a typical 'side-to-side' orientation growth of the rod type crystals. On the other hands when apatite was prepared in the GEL matrix without using fluoride, most of the phases belonged to HAp crystal, in which the morphology was tiny needle-type with high aspect ratio (Fig. 3a) and sometimes we could observe plate-type crystallites, believed to be mainly OCP phase (Fig. 3b). The morphology of protein template in the GEL matrix showed a strong relevance with the formation of apatite phase. In the fluorinated sample it was hard to find fine needles or wide plates in Fig. 3 beside the typical rod type crystals in Fig. 2. Table 1 shows the indexing of the inter-planer spacing from the ED patterns in Figs. 2a and b, and d_{hkl} values were compared with the JCPDS data of $Ca_5(PO_4)_3F$ (FAp). The crystal phase in Fig. 2 corresponds to the FAp.

HAp/GEL, prepared by the normal coprecipitation, shows broad XRD pattern (Fig. 4) when it is compared with pure hydroxyapatite [HAP]. In the spray-HAp/GEL sample, XRD diffraction of (002) peak is more diffusive and the intensity is smaller, compared to that of HAp/GEL, even if it is hard to compare entire diffraction peaks. On the contrary the spray-FAp/GEL powders showed the stronger crystal growth. The (202) and (300) lattice planes were critically developed and down-shifted to lower degree because of F^- incorporation into OH^- site in HAp phase. The (002) peak was well developed, indicating the growth of better crystallites.

3.2 Crystal morphology in the GEL matrices

We suggest that different types of apatitic phase between OCP and HAp [18] could be induced by the morphology of protein template. The concept is completely based on the heterogeneous nucleation of apatite on GEL macromoleclules, in which the number of nucleation sites is directly

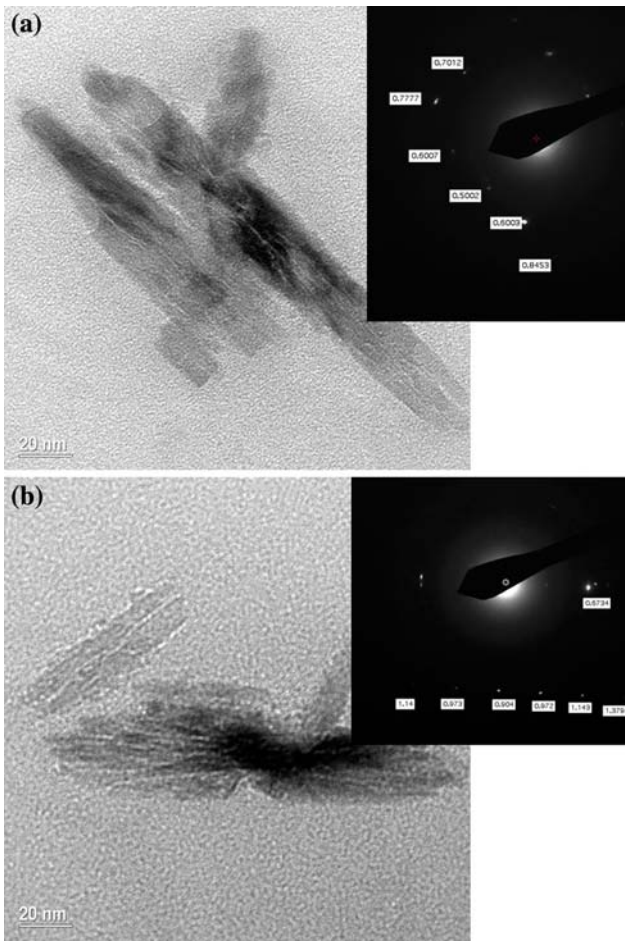

Fig. 2 TEM of (F, OH)apatite crystals showing typical 'side-to-side' orientation in spray-FAp/GEL. The scale bar indicates 20 nm. ED patterns (**a** and **b**) reveal the mostly fluoroapatite phase. The single crystalline particles with good aspect ratio were aligned by side-to-side orientation. The incorporation of F^- tremendously stabilized the apatite structure and contributed to the highly directional growth of apatite crystal on the GEL template. It seems that the driving force of F^- for apatite crystal structure was actively working even though it was the initial stage of crystal growth

