Available online at www.sciencedirect.com
SCIENCE DIRECT®
Current Applied Physics 5 (2005) 526–530
Current Applied Physics
Physics, Chemistry and Materials Science
www.elsevier.com/locate/cap

Coprecipitation of cytochrome C with calcium phosphate on hydroxyapatite ceramic

Yu Sogo [a,*], Atsuo Ito [a], Koshiro Fukasawa [b], Nao Kondo [c], Yusuke Ishikawa [c], Noboru Ichinose [b], Atsushi Yamazaki [c]

[a] *Institute for Human Science and Biomedical Engineering, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan*

[b] *Department of Materials Science and Engineering, School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan*

[c] *Department of Resources and Environmental Engineering, School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan*

Received 18 October 2004; received in revised form 8 November 2004
Available online 19 February 2005

Abstract

Cytochrome C (cyt C), which has a molecular weight and an isoelectric point similar to those of fibroblast growth factor (FGF-2), was coprecipitated with calcium phosphates in the presence or absence of a hydroxyapatite ceramic (HAP) using supersaturated calcium phosphate solutions that can be prepared by mixing infusion fluids. The precipitates formed in the solution consisted of amorphous calcium phosphate in the absence of a HAP. In the presence of a HAP, precipitates morphologically identical to bone-like apatite formed a calcium phosphate layer on the surface of HAP. The optimum conditions for coprecipitation of cyt C with calcium phosphate on HAP were a Ca/P molar ratio of 1.5 and a $NaHCO_3$ concentration of 7.90 mM. The release of cyt C from the calcium phosphate layer continued for at least 10 days in the physiological salt solution. The results suggest that FGF-2 is also expected to be coprecipitated with calcium phosphates onto HAPs using infusion fluids.

PACS: 87.83.+a

Keywords: Cytochrome C; Fibroblast growth factor; ACP; Coprecipitation

1. Introduction

A calcium phosphate layer incorporating biologically active proteins is promising as a surface layer to be formed on biomaterials. Calcium phosphate layers have been formed on the surfaces of various biomaterials including hydroxyapatite ceramics, polymers and titanium alloys by immersing biomaterials in solutions supersaturated with respect to calcium phosphates [1–5].

When the supersaturated solution contains proteins, the proteins can coprecipitate with calcium phosphates and consequently be incorporated in the calcium phosphate layer. So far, proteins coprecipitated with calcium phosphates include laminin [6,7], fibronectin [8], albumin [9] and recombinant human bone morphogenetic protein 2 [10]. These proteins have isoelectric points varying from 4.7 to 8.5. However, it is less clear whether highly basic proteins including fibroblast growth factor-2 (FGF-2) and cytochrome C (cyt C) can coprecipitate efficiently with calcium phosphates to form a protein-containing calcium phosphate layer on biomaterial surfaces. Previously, only the spontaneous coprecipitation

* Corresponding author. Tel.: +81 29 861 6072; fax: +81 29 861 6149.
E-mail address: yu-sogou@aist.go.jp (Y. Sogo).

1567-1739/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.cap.2005.01.025

of FGF-2 with calcium phosphate without substrates was reported [11]. Although FGF-2 was adsorbed on hydroxyapatite powder using Tris-buffered saline, the amount of FGF-2 adsorbed on the powder was about 0.8 μg/m^2 and the release of FGF-2 continued for 60 min [12].

Calcium phosphate solutions or their raw reagents previously used for the coprecipitation are yet to be officially approved for clinical use. At present, calcium- or phosphorus- containing solutions available for clinical use are limited to several medicines including infusion fluids. Mixing a calcium-containing infusion fluid and a phosphate-containing one leads to calcium phosphate precipitation under certain conditions. This fact suggests that mixed infusion fluids containing calcium and phosphate are supersaturated with respect to a calcium phosphate. Hence, biomaterial surfaces could be coated with a calcium phosphate layer incorporating proteins such as growth factors using infusion fluids.

