JOURNAL OF MATERIALS SCIENCE **40** (2005) 6417–6422

In vitro simulation of vascular calcification by the controlled crystallization of amorphous calcium phosphates onto porous cholesterol

S. V. DOROZHKIN, E. I. DOROZHKINA
Kudrinskaja sq. 1-155, 123242 Moscow D-242, Russia
E-mail: sedorozhkin@yandex.ru

Published online: 18 October 2005

The *in vivo* process of vascular calcification was simulated *in vitro* by a slow crystallization of a non-stoichiometric poorly crystallized carbonateapatite from Kokubo's revised simulated body fluid (rSBF) on the surface of a porous pellet made of pure cholesterol. To achieve this, the crystallization experiments were performed under strictly controlled conditions (similar to physiological ones) provided by a constant-composition double-diffusion (CCDD) device. To perform a closer match to *in vivo* conditions, rSBF was enriched by addition of glucose and bovine serum albumin (BSA) in physiological amounts. Precipitation took place on the surface of cholesterol and the precipitates consisted of poorly crystalline (almost amorphous) non-stoichiometric, sodium- and magnesium-contained carbonateapatite.

1. Introduction

Biological calcification is the process of *in vivo* formation of inorganic crystals by living organisms. For vertebrates, the calcification can be either normal (e.g., formation of bones and teeth) or pathological (vascular and soft-tissue deposits; dental and urinary calculi; salivary stones; etc.). For humans and other mammals most types of calcifications consist of calcium phosphates, mainly of so-called "biological apatite" [1–5]. The latter is a poorly crystalline, non-stoichiometric, sodium- and magnesium-containing carbonateapatite (synonym: dahllite) with approximate chemical formula $(Ca,Mg,Na)_{10-x}(HPO_4,CO_3)_x(PO_4)_{6-x}(OH,CO_3)_{2-x}\cdot yH_2O$ [3, 6, 7]. As blood serum is slightly supersaturated with respect to precipitation of calcium phosphates, both normal and pathological calcifications can be considered as *in vivo* crystallization of biological apatite.

As calcium phosphates are sparingly soluble in water, it is not difficult to perform crystallization of these compounds. It is just necessary to mix aqueous solutions containing ions of calcium and phosphate and a precipitate will form. This process is called "chemical crystallization" and was done many times before, often at physiological conditions (temperature $37.0 \pm 0.5^{\circ}C$ and solution pH $= 7.3 \pm 0.1$) for different Ca/P molar ratios [8–10]. However, the precipitates obtained by chemical crystallization are always different from the ones found in vascular calcifications. This is mainly due to:

1. dissimilarities in the solution composition: the ionic concentration is constant in biological systems, whereas the solution is permanently depleted by ions of calcium and phosphate during chemical crystallization; the same applies to solution pH.
2. differences in the crystallization kinetics: it is fast (time scale of minutes and hours) for chemical crystallization and slow (time scale of months and years) for the biological one;
3. the influence of bioorganic compounds present in plenties inside the human body. Each of them may act as either a promoter or an inhibitor of nucleation and crystallization, not to mention various adsorption/desorption phenomena of biomolecules on crystal surfaces.

In order to overcome the first problem, the constant-composition (CC) technique was invented. Initially it had been created for keeping constant only the solution pH [11, 12] but after an ion-selective electrode for calcium became available, the dual CC technique was proposed [13, 14]. The CC technique allows one to study the processes of dissolution and crystallization from bulk solutions while keeping constant the ionic concentrations. Therefore, the solution supersaturation is also constant and the crystallization occurs at a constant rate. The CC technique is well suited for chemical crystallization that is a fast process (minutes and hours), while vascular calcification is a much slower process (months and years).

