Apatite formation on collagen fibrils in the presence of polyacrylic acid

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Recently, we reported the formation of bone-like apatite on collagen fibrils by biomimetic method. Compounds containing carboxyl moieties are believed to be effective in the formation of apatite. Polyacrylic acid $[-CH_2CH(COOH)-]_n$ (PAAc) is widely used in dentistry. In the present study, the effect of PAAc in the formation of apatite from revised simulated body fluid (R-SBF) on collagen fibrils was studied. Two different experimental approaches were tried to study the effect of PAAc present in the collagen and in the R-SBF solution. In the first, collagen gel was soaked with 1 mg/ml PAAc (molecular weights 25 000 and 100 000) for the time intervals of 30 min and 6 h. The gels were then dried in air and incubated in R-SBF. Characterization of the precipitates formed on the collagen fibrils in gel showed that the formation of apatite was inhibited when soaked in PAAc for 6 h. In the second experiment, when PAAc (0.1 and 1.0 mg/ml) was mixed with R-SBF the microstructure of the precipitates formed on collagen fibrils was affected partially. Hence, it is confirmed that the presence of PAAc in the biomimetic environment of collagen affects the mineralization of apatite.

Introduction

Human bone and dentin are biologically formed composites of hydroxyapatite (HAP) and collagen. Synthetic HAP having the formula $[Ca_{10}(PO_4)_6(OH)_2]$ resembles more closely to the inorganic constituent of mineralized bone tissue. It has been the most studied material and is demonstrated to be a bioactive biomaterial [1–4]. HAP combined with gelatin and HAP immersed in collagen has shown excellent bioactivity [5–8]. In our pervious reports, we had shown that reconstituted collagen fibrils form bone-like carbonated apatite from SBF solutions [9, 10].

It has been shown that all calcified tissues such as bone, dentin and enamel contain polyanionic macromolecules such as phosphoproteins [11, 12]. Calcificability of collagen matrix *in vivo* has been attributed to many non-collagenous proteins that could mediate between mineral and collagen [13, 14]. These interactions with calcium phosphate mineral phase play important roles in biological systems [15]. Compounds containing the functional groups such as phosphate, carboxyl, citrate, pyrophosphate, etc. are implicated in the adsorption and crystallization of calcium phosphates [16–18]. The carboxylate groups of collagen are supposed to be responsible for the nucleation of HAP on collagen from SBF solution [19]. Presence of carboxyl moieties in PAAc makes it a preferable material for the simulation system of biological calcification in the presence of additives.

Various researchers had studied the effect of PAAc present in the mineralizing environment of apatite in solution as well as in the substrate. Taguchi *et al*. [20] had shown that the amount of apatite formed on PAAc grafted polyethylene films increased with an increase in grafting density up to $30\,\mu g/cm^2$. Also, hot pressing of an acrylic acid copolymer with tetracalcium phosphate powder has resulted in a biocomposite, in which HAP crystals were embedded in a network of calcium salt of the polymer [21]. Conversely, inhibition of apatite formation in SBF was observed by Kamitakahara *et al*. [22], in the investigation of the possibility of obtaining a bioactive glass-ionomer cement. It was confirmed that the release of PAAc present in the glass-ionomer cement into SBF inhibits the formation of apatite on their surface. Also, the suppression of HAP formation on a polymeric substrate in the presence of PAAc in the reaction solution was reported by Yokogawa *et al*. [23].

Polyalkenoic acids such as PAAc are extensively used in biomedical applications. PAAc is widely used in dental restorative materials, as they form both molecular and mechanical bonding with native tooth materials [24]. Collagen and HAP are the native component materials of bone and dentin. Therefore, a biomimetic study of the effect of PAAc in the mineralization of HAP on collagen *in vitro* can be useful to understand the *in vivo* phenomenon.

The objective of the present *in vitro* investigation was to study the influence of PAAc in the formation of HAP

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on reconstituted collagen fibrils from R-SBF solution by biomimetic method. Two different experimental approaches were tried to study the influence of the presence of PAAc in the substrate (in the collagen gel medium) and in the solution (in R-SBF).