In the present study, cyt C was coprecipitated with calcium phosphates in the presence or absence of a hydroxyapatite ceramic (HAP) using aqueous solutions with ionic concentrations that are identical to those of commercially available infusion fluids. Cyt C was used as a dummy protein for FGF-2 since the molecular weight and the isoelectric point of cyt C (12.3 kDa, pI = 10) are almost the same as those of FGF-2 (18 kDa, pI = 9.6). It is expected that the optimum conditions for the coprecipitation of cyt C with calcium phosphates are equal to those of FGF-2.

2. Materials and methods

Three aqueous solutions with ionic concentrations identical to those of Ringer's solution (2.25 mM Ca^{2+}), Klinisalz B (10 mM PO_4^{3-}) and an alkalinizer (166 mM $NaHCO_3$) (Table 1) were used. Each solution was sterilized using a membrane with a pore size of 0.22 μm before the experiments. The calcium-containing, phosphorous-containing solutions and $NaHCO_3$ solution were mixed to prepare aqueous solutions with Ca/P molar ratios ranging from 0.5 to 2.5 and $NaHCO_3$ concentrations from 0.00 to 47.43 mM. Cyt C was dissolved in the physiological salt solution at a concentration of 250 μg mL^{-1}. The physiological salt solution containing cyt C was mixed with the aqueous solution at a volume ratio of 1–9 to obtain the supersaturated calcium phosphate solution. The supersaturated calcium phosphate solution finally contained 1.00–1.85 mM Ca^{2+} and 0.53–2.79 mM PO_4^{3-}. The initial pH of the supersaturated calcium phosphate solution was in the range of 5.85–6.15.

Table 1
Chemical compositions of Ringer's solution, Klinisalz B and alkalinizer

	Ringer's solution (mM)	Klinisalz B (mM)	Alkalinizer (mM)
pH	5.0–7.5	5.0–6.5	8.28[a]
Na^+	147.00	45.00	166.00
K^+	4.00	25.00	–
Mg^{2+}	–	2.50	–
Ca^{2+}	2.25	–	–
Cl^-	157.00	45.00	–
$H_2PO_4^-$	–	10.00	–
HCO_3^-	–	–	166.00
CH_3COO^-	–	20.00	–
Xylitol	–	333.00	–

[a] The pH value was obtained experimentally at room temperature.

The supersaturated calcium phosphate solutions at 2 mL each were allowed to stand at 37 °C for 2 days in the presence or absence of HAP (1 mm thick and ϕ 13 mm). After the period, the supersaturated calcium phosphate solutions were filtered using a membrane with a pore size of 0.22 μm to separate the precipitates from the solutions. When the ceramics were immersed in the solutions, they were washed with 1 mL of ultrapure water twice and dried at room temperature.

The X-ray diffraction (XRD) patterns of the precipitates were observed using a powder X-ray diffractometer (Model Rint 2400; Rigaku, Japan). The amounts of residual cyt C in the supernatant were analyzed by colorimetry using a micro BCA™ protein assay reagent kit (Pierce Chemical Co., USA) to determine the amount of cyt C that coprecipitated with calcium phosphate. The coprecipitate on the HAPs was observed using a scanning electron microscope (SEM: JSM-5400, JEOL, Japan).

The HAPs with the coprecipitate were immersed in 1.5 mL of physiological salt solution and allowed to stand at 37 °C. The amounts of cyt C released in the physiological salt solution were analyzed by colorimetry.

3. Results

The final pH of the solution rose to 8.3–9.1 depending on the $NaHCO_3$ concentration. However, pH remained almost unchanged when no $NaHCO_3$ was added to the solution (Table 2). The increase in the pH of the supersaturated calcium phosphate solutions caused calcium phosphate precipitation. In the absence of a HAP in the solution, the precipitate was in a non-crystalline phase regardless of whether cyt C existed in the solution or not (Fig. 1). The precipitates were identified as amorphous calcium phosphate (ACP) since a broad background swelling of around $2\theta = 30°$ appeared in their XRD profiles. It was considered that cyt C was coprecipitated with ACP since cyt C concentrations in the solutions decreased by at most 44 ± 4% after 2 days at 37 °C.