0022-2461
DOI: 10.1007/s10853-005-2154-x

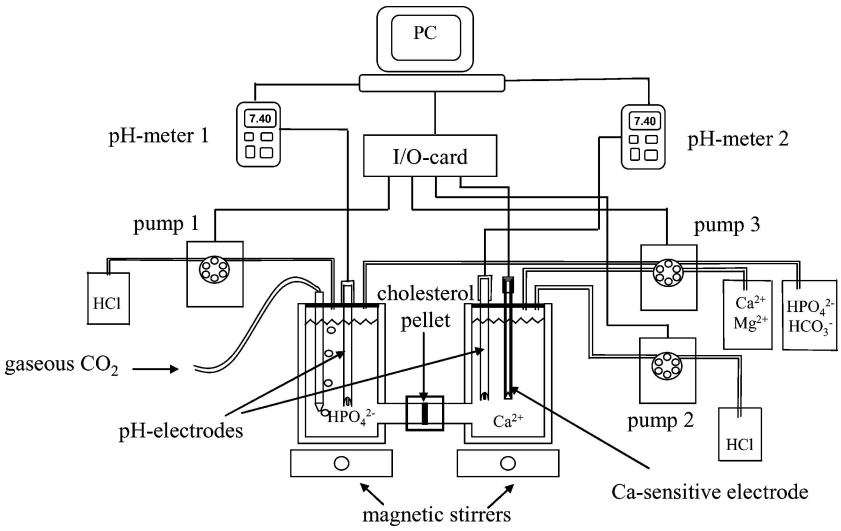


Figure 1 A schematic setup of the CCDD device.

A restrained diffusion is one of the easiest ways to reduce the crystallization rate. For example, crystallization of calcium phosphates can be performed in highly viscous systems like hydrogels, gelatin, collagen, or agar-agar. In this case, the amount of calcium phosphate precipitated depends on the rate of diffusion [15–19]. Another method consists of a separation of calcium- and phosphate-containing solutions from each other by a permeable membrane. In this case, the solutions diffuse to each other. This was realized in a double diffusion (DD) device for crystallization of fluorapatite using a collagen matrix [20, 21], as well as of fluorapatite and hydroxyapatite using a matrix made of a porous polyester [22]. In all above cases, the crystallization kinetics was strongly reduced, making it more similar to biological crystallization. However, as the crystallization progressed, the solutions surrounding the matrix were permanently depleted by ions of calcium, phosphate, and hydroxide (the latter is easily detected by decreasing of the solution pH). Therefore, the crystallization conditions were never constant and, finally, the crystals were different compared to those found *in vivo*.

The aforementioned facts reflect the great difficulties to investigate the fundamentals of vascular calcification because this is a slow process occurring *in vivo* under the strictly controlled parameters of:

1. solution supersaturation
2. temperature (37°C)
3. chemical composition (amounts of additives, like biomolecules)
4. solution hydrodynamics.

In order to simplify the task, various *in vitro* simulation techniques have been developed. Two main experimental approaches have been discussed above and none of them appears to be able to fulfill all necessary crystallization conditions. Earlier we applied a combination of the CC and DD devices (as a constant composition double diffusion (CCDD) device [23–25 and references therein]) to perform crystallization of a non-stoichiometric carbonateapatite from the Kokubo's revised simulating body fluid (rSBF). In the CCDD device, a porous membrane separates solutions containing ions of calcium and phosphate, like in a DD device, and the concentrations of both solutions are kept constant, like in a CC device. This provides an opportunity to perform a slow crystallization under the strictly controlled conditions resulting in a good simulation of *in vivo* conditions. Using Kokubo's rSBF allows us to become even closer to the biological conditions, as this simulating solution perfectly matches the ionic composition of human blood plasma [26]. In addition, rSBF has both the ionic strength and Ca/P molar ratio similar to those of the plasma. However, the previous crystallization experiments of us [23–25] were performed on the surface of a cellulose filter that did not simulate the process of vascular calcification. Therefore, similar experiments but carried out on the surface of a porous pellet made of pure cholesterol have been done and the results are discussed in this paper.