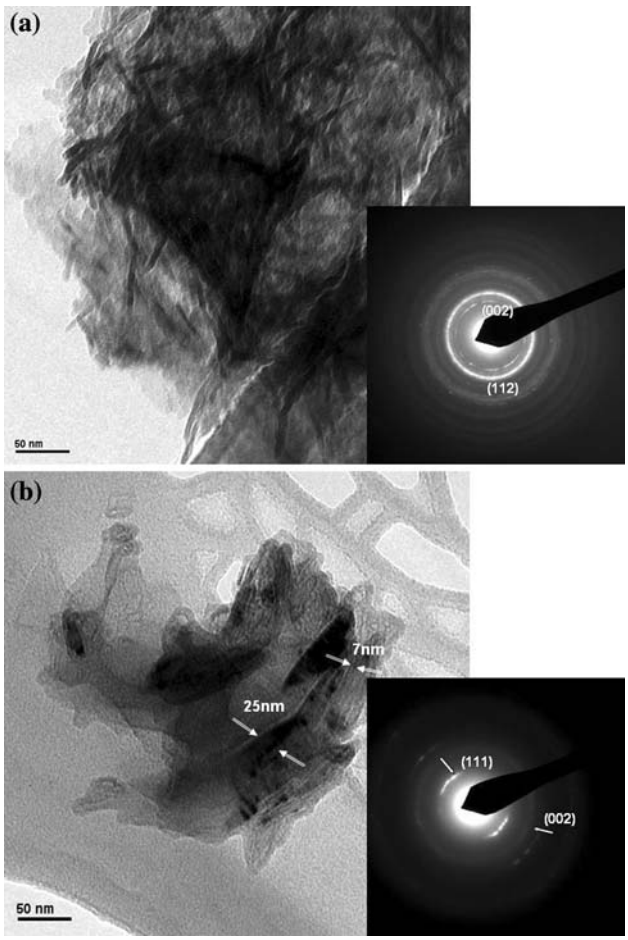

Fig. 3 TEM of apatite crystals in HAp/GEL showing needle-type crystal (**a**) and plate-type crystal (**b**). The scale bar is 50 nm. (**b**) We can know that the developed crystals are parallel to collagen band from the dark contrast along the worm-like fibril. From ED pattern HAp is identified in (**a**), while OCP prevails in (**b**)

Table 1 Indexing of interplaner distances (*d*-value) of ED patterns in Fig. 2

d (FAP/GEL)	*hkl*	*d* (JCPDS)
0.4648	311	0.4671
0.5002	203	0.5004
0.6003−7	322	0.6102
0.6734	502	0.6808
0.7012	511	0.7007
0.7777	423	0.7837

From the comparison between ED patterns and JCPDS the phase corresponds to $Ca_5(PO_4)_3F$

related with $[Ca^{2+}]$ or $[H_xPO_4^{-(3-x)}]$. H_3PO_4 is a triprotic acid [19], indicating the existence of $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}. If carboxyl group is a good binding site for Ca^{2+}, the number of carboxyl groups in the protein template will be one of factors to control $[Ca^{2+}]$ under the constant supplying of Ca^{2+} and GEL-phosphate solution. Ca^{2+} ions, supplied by the peristaltic pump are mixed with the phosphorylated GEL solution, and bound with carboxyl group to result in an apatitic phase through the reaction with the surrounding phosphates. Ca^{2+} ions can react with both of $H_xPO_4^{-(3-x)}$ ions and carboxyl sites of aqueous GEL macromolecules. Even if Ca^{2+} ions first meet with phosphates, the homogeneous clusters can be formed before growing into nuclei, and will have many chances to be pulled onto the carboxyl sites of the GEL molecules, which are the active sites for the heterogeneous nucleation.