Experimental

Wako chemicals, Tokyo, Japan, supplied all the chemicals used in this experiment.

Preparation of collagen gel

Type I collagen solution (porcine skin, 3 mg/ml in 1 N HCl, pH 3) was adjusted to pH 7.2 by adding appropriate amounts of 1.5 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M NaOH. Concentration of collagen after pH adjustment was about 1 mg/ml and was allowed to polymerize at 36.5 °C for one day. Polymerized gels were cross-linked in glutaraldehyde vapor atmosphere. The resultant gel thickness was approximately 1 mm.

Preparation of R-SBF

The R-SBF solution was prepared by dissolving NaCl, $NaHCO_3$, Na_2CO_3, KCl, K_2HPO_4, $MgCl_2$, 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethane sulfonic acid (HEPES), $CaCl_2$, Na_2SO_4 and 1 M NaOH in distilled water [25].

Incubation in R-SBF

The gels were immersed in R-SBF solutions contained in plastic bottles with airtight lids and maintained at 36.5 °C. The effect of PAAc was studies in two different experimental approaches.

The effect of PAAc present in the substrate

Cross-linked gel was kept in contact with the solution of 1 mg/ml PAAc in water. Individual experiments were performed with PAAc of molecular weights 25 000 and 100 000 for the soaking periods of 30 min and 6 h. The gel was removed from the additive solution and dried in air at room temperature for 1 h before incubating in R-SBF solution. Incubation was done for the soaking periods of 1, 3, 5 and 7 days.

The effect of PAAc present in the solution

Experiments were conducted by mixing PAAc (molecular weight 25 000) directly with R-SBF solution (0.1 and 1.0 mg/ml). Incubation was done for the soaking periods of 1, 3, 5 and 7 days. The pH value of the solution was measured for each soaking period.

After incubation, the gels were washed with water and dried by critical point drying and a thin film of collagen gel was obtained.

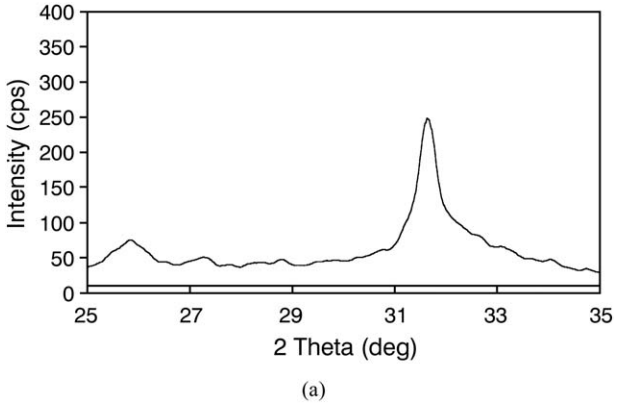
Characterization

Thin film X-ray diffraction (TF-XRD) pattern of the gels were recorded on a MAC Science MXP diffractometer using monochromated CuKα radiation at 40 kV and 20 mA. Scanning electron microscopy (SEM) was performed using a Hitachi S-3000N SEM. Micro FT-IR spectra was recorded on a Jasco Micro-FT-IR Jansen Fourier transform infrared spectrometer by encasing the sample in a transparent KBr matrix. pH measurements were done using Mettler Toledo MP220 pH meter.