2. Materials and methods

The schematic setup of the CCDD device is shown in Fig. 1. It consists of 2 thermostatted glass vessels, 2 magnetic stirrers, 2 pH electrodes, 2 pH meters, 1 calcium-selective electrode, 3 peristaltic pumps, and 4 plastic vessels containing stock solutions of HCl (2 vessels), calcium and phosphate ions (1 vessel for each stock solution). The whole system is operated and controlled by a computer under the framework of the VISIDAQ (Advantech) software. The CCDD device keeps constant both the solution pH (±0.05 pH units) independently in two glass vessels and the ionic concentration of calcium in the calcium vessel (right vessel in Fig. 1). As, a phosphate-selective electrode is not yet available on the market, the concentration of phosphate ions in the left vessel is continuously calculated according to the chemical equation[1] :

$$10Ca^{2+} + 6HPO_4^{2-} + 8OH^- \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (1)$$

[1] In fact, a non-stoichiometric magnesium containing carbonateapatite is always precipitated from rSBF [23–26]. Unfortunately, it is not possible to take this into account quantitatively.

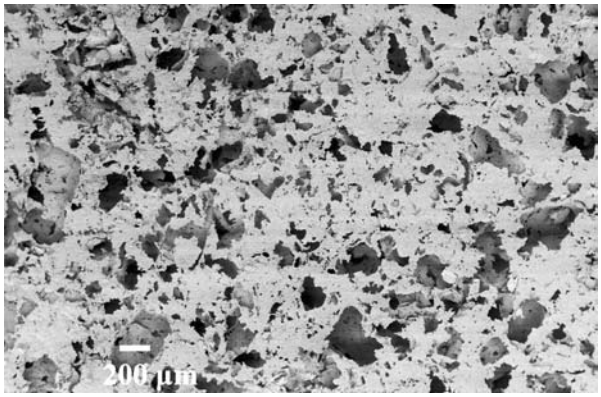


Figure 2 A SEM image of an initial porous cholesterol pellet. Magnification: ×25. Bar is 200 μm.

The necessary amount of the stock solution of phosphate is added to the phosphate vessel simultaneously with addition of the stock solution of calcium to the calcium vessel. To perform this, both calcium- and phosphate-stock solutions are transported by the same peristaltic pump. Further details of the CCDD device have been published previously [23 and references therein].

A porous pellet made of pure cholesterol was prepared. To do so, powdered cholesterol (purchased from Fluka) was mixed with crystals of sodium chloride in proportions 1:3 to 1:5 (by weight) followed by pellets preparation using a mechanical press. The mixtures were placed in a die and compressed into dense pellets at pressure of 3000 kg/cm^2. As cholesterol is insoluble in water while sodium chloride is soluble, the dense pellets were kept in doubly distilled water for 48 h to dissolve sodium chloride. After the entire amount of sodium chloride was dissolved, the porous pellets were dried at ambient temperature and used for crystallization experiments (Fig. 2).

One porous pellet was clamped between two glass vessels of the CCDD device. Revised simulated body fluid (rSBF), as introduced by Kim, Kokubo *et al*. [26], was used as the source of both calcium and phosphate ions. The chemical composition of rSBF is shown in the second row of Table I. However, the following changes in the chemical composition of rSBF were made:

1. rSBF does not contain any bioorganic compounds. In order to be closer to biological conditions, 4 g l^{-1} of glucose and 80 g l^{-1} of bovine serum albumin (BSA) were added to rSBF [24, 25];

2. to avoid possible growth of bacteria 0.1 g l^{-1} of NaN_3 was always added.

Initially we started working with rSBF. However, the crystallization kinetics appeared to be similar to those *in vivo*, i.e. very slow (due to the small supersaturation in rSBF) and each experiment took very long (time scale of several months). Therefore, we decided to work with solutions containing 4 times the ionic concentrations of rSBF (see Table I). Such condensed solutions cannot be prepared in one vessel because crystallization starts during preparation. Fortunately, the CCDD device requires separation calcium and phosphate ions from each other (Fig. 1). Therefore, two solutions simulating 4 times rSBF were prepared and used for the experiments: rSBF-Ca containing double amounts of calcium and magnesium ions but zero amounts of phosphate and carbonate ions, and rSBF-PO_4 containing double amounts of phosphate and carbonate ions but zero amounts of calcium and magnesium ions (the fourth and fifth rows in Table I). After mixing of equal amounts, rSBF-Ca and rSBF-PO_4 gave rise to 4 times rSBF [23–25].