The GEL aqueous solution is essentially not homogeneous in the protein conformation and the sorts of protein macromolecules. The precursor of the GEL contains

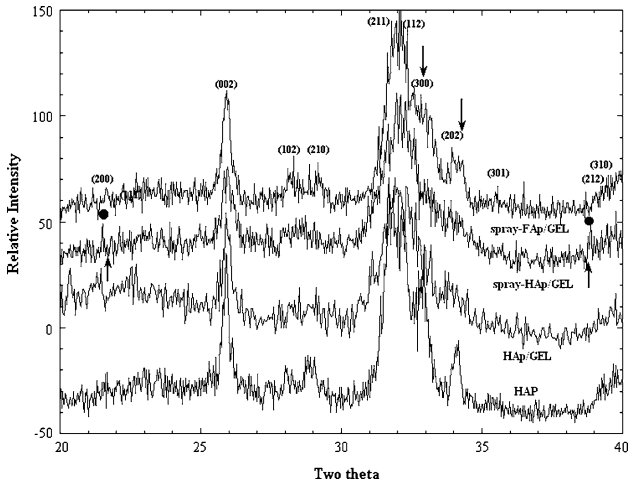

Fig. 4 XRD for spray-FAp/GEL, spray-HAp/GEL, HAp/GEL and pure HAP. The arrows indicate the crystal lattice shift by the incorporation of F^- into OH^-

various types of collagens with small amount of non-collagenous proteins [13]. The denaturation process produces a variety of the macromolecules conformation from fully disintegrated individual glands to collagen bundles. So we consider that the aqueous GEL solution contains (1) finely divided individual glands, which are frequently observed, (2) collagen fibrils, and (3) collagen bundles. The latter morphology of collagen fibrils or bundles is comprised of a weakly broken or disintegrated collagen, and observed less frequently. Especially collagen bundles, which were not sufficiently disintegrated in spite of the denaturation treatment, form the 2-D sheet having a versatile size distribution. The skeleton of unbroken collagen molecules bundles, and the large sheet comprised of the COL bundles can be an energy favorable template for the precipitation of OCP phase. In this experiment we vigorously stirred the reactants during the coprecipitation process. Under the static preparation condition without stirring, more amount of OCP phase can be expected [18]. It is supposed that fully disintegrated GEL gland (1) has more amounts of active functional groups for the apatite nucleation reaction compared to COL fibrils (2) or bundle (3). If a COL bundle forms an interconnected sheet it will have relatively less amount of functional groups. Therefore, under the condition of the continuously supplied Ca^{2+} and PO_4^{3-} to keep pH as 8.0, a large sheet of COL bundles will have a relatively high concentration of ions per a functional group which will favorably result in the formation of a stable OCP. On the contrary finely dispersed individual glands have higher amount of functional groups. Therefore, the resultant ion concentration per a functional group is relatively lower, which will induce the formation of HAp phase. As a result we can say that morphology of the GEL molecules template was strongly related with the development of OCP. However, it is noted that the fluoride incorporation into the apatitic phase greatly suppressed the existence of OCP phase. The existence of F^- during the coprecipitation reaction surpassed the influence of the ion concentration per a functional group, which was caused by the protein template morphology. The incorporation of F^- into apatitic crystal gave the very strong driving force to stabilize the normal apatite crystal. The energy minimization by the F^- incorporation into the Ca^{2+} triangle induces the most stable apatite structure [5, 16, 17]. It is known that low concentration of ppm order F^- helps to induce the OCP phase, while higher concentration of F^- strongly suppresses the formation of OCP [15, 16]. It seems that there is a turning point of $[F^-]$ for the energy minimization by F^- incorporation into apatite, and this point is related with the lattice relaxation of Ca^{2+} triangle. Fluoride ions promote the conversion of OCP to fluoroapatite (FAp), and the concentration strongly affects the precipitation and dissolution reaction of FAp [16, 17]. Increasing fluoride concentration tends to reduce the aspect ratio of crystallites produced. In general it is considered that OCP is formed in the initial stage of precipitation in vivo or in vitro, and a less stable phase to transform to the stable HAp phase with the aging [14]. However, it is not clear how long the lifetime scale of the OCP phase in artificial synthesis or biological condition. In our experimental condition, the OCP phase is long-lasting if it is embedded in the sheet type template of GEL matrix, while it is assumed that OCP is transformed to HAp rapidly if it is embedded in the well-dispersed GEL glands. It may be more reasonable that HAp crystals are directly precipitated in the homogeneous solution of single GEL glands [16]. The driving force of phase stability of OCP can be affected by the template morphology of the GEL matrix under the constant ion concentration, but we can say that the morphological driving force is rapidly suppressed by the fluoride incorporation.

