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Protein release behavior from carbonate apatite hydrogel

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Abstract

Incorporation of protein in poorly crystalline carbonate apatite hydrogel, the protein release behavior from the cake and the dissolution of the cake in aqueous solution were studied. Poorly crystalline apatite was prepared by precipitation of saturated solution of calcium and phosphate in air or under N_2 atmosphere. The carbonate content and particle size of obtained hydrogel depends on the maturation period and atmospheric condition. The mixture of apatite hydrogel and protein was dried for 4 days in air at 40% of relative humidity to yield cakes. The quantity of loaded cytochrome *c* in apatite hydrogel formed in air was 0.490 wt.%, while that in apatite hydrogel formed under N_2 atmosphere was 0.305 wt.%. But the quantity of loaded albumin in apatite hydrogel formed in air was less than that in apatite hydrogel formed under N_2 atmosphere. For the cakes containing protein immersed in aqueous solution, the protein release was less than the decrease in weight of apatite cake, which may be associated with apatite recrystallization and protein re-incorporation. The quantity of released protein may depend on the quantity of loaded protein in apatite hydrogel.

Keywords: Drying; Inclusions; Biomedical applications

1. Introduction

Hydroxyapatite (HAp) and related calcium phosphate have good biocompatibility and osteoconductive properties, and have been widely used as bone filler, spacer, and bone graft. With the osteoconductive properties, HAp ceramics has been also investigated as scaffolds for cell delivery and tissue engineering.[1] The interaction between cells and surfaces should play an important role in cellular behavior, and is mediated through adsorbed proteins and biologically active molecules.[2] The biologically active molecules stimulate the cells to proliferate and differentiate to form specific tissues. The adsorption of proteins onto HAp is important in a variety of oral or osseous biological events. HAp has also adsorptive capabilities with respect to proteins and biologically active molecules, such as osteogenic agents and growth factors.

Extensive studies on incorporation of such molecules on/in calcium phosphate matrices and their release behavior were reported.[3–10] The way of adsorption of the molecules on the calcium phosphate matrices has been employed for the delivery system and coatings on implant substrate.[3,4] Suspended proteins are precipitated on the calcium phosphate matrices, then most of the loaded molecules dissociate within the first 48 h, so called "high initial burst" release.[5,6] The total amount of loaded molecules is also dependent on the surface characteristics of calcium phosphate matrices, and the period of release was limited to 2–3 weeks. A number of studies on calcium phosphate cements in which therapeutic compounds were incorporated were performed, and a variety of compounds could be immobilized, and the period of release was dependent on the molecular weight of the species diffusing from the cement and the interaction of the species with HAp. Carbonate apatite is more soluble in vivo than HAp[7] and may have different manner of protein adsorption and desorption behavior. Barralet et al.[10] reported that proteins were incorporated in carbonate apatite or amorphous calcium phosphate suspension, which was filtered to form a

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cake. The rate of short-term release of albumin was lower from carbonate apatite than microcrystalline HAp, but the period of release was limited, which may have been associated with recrystallization of the apatite.

Biological apatite is poorly crystalline carbonate apatite with high surface reactivity due to their nanometric crystal size and the hydrated layer rich in active environments of mineral ions on the crystal surface. Poorly crystalline carbonate apatite can be obtained by precipitation from saturated solution of calcium and phosphate.[11] The authors reported the preparation of poorly crystalline apatite materials by caking of the precipitates.[12] The carbonate content of apatite was found to be changed by maturation period and atmospheric conditions. The caking behavior mainly depends on the relative humidity around the cake, as well as particle size of precipitates.[12] The purpose of this study is to investigate the protein loading on carbonate apatite hydrogel with different carbonate content and particle size, and the release profile of proteins and the dissolution of the cake containing proteins stored in aqueous solution.