To keep the ionic concentrations of calcium and phosphate constant during the experiments, calcium- and phosphate-containing stock solutions were prepared as well. Both stock solutions contained 4 times the ionic concentrations of rSBF for Na^+, K^+, Cl^-, and $SO_4{}^{2-}$ but 16 times the ionic concentrations of rSBF for $Ca^{2+} + Mg^{2+}$ and $HPO_4{}^{2-} + HCO_3{}^-$, respectively (last two rows in Table I). Solution pH of the calcium stock solution was adjusted to 7.4 with NaOH whereas the pH of the phosphate solution was about 10. This was necessary to keep the full amount of carbonates in this solution, as evolution of CO_2 would have occurred otherwise. The additives (4 g l^{-1} of glucose, 80 g l^{-1} of BSA and 0.1 g l^{-1} of NaN_3) were also added to the stock solutions.

Each crystallization experiment was performed for 7 days. After the experiment was over, the installation was disassembled; the cholesterol pellet was removed, washed with doubly distilled water, dried, and weighed. Afterwards, the chemical and structural composition of the precipitates (about 10 to 20 mg were crystallized) formed on the pellet were studied by scanning electron microscopy (SEM; LEO 1530; gold-sputtered samples), infra red (IR) spectroscopy (1720X, Perkin

TABLE I Ionic concentrations of the rSBF solutions used in this study, mM

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	pH	NaN_3,g1^{-1}	HEPES, g1^{-1}
Blood plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	7.25–7.40	–	–
rSBF [26]	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	7.40 ± 0.01	–	12
4 times rSBF**	568.0	20.0	10.0	6.0	412.0	108.0	4.0	2.0	7.40 ± 0.02	0.1	48
4 times rSBF-Ca	568.0*	20.0	20.0	12.0	412.0	–	–	2.0	7.40 ± 0.02	0.1	–
4 times rSBF-PO_4	568.0*	20.0	–	–	412.0	216.0	8.0	2.0	7.40 ± 0.02	0.1	48
Ca-stock solution	568.0*	20.0	40.0	24.0	412.0	–	–	2.0	7.4 ± 0.1	0.1	–
PO_4-stock solution	568.0*	20.0	–	–	412.0	432.0	16.0	2.0	∼10	0.1	–

*contains extra 1.5 mM of sodium due to presence of NaN_3.
**very unstable (precipitation starts during preparation).

Elmer), X-ray diffraction (XRD; D8 Advance, Bruker AXS; Cu Kα radiation), as well as by energy dispersive X-ray (EDX) spectroscopy (ISIS, Link Analytical, Oxford Instruments). To confirm the stability of the crystallization conditions, calcium and magnesium concentrations (titration with sodium ethylendiaminetetraacetate; EDTA), as well as the ionic concentration of phosphate (photometrically via formation of the blue ammonium phosphate-molybdate complex) were always measured in the rSBF-Ca solution after each experiment was over [23–25].

All experiments were carried out at physiological temperature (37.0 $\pm$ 0.2°C) and solution pH = 7.40 $\pm$ 0.05. All solutions were prepared with compounds of *pro analysi* quality obtained from Merck and double-distilled water. BSA (Fraction V, MW=67,000 g mol^{-1}) was obtained from Biomol Feinchemikalien GmbH, Hamburg, Germany.

3. Results and discussion

Experimental results on the stability of the CCDD device are shown in Fig. 3. As can be seen from the plots, the CCDD device keeps the crystallization environment within the very narrow limits and crystallization occurs under the conditions similar to biological ones [23–25].