Results and discussion

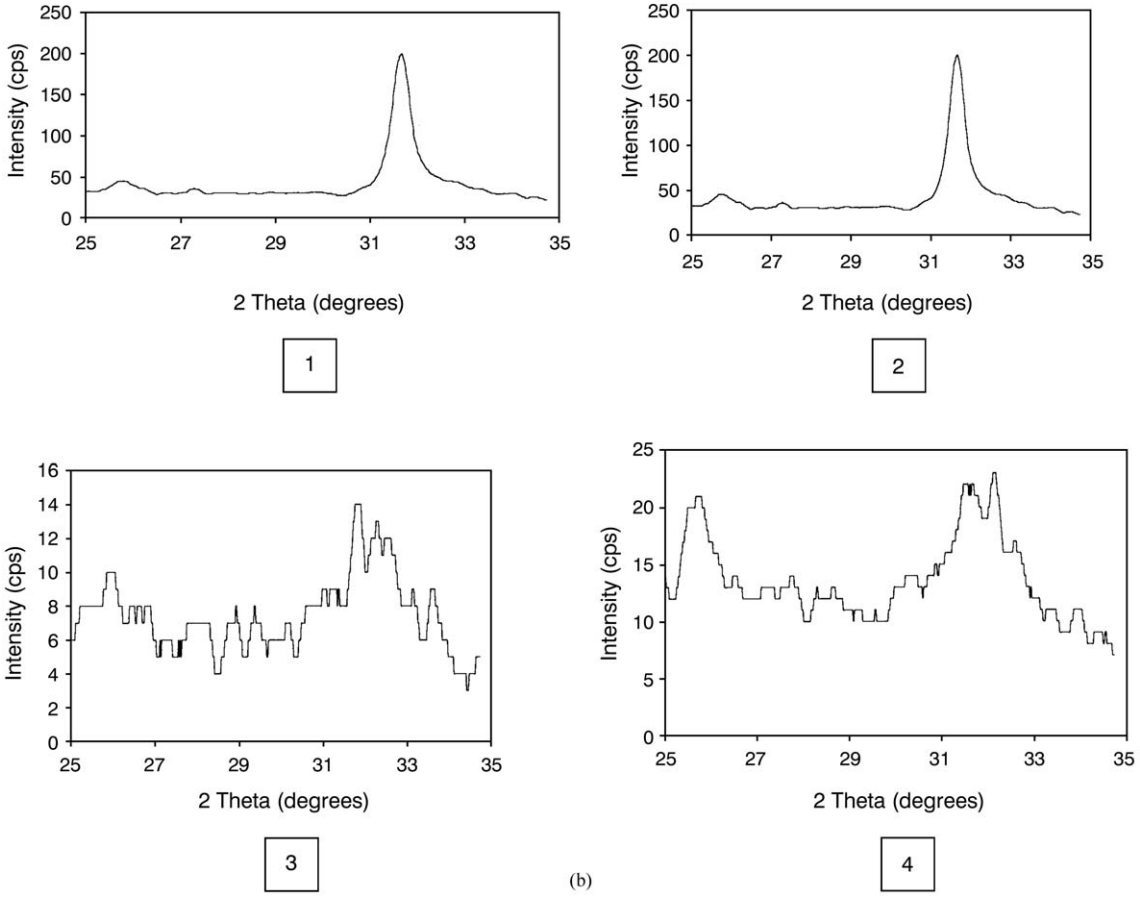
TF-XRD analysis

Fig. 1(a) is the TF-XRD pattern obtained for the samples in the absence of PAAc. Fig. 1(b) and (c) are those obtained for the first and second approaches, respectively. The broad peak in between 31 and 33° is due to the 211, 112 and 300 reflections characteristic to apatite [26]. The broadness of the peaks may be due to the low crystallinity or the small crystallite size of the apatite. The intensity of these reflections reduced drastically for the precipitates obtained on the gels soaked for 6 h in the solution of PAAc. The reflection was recognizable when the full scale was reduced (Figs. 1(b)(3) and (4)]. The remarkable reduction in the intensity indicated that the long time soaking in PAAc inhibited the precipitation of apatite. A non-apatite extra peak is seen at 27.38°. This

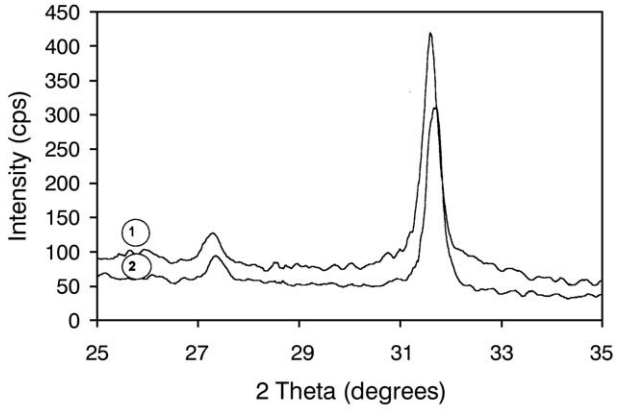


(a)