2. Materials and methods

2.1. Materials

All chemicals used in this study were supplied by Wako Pure Chemical Industries, Ltd. or Katayama Chemicals and used without further purification. N_2-purged water obtained from distilling water previously passed through an ion-exchange resin was used in the preparation of all aqueous solutions. The preparation of apatite hydrogel was the same as in the previous report.[12] Di-sodium hydrogen phosphate dodeca-hydrate ($Na_2HPO_4{\cdot}12H_2O$) and calcium chloride 2 hydrate ($CaCl_2{\cdot}2H_2O$) were separably in distilled water. The latter solution was put into the former solution, and pH was adjusted to 7.40 using sodium hydroxide solution. The mixed solution was stirred at 500 rpm at room temperature for 14 days in air or under N_2 atmosphere. A certain amount of protein was added into the mixed solution and kept for 30 min at room temperature. Cytochrome *c* (ca. 12 kDa) from equine heart and albumin (ca. 66 kDa) from bovine serum (BSA) were used in this study. The precipitates containing protein filtered off and washed with distilled water were dried in air to make cakes. The washed solution was kept, and the amount of washed out protein was measured. The filtered apatite hydrogel containing protein was put in plastic container of 33 mm diameter and 5 mm in depth, the specimen was taken outside plastic container 1 day after. The specimens were dried for 4 days at 40% of relative humidity to make cakes.

2.2. Determination of adsorbed amount

The protein loaded materials were soaked in aqueous solution (pure water or phosphate buffer saline, PBS) at room temperature, and the released protein into solution and the amount of washed out protein was measured using HPLC (Shimadzu LC-10A system) with UV–vis detector and hydrophobic column. The cakes after a certain period of soaking were taken from solution, and washed with distilled water. The washed cakes were dried and weighed, and the size was measured.

2.3. Characterization

The morphologies of the samples were observed by scanning electron microscopy, and EDX analyses were performed. All micro-FTIR spectra were recorded using samples encased in a transparent KBr. The X-ray powder diffraction patterns of the materials were recorded on a MAC Science MXP[3] diffractometer using Cu Kα radiation at 40 kV and 20 mA and monochrometer. TEM observation of the products was conducted using JEOL, JEM-4000FX at 300 kV. The determination of the Ca and P content of the products were done using an inductively coupled plasma emission spectroscopy, Seiko, SPS7000. The surface potential of apatite hydro gel was measured using Laser zeta potential analyzer. The porosity and Vickers hardness of the dried specimen were also measured.

3. Results and discussions

3.1. Materials

The precipitates were formed from a mixed solution of $Na_2HPO_4{\cdot}12H_2O$ and $CaCl_2{\cdot}2H_2O$ and maturated in air or under N_2 atmosphere. The X-ray diffraction data of the precipitates showed an apatitic pattern, and broadness of peaks were observed, which is analogous with bone minerals.[11] These precipitates formed from an aqueous solution are hydrated layer rich, and apatite hydrogel. The FTIR spectroscopy indicated that the apatite hydrogel was carbonate apatite. The absorbance ratio of carbonate/phosphate of the apatite hydrogel prepared under N_2 was low compared to those prepared in air.[10] The CO_2 content of apatite hydrogel was analyzed by using CO_2 analyzer. Both increase with an increase of maturation period, but that prepared in air was higher than that in N_2 atmosphere as shown in Fig. 1. The apatite hydrogel was precipitated from "carbonate-poor" solutions,[11] that is carbonate ions were not included in starting materials, and the CO_2 should be coming from air.

TEM images of the crystals in apatite hydrogel indicated that the crystals formed in 1 day were angular and irregular in shape. Those formed both in air and in N_2 for several days were rod-like in shape and 100–200 nm long, and they seemed to grow somewhat with an increase of maturation period, but almost be the same in size, independent of maturation period and atmospheric condition. Fig. 2 shows the particle size and specific surface area of apatite hydrogel formed in air. The particle size of apatite hydrogel formed in air for 1 day was