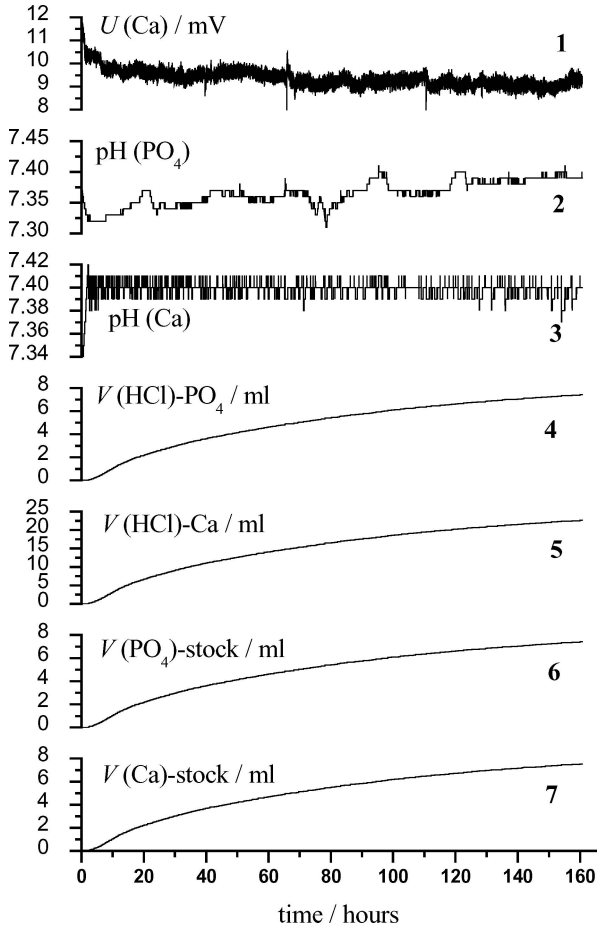


Figure 3 Experimental data of the stability of the CCDD device: 1—data recorded from the calcium-selective electrode; 2—pH in the phosphate vessel; 3—pH in the calcium vessel; 4—amount of 0.5 M HCl added to the phosphate vessel to keep the pH constant, ml; 5—amount of 0.05 M HCl added to the calcium vessel to keep the pH constant, ml; 6—amount of phosphate stock-solution (16 times rSBF) added to the phosphate vessel to keep the concentration of phosphate and carbonate ions constant, ml; 7—the same for the calcium side.

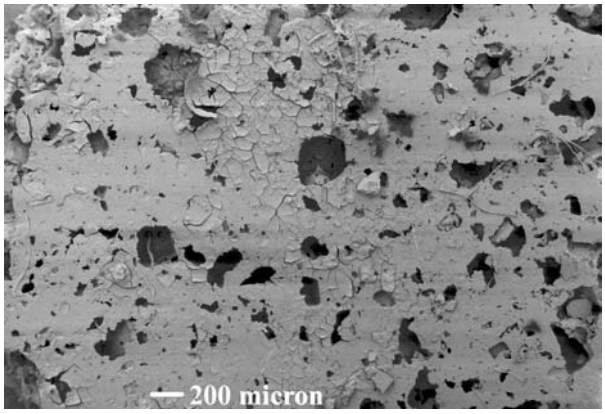
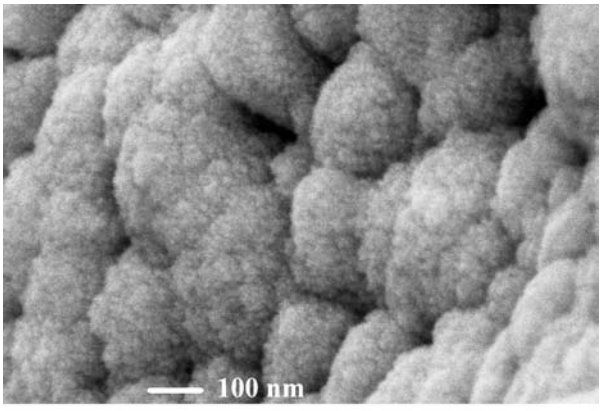
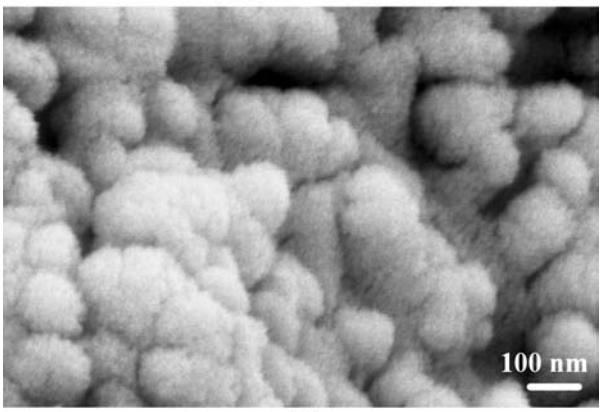


Figure 4 A typical scanning electron micrograph of the precipitates formed in CCDD device from 4 times rSBF solutions on the surface of a porous cholesterol pellet. Magnification: ×25. Bar is 200 μm.