Figure 1 (a) TF-XRD pattern of the sample soaked in RSBF in the absence of PAAc. (b) TF-XRD pattern obtained for the samples in the first approach: (1) 25 000; (2) 1 00 000 M.W. PAAc for 30 min; (3) 25 000; (4) 100 000 M.W. PAAc for 6 h, and (c) TF-XRD pattern obtained for the samples in the second approach: (1) 0.1 mg/ml; (2) 1.0 mg/ml.



(b)



(c)

Figure 1 (Continued)

might represent the precipitation of traces of calcium carbonate [27].

SEM Analysis

The SEM observation of the gels soaked in SBF solution reveals a thin membrane-like layer on the top and bottom encasing bundles of lengthy reconstituted collagen fibrils with spherical deposits of calcium phosphate along them (Fig. 2). The underlying fibrils are exposed through the cracks and the voids of the surface layer. In the present study, the samples obtained in the first experimental approach revealed a similar structure with lumps of spherical deposits on the surface layer of the gel (Fig. 3). In the second approach different microstructures were observed. Some samples exhibited the previous microstructure (Fig. 4(a)). While others were spherical precipitates with fibrils mixed in the surface layer or collagen fibrils with thick coating as shown in Fig. 4(b) and (c) respectively.

FT-IR analysis

Fig. 5(a) is the FT-IR spectra for the samples in the absence of PAAc. Fig. 5(b) and (c) are for the first and second approach, respectively. The band around 601 cm^{-1} is due to the γ_4 vibrations of the PO_4 group in apatitic structure precipitated from solution [28]. The out of the plane mode of $(CO_3)^{2-}$ ion is observed at 875 cm^{-1}. The broad band in the region 968–1200 cm^{-1} is due to the γ_3 vibrations of the PO_4 group [29]. The stretching mode of $(CO_3)^{2-}$ is observed around 1428 and 1470 cm^{-1}. The peak around 1670 cm^{-1} arises from the C=O of the peptide bond of the collagen. The carbonate peaks at 875, 1428 and 1470 cm^{-1} suggest that the carbonate substitution has occurred in the B site [30]. The additional peak around 1560 cm^{-1} and also the peak around 1428 cm^{-1} are assigned to the vibrations of $(COO)_2Ca$ [21, 23]. This confirms the formation of chelation complex from the carboxylic acid group of PAAc and the calcium ion from

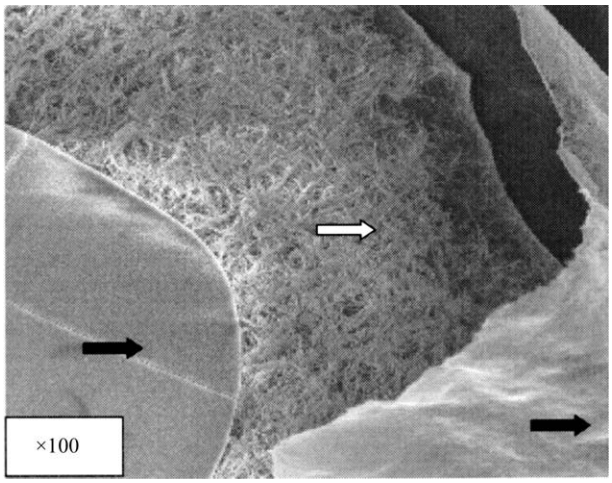


Figure 2 SEM micrograph showing the microstructure of the collagen gel soaked in SBF.