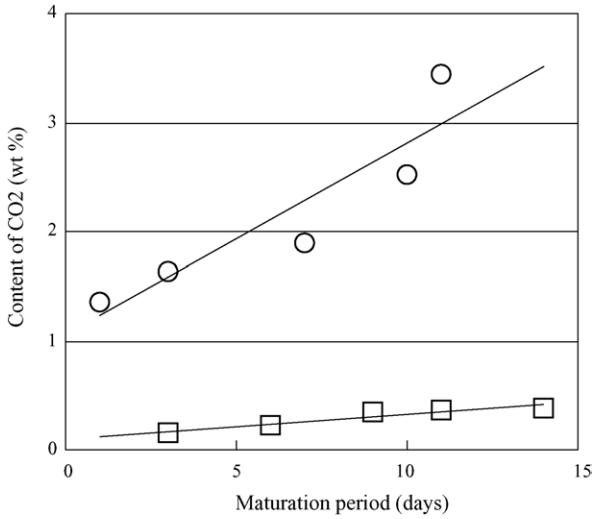

Fig. 1. CO_2 content of apatite hydrogel formed in air (open circle) and under N_2 atmosphere (open square) as a function of maturation period.

2–3 μm, and decreased to 0.4 μm after 15 days of maturation period. But those increased in N_2 from 1 to 4–5 μm with an increase of maturation period from 1 to 15 days. The particle size of apatite hydrogel should depend on the dispersibility of apatite crystallites, and the aggregate of apatite crystallites may be strongly affected by the carbonate ions in apatite hydrogel.

3.2. Protein adsorption and caking

The quantity of loaded cytochrome *c* in apatite hydrogel formed in air was 0.49 wt.%, while in apatite hydrogel formed under N_2 was 0.305 wt.%. The quantity of adsorbed protein should depend on the specific surface area of apatite hydrogel. The particle size and specific surface area of the former were around 0.4 μm and 80–100 m^2/g, and those of the latter were around 4 μm and 20–30 m^2/g. But the quantity of loaded alubmin in apatite hydrogel showed different

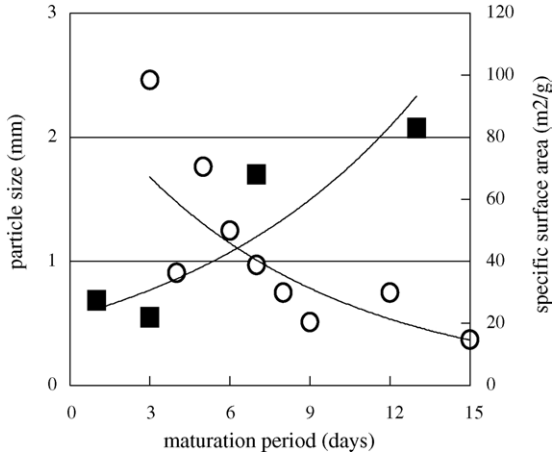

Fig. 2. Particle size (open circle) and specific surface area (filled square) of apatite hydrogel formed in air as a function of maturation period.

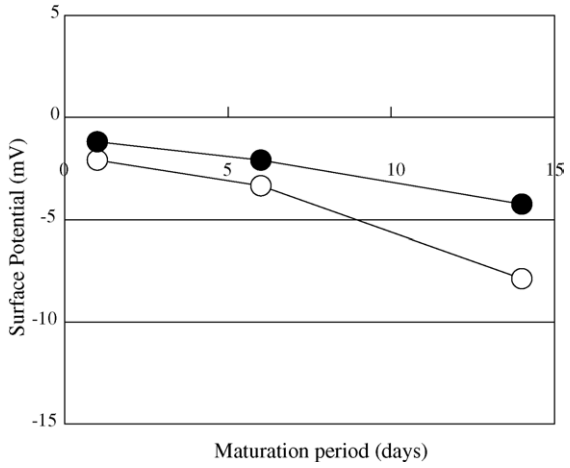

Fig. 3. Surface potential of precipitates formed in air (open circle) and under N_2 atmosphere (filled circle) at pH value of 7 as a function of maturation period.