3.3 FT-IR and organic–inorganic interaction

The organic coordination of apatite phase was analyzed from FT-IR spectra in Fig. 5. From PO_4 spectra between 1,200 and 900 cm^{-1}, the calcium phosphate (Ca–P) formation in the GEL was greatly affected by the spray precipitation process. PO_4 v_3 domain indicates the mineralization, stoichiometry, symmetry of tetrahedral PO_4, and the existence of HPO_4^{2-} [5]. 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 [5, 10]. The mineralization of spray-HAp/GEL was more diversified, and

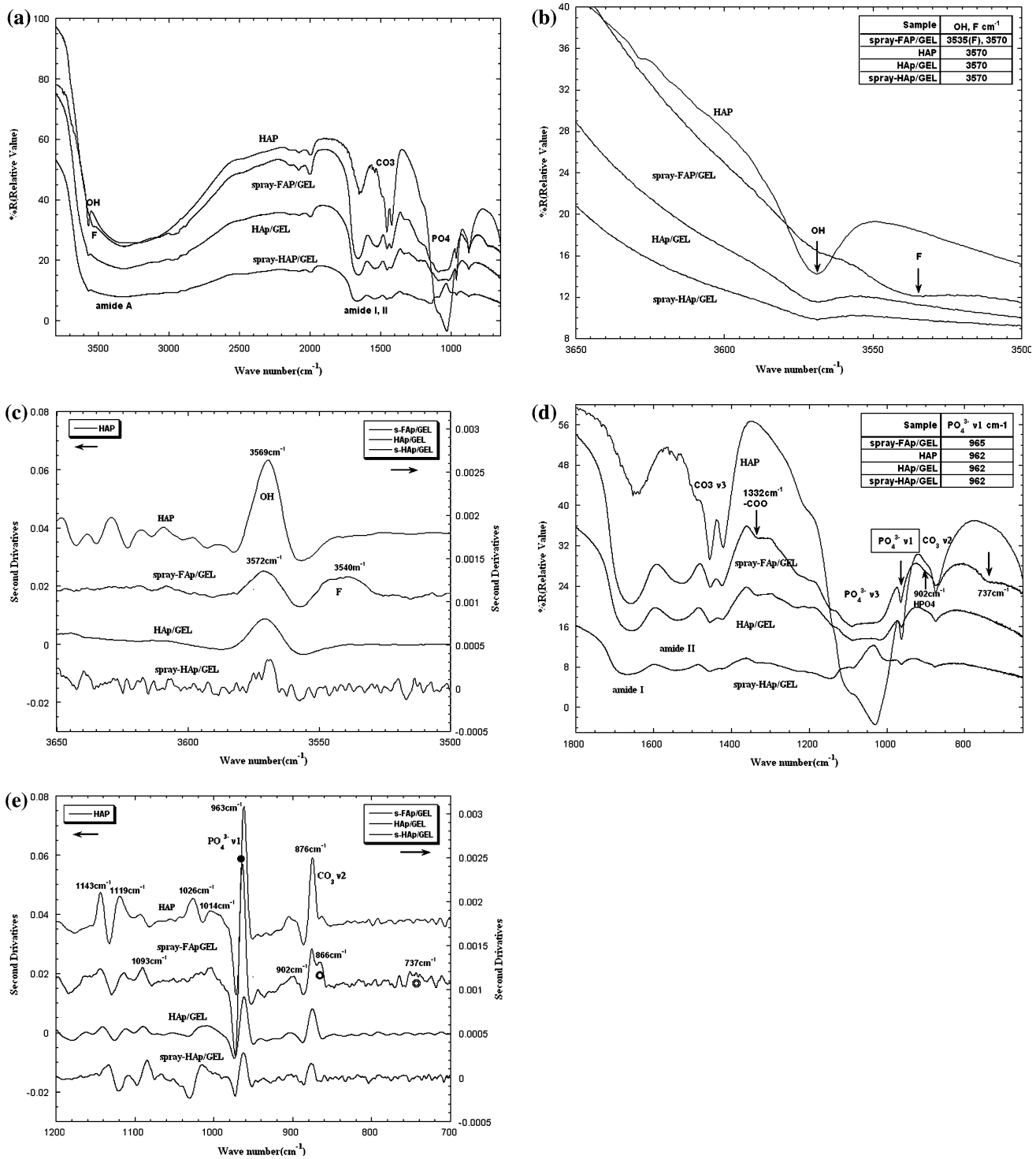

Fig. 5 FT-IR spectra for spray-FAp/GEL, spray-HAp/GEL, HAp/GEL and pure HAP. (**a**) Entire scan between 650 and 3,800 cm^{-1}. (**b**) OH^- and F^- band spectra. (**c**) Second derivatives for (**b**) spectra pattern. (**d**) Amide band and PO_4 band spectra. (**e**) Second derivatives for (**d**) spectra pattern

Ca–P peaks with HAp were developed in the spectra, compared to a normal HAp/GEL. It is noted that spray-FAp/GEL shows the typical apatite/GEL nanocomposite spectra pattern for more crystallized phase, which was induced by the incorporation of F^- into OH^- site in HAp. The incorporation of F^- in the composite was confirmed from the increase of F^- peak intensity and the decrease of OH^- peak intensity (Fig. 5b and c). The number of OH^-