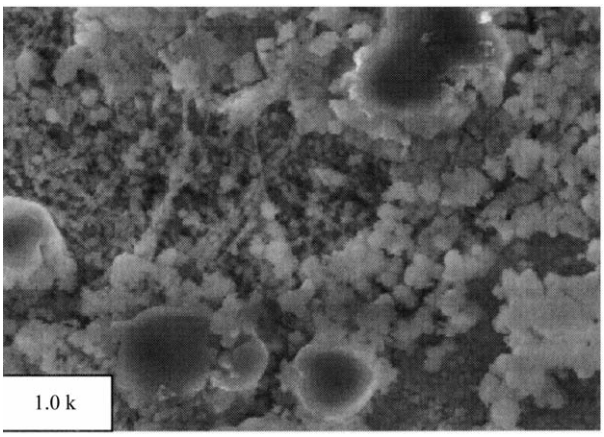


Figure 3 SEM micrograph showing the microstructure of the samples in the first approach.

R-SBF. Although the intensity of the bands varies enormously, the presence of phosphate, carbonate and chelation complex in all the samples of the first and second approach is apparent.

Fig. 6(a)–(c) show the change in the pH of the R-SBF solution in the absence of PAAc and of the second approach in which PAAc was directly mixed with the R-SBF solution. The R-SBF solutions used were prepared in two different batches and had a slightly different pH. Therefore, there was a slight difference in the initial pH of the solutions for the PAAc absent and PAAc present (0.1 and 1.0 g/ml) experiments. The pH of the solution decreased with increase in the incubation time of the collagen gel when PAAc was absent. This is an indirect proof of apatite precipitation, that involves consumption of OH ions, and hence the pH decrease. Dissolution of PAAc into R-SBF solution resulted in the formation of a chelation complex. Also, the inhibition effect on the apatite formation caused by the addition of PAAc might be the reason for the increase in pH of the solution.

The rationale behind the use of R-SBF over the conventional SBF (C-SBF) for the present study was that the ionic concentration of HCO_3^- and Cl^- are also the same to those of human blood plasma. The pH of the collagen solution was adjusted to 7.2 by the addition of 1.5 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M NaOH in a ratio 0.5 : 9 : 0.5. Though the gels were washed thoroughly in distilled water after polymerizing and cross-linking, the sodium, chloride and phosphate ions might not have been removed completely from the gel before incubation in R-SBF solution. Formation of bone-like carbonate apatite in R-SBF by reconstituted collagen fibrils had been reported in our previous study [10]. As the Cl^- content was less in the precipitates formed from R-SBF than that from C-SBF on collagen fibrils it was clear that the intention of use of R-SBF was not impaired.

The precipitates formed along the collagen fibrils did not loose the morphology or crystallinity by treating the collagen gel with 1 mg/ml PAAc of both molecular weights for 30 min. But a remarkable inhibition of apatite precipitation was observed when the gel was treated with the above PAAc solutions for 6 h. While the morphology of the deposits were affected partially by the direct mixing of PAAc with R-SBF.