behavior. The quantity of loaded albumin in apatite hydrogel formed in air was 0.103 wt.%, and that under N_2 atmosphere was 0.237 wt.%. Carbonate ions in HAp should be related to albumin adsorption on to HAp.[13] Fig. 3 shows the change of zeta potential of apatite hydrogel formed in air or under N_2 atmosphere. With an increase of maturation period, the zeta potential of the gel decreased in air from around −2 to −8 mV in 14 days at pH value of 7. The isoelectric point of the gel slightly shifted to lower value for longer period of maturation, which is due to the increase of carbonate ions in apatite structure. The adsorption and elution of the ions within the surface of HAp may lead to the new phase formation on the surface, and be attributed to the shift of surface potential.[14] The surface potential of the gel also decreased in N_2 atmosphere, but the decrease is less than that of those of the gel formed in air. The zeta potential of the gel formed in air at pH value of 7 was negatively larger than that of the gel formed under N_2 atmosphere. Surface potential of cytochrome *c* is positive and that of albumin is negative. The quantity of protein adsorbed could be due to the relationship between isoelectric point of protein and apatite hydrogel as well as specific surface area of apatite hydrogel.

The apatite hydrogel was put in plastic container, and dried at 40% of relative humidity. The volume of the specimen dropped within 2 days, and it seemed to stabilize in 3 days of drying period. Specimens obtained by drying apatite hydrogel prepared in air or under N_2 atmosphere show similar shrinkage behavior. Both specimen obtained in air or under N_2 atmosphere reached around 0.1 in volume ratio against the green apatite cake. The vicker's hardness also increased with a decrease in volume as shown in Fig. 4.

3.3. Protein release in vitro

The protein loaded specimen was immersed in aqueous solution (pure water or phosphate buffer solution) at room temperature. Fig. 5 showed the weight change of apatite

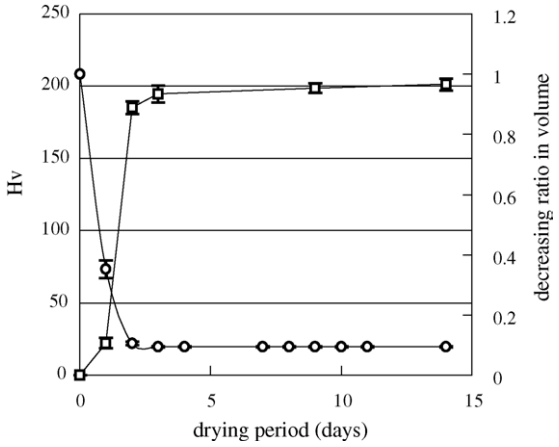

Fig. 4. Vickers hardness and volume change of apatite hydrogel dried at 40% of relative humidity as a function of drying period.

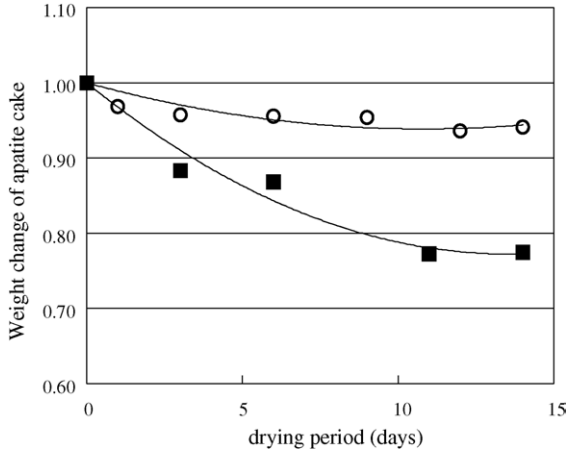

Fig. 6. Weight change of apatite cake, prepared from hydrogel formed in air, containing albumin, in PBS (open cirlce) and in pure water (filled square).

cake, prepared from hydrogel formed in air or under N_2 atmosphere, containing albumin (a) or cytochrome *c* (b), in aqueous solution. With an increase in soaking time, the weight of specimen soaked in aqueous solution gradually decreased, which may be due to dissolution into aqueous solution. The weight decrease of apatite cakes prepared from hydrogel formed in air was larger than that from hydrogel formed under N_2 atmosphere. The FT-IR absorbance ratio (CO_3-band/PO_4-band) of the apatite hydrogel prepared in air was higher than those under N_2.[12] Apatite hydrogel formed in air was carbonate rich apatite, while that under N_2 atmosphere was carbonate poor apatite. The apatite cake prepared from the former was more soluble in aqueous solution than that from the latter. The concentration of protein in solution increased with the dissolution of apatite cakes. Around 10–20% of apatite cakes containing albumin were dissolved in 2 weeks, and the released albumin was around 5 wt.% of loaded protein. These may be associated with recrystallization of apatite crystals.