site in apatite phase was greatly reduced in the spray process. In spray-FAp/GEL composite the population of OH^- site was nearly negligible. So, the formed crystal phase can be expressed as FAp in stead of (OH, F)Ap. In spray-HAp/GEL the formed crystal phases could be versatile such as HAp and Ca-P (Fig. 5d and e). The stable phase formation of spray-FAp/GEL could be confirmed from the 2nd derivative spectra (Fig. 5e), which are similar with that of HAp/GEL. The 2nd derivative spectra pattern of spray-HAp/GEL shows the very versatile peaks. The organic-inorganic interaction of these nanocomposites can be analyzed from amide I, II and PO_4 band in Fig. 5d. The 737 cm^{-1} band well appeared in spray-FAp/GEL nanocomposite structure, and the incorporation of F^- induced the relatively stronger bands such as Ca^{2+}-carboxyl band at 1,332 cm^{-1} and HPO_4 band at 902 cm^{-1}. Band frequency of PO_4 v_1 for spray-FAp/GEL was down-shifted, indicating the lattice change by the F^- incorporation. PO_4 tetrahedral configuration is distorted by the introduction of F^- into the Ca^{2+} triangle in HAp structure because the fluoride group (Ca–F bond) has a larger ionic radius than that of the OH group (Ca–OH bond) [5, 17]. The distortion can also be confirmed from the difference in lattice change of a-axis and c-axis (Fig. 4). As commented in XRD, (200) and (300) peaks were shifted to lower degree as shown in the arrows, but the shift of (002) peak was negligible.