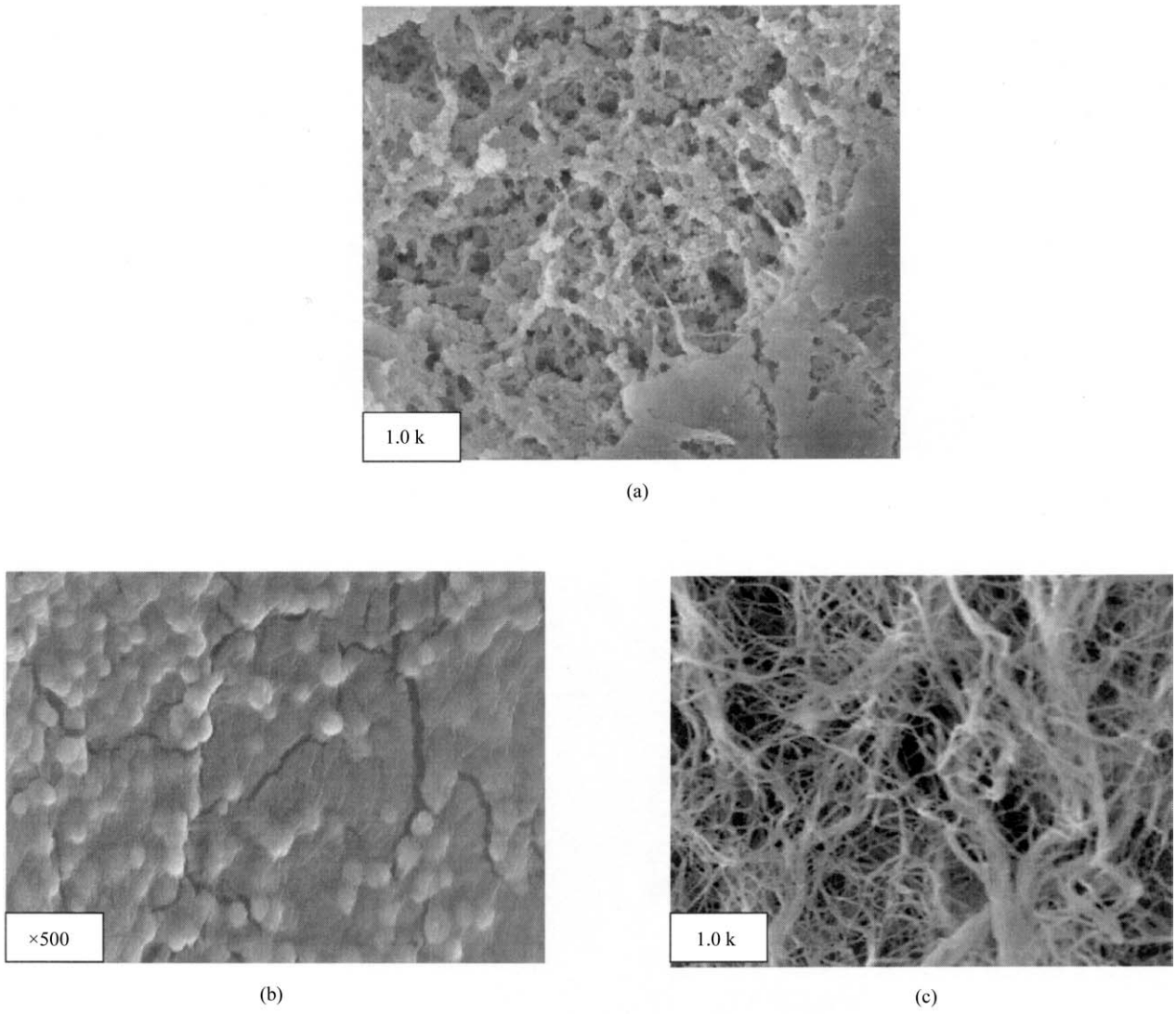


Figure 4 (a), (b) and (c) SEM micrograph of the microstructures observed in the second approach.

In all the samples obtained by both approaches, the peak around 1570 cm^{-1} in the FT-IR spectrum is appreciable, proving the formation of $(COO)_2Ca$ chelation complex. This explains the inhibition of apatite formation and change in morphology of the precipitates in presence of PAAc.

Comparing to the precipitates obtained on collagen fibrils from R-SBF in the absence of PAAc [10], the results obtained in our present study are summarized below. Presence of PAAc

1. in the substrate, inhibited the apatite formation for both the molecular weights (25 000 and 100 000) when soaked for 6 h;
2. in the solution, both the concentrations (1 and 0.1 mg/ml) have changed the morphology of the deposits, but not completely inhibited the apatite formation.

From the present investigation, it was concluded that in the first approach, long time exposure of the collagen gel to PAAc solution may lead to adsorption of more PAAc molecules and hence resulted in the inhibition of apatite precipitation. In the second approach when PAAc was mixed with R-SBF, chelation complex formed in the solution and deposited along the fibril or on the surface of the gel. Also, the carboxyl molecule along the collagen fibril interacted with the Ca^{+} and $(PO_4)^{-}$ ions in R-SBF and formed apatite.