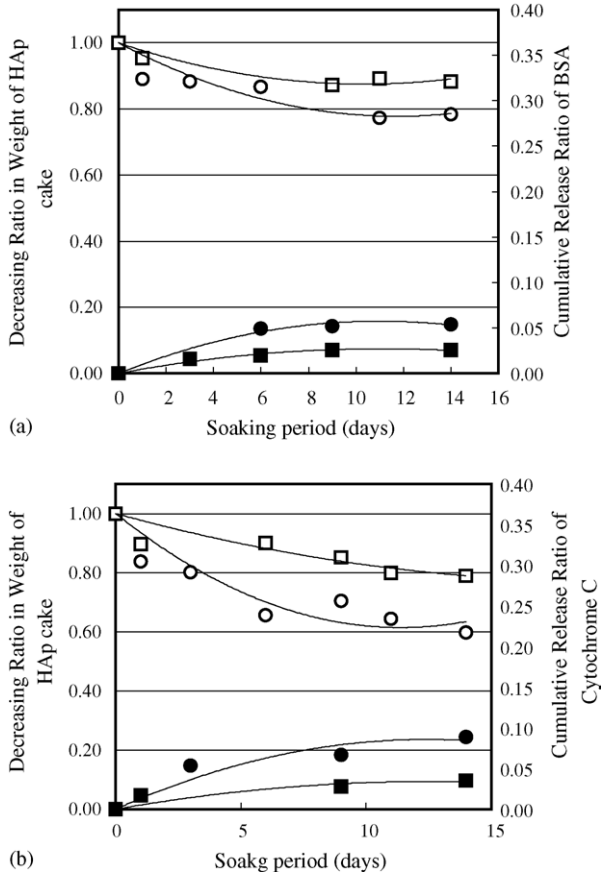

Fig. 5. Weight change of apatite cake, prepared from hydrogel formed in air (open circle) or under N_2 atmosphere (open square), containing albumin (a) or cytochrome *c* (b), in aqueous solution. The cumulative release ratio of protein from HAp cakes prepared from hydrogel formed in air (filled circle) or under N_2 atmosphere (filled square).

About 40% of apatite cake, containing cytochrome *c*, prepared from hydrogel formed in air was dissolved in aqueous solution in 2 weeks, while around 20% of apatite cake, containing albumin, prepared from hydrogel formed in air was dissolved in aqueous solution in 2 weeks. The apatite cake containing more amount of protein seems to have higher solubility in aqueous solution. The size of apatite cake containing more amount of protein was larger than that of apatite cake containing less amount of protein, so the apatite cake containing more amount of protein should have large contact surface and be more soluble in aqueous solution. In PBS, the solubility of the apatite cake was less, as shown in Fig. 6, as phosphate ions is rich in PBS and the dissolution is depressed.

4. Conclusion

Protein loading on apatite hydrogel cake and its solubility was studied. The quantity of loaded cytochrome *c* in apatite hydrogel formed in air was 0.49 wt.%, while in apatite hydrogel formed under N_2 atmosphere was 0.305 wt.%. The difference may be due to the relationship between isoelectric point of protein and apatite hydrogel. The apatite hydrogel

containing protein was dried for 4 days in air at 40% of relative humidity to make cakes, and the cakes was immersed in aqueous solution. The dried specimen slowly dissolves in aqueous solution, and the decrease in weight of apatite cake prepared from hydrogel formed in air was larger than that from hydrogel formed under N_2. The apatite cake containing more amount of protein seems to have high solubility in aqueous solution, due to the large contact surface area to solution. Compared to the quantity of the dissolved apatite, the released protein was less, which may be associated with recrystallization of the apatite hydrogel.

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