3.4 Reaction model

In this spray process we suggest the schematic reaction models (Fig. 6) for the formation of (OH)Ap and (F, OH)Ap. The free ions of Ca^{2+} are released from $Ca(OH)_2$ in water and the releasing kinetics is modeled (Fig. 6a). The schematic region of a $Ca(OH)_2$ powder can be hypothetically classified as hydrolyzed cluster, buffer layer and solvated layer. The triprotic ions from H_3PO_4 will react with the released Ca^{2+} and form the apatite phase. Actually free calcium ion Ca^{2+} will coordinate with the phosphorylated GEL macromolecules and so apatite/GEL nanocomposite will be formed. The hydrolysis of $Ca(OH)_2$ was promoted by the introduction of F^- (Fig. 6b), and so the crystals were highly developed. Ca^{2+} ion releasing was accelerated by the presence of F^- and so the reaction kinetics was very fast.

In the coprecipitation process under limited humid atmosphere, the formation and stability of apatitic phase was decided by the reaction kinetics. It was not easy to form pure hydroxyapatites in the GEL matrix, even after 24 h aging of the precipitates in the water. The initial kinetics in this mist reaction was very important for the stability of phase formation. However, the existence of fluoride ions in calcium hydroxide slurries did a very strong role to make a highly crystalline phase of FAp. The

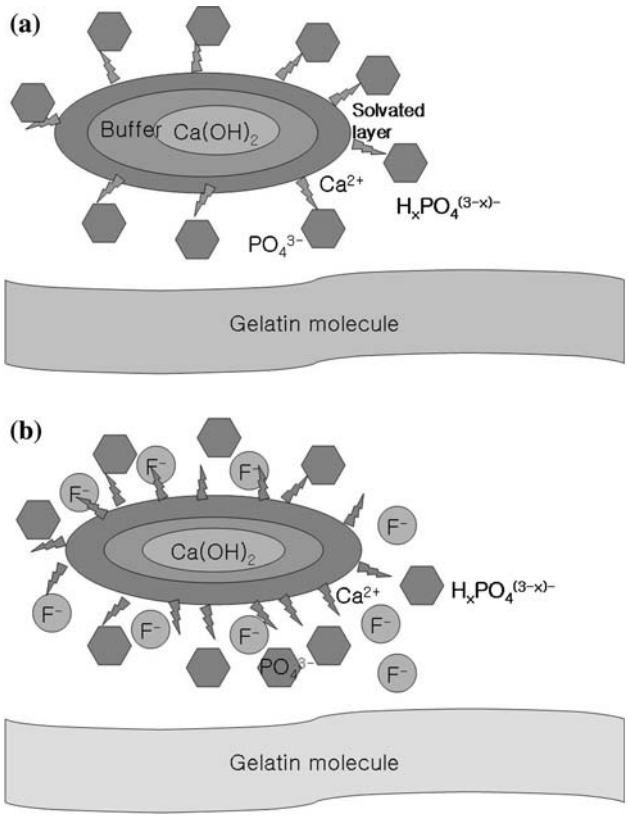

Fig. 6 Hypothetic schematic model of reaction kinetics for HAp/GEL (**a**) and FAp/GEL (**b**)

hydrolysis of $Ca(OH)_2$, releasing Ca^{2+} ions into the solution, was an important control factor to develop a good apatite crystal. The supply of Ca^{2+} ions is a rate-determining step for the formation of apatite crystals (Fig. 1b).

4 Conclusion

The incorporation of F^- into HAp/GEL nanocomposite greatly induced the stable phase formation and crystal development of FAp in apatite complex phase. The designed spray-process was very effective to compare the driving force difference between HAp/GEL and FAp/GEL nanocomposites. The mist of sprayed droplets of fluorinated solution resulted in the stable crystallites of FAp/GEL composition.

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