Conclusion

Mineralization of calcium phosphate from R-SBF solution on collagen fibrils in presence of PAAc in the gel as well as in the solution was studied. The precipitates formed were analyzed by TF-XRD, SEM, EDX and FT-IR. The inhibition of apatite formation was observed when the gels were treated with PAAc solution for 6 h. The PAAc present in the solution was found to affect the microstructure partially. Formation of $(COO)_2Ca$, the chelation complex from the carboxylic group of PAAc and the Ca^{+} ion from the R-SBF was confirmed by FT-IR. This study shows that the presence of polyacrylic acid affected the mineralization of apatite on collagen.

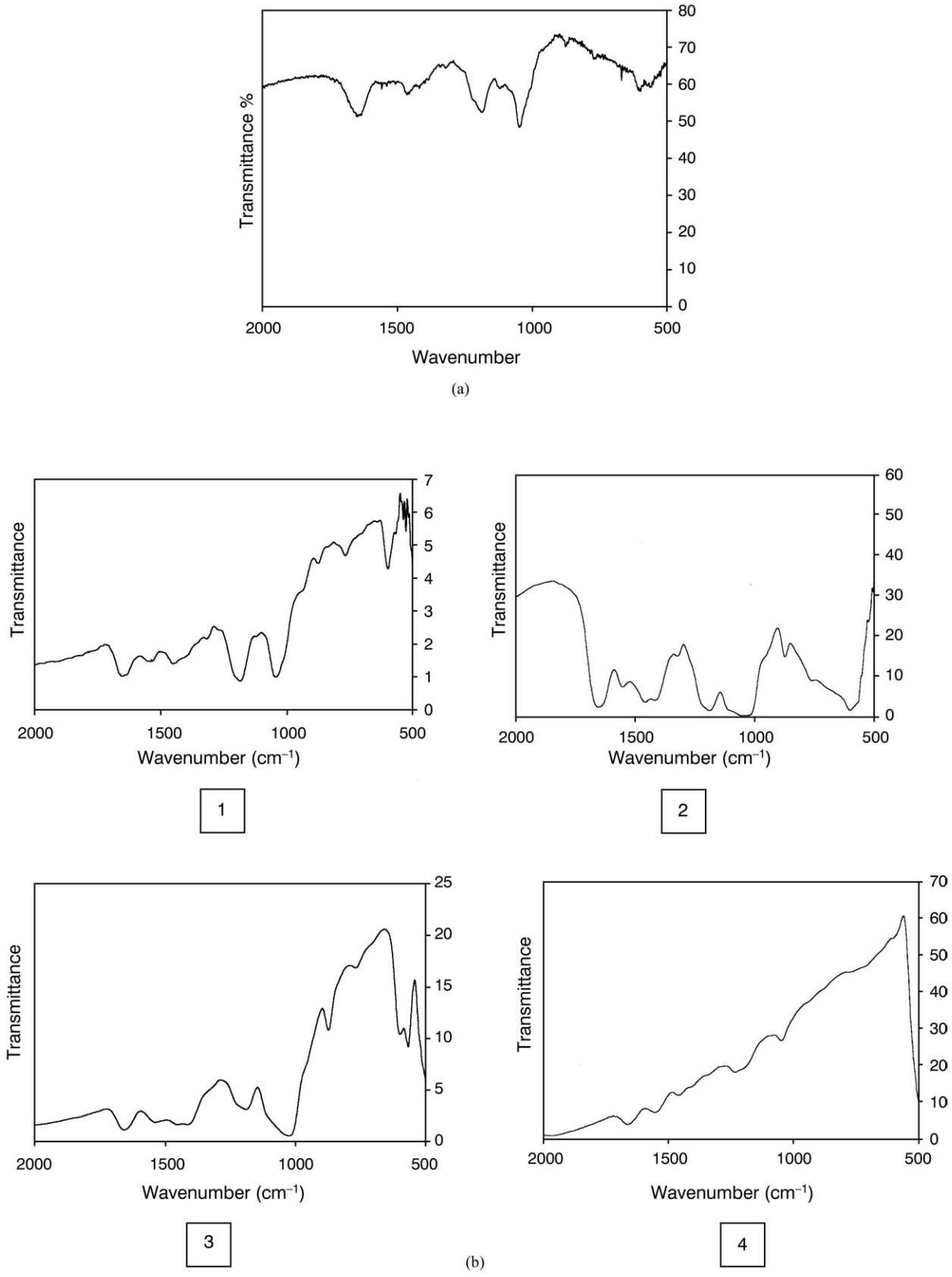
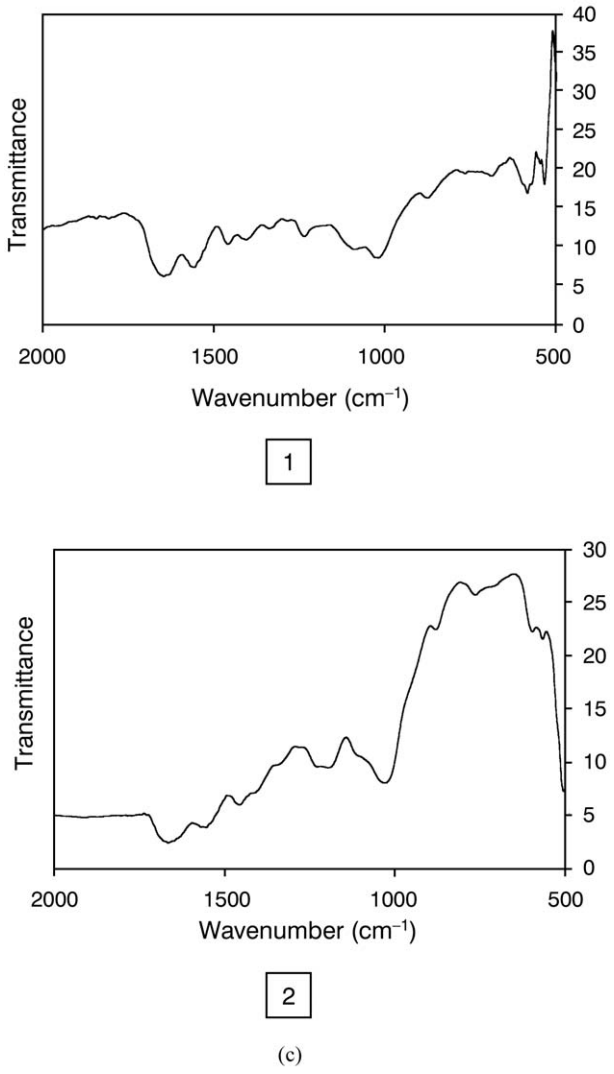


Figure 5 (a) FT-IR spectra of the sample soaked in R-SBF in the absence of PAAc. (b) FT-IR spectra of the samples obtained from: (1) 25 000; (2) 100 000 M.W. PAAc for 30 min; (3) 25 000; (4) 100 000 M.W. PAAc for 6 h and (c) FT-IR spectra obtained for the samples in the second approach: (1) 1.0 mg/ml PAAc; (2) 0.1 mg/ml PAAc.



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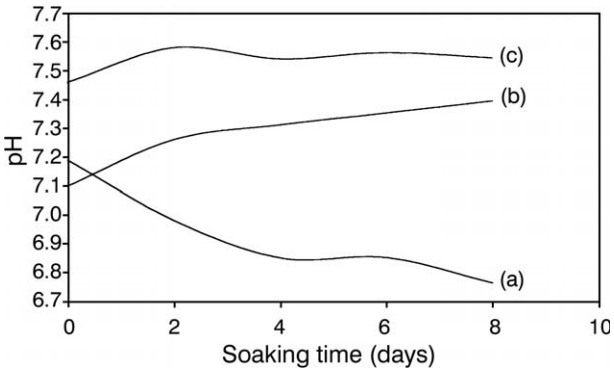

Figure 6 Change in pH of the R-SBF solution with soaking time: (a) in the absence of PAAc; (b) in the presence of 1 mg/ml and (c) 0.1 mg/ml PAAc.

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