Typical precipitates obtained in the CCDD device from 4 times rSBF enriched with glucose and BSA are shown in Figs 4 (the surface, a low magnification) and 5 (both the surface and inner part, a high magnification). The precipitates were found to be similar on both sides of the cholesterol pellet and were on bead-like shape, which corresponded well with the shape of the precipitates found in cardiovascular calcifications [5].



(a)



(b)

Figure 5 Typical scanning electron micrograph of the precipitates formed in CCDD device from 4 times rSBF solutions on a porous cholesterol pellet: (a)—the surface, (b)—an inner part (to do so, a pellet was cut in halves). Magnification: ×100,000. Bar is 100 nm.

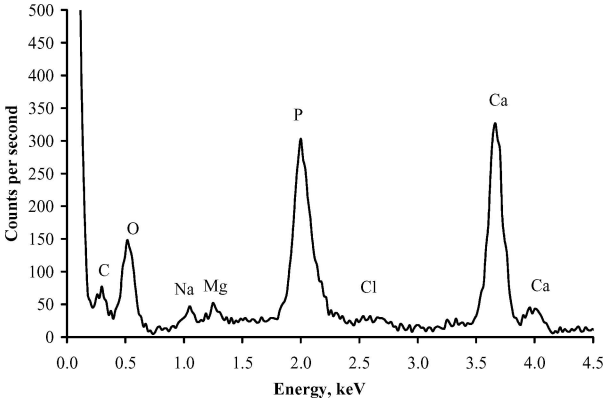


Figure 6 Energy dispersive X-ray spectroscopy of the precipitates from 4 times rSBF solutions on cholesterol. Bands of main chemical elements are marked.

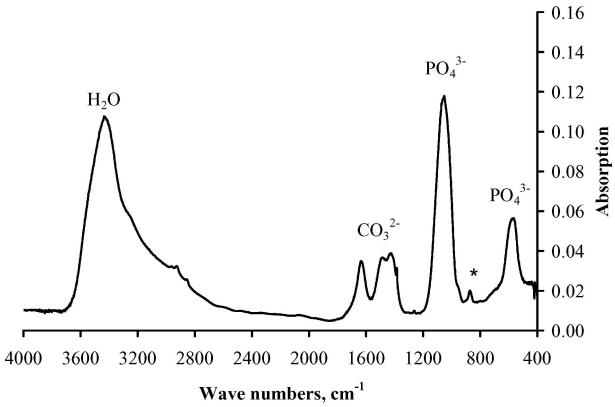


Figure 8 A representative infrared spectrum of the precipitates formed on cholesterol. Bands of main chemical ions and compounds are marked. The band marked by asterisk corresponds to either hydrogenphosphate or carbonates [3, 7].

The chemical composition of the precipitates was qualitatively determined by EDX technique: intensive peaks of calcium and phosphorus revealed that the precipitates consisted of calcium phosphates (Fig. 6). Intensive peaks of calcium, phosphorus, oxygen and carbon, as well as small peaks of sodium, magnesium and chloride could be distinguished. Peaks of sodium and chloride are likely due to unwashed traces of rSBF (NaCl is the major component of rSBF—see Table I). The strong peak of carbon comes from either cholesterol or carbonateapatite, while EDX peaks of other chemical elements qualitatively represent that the precipitates consist of calcium phosphates with minor incorporation of magnesium and, probably, of sodium. This finding perfectly correlates with the chemical composition of biological apatite [3, 6, 7].

In addition, the cholesterol pellets were studied by XRD (Fig. 7). The diffraction patterns of pure cholesterol and of well crystallized hydroxyapatite (HA, chemical formula $Ca_{10}(PO_4)_6(OH)_2$) are displayed for comparison. As seen in Fig. 7, the specific diffraction peaks of cholesterol covered with calcium phosphates (within $10 < {}^\circ 2\Theta < 25$) had lower intensities when compared to those of pure cholesterol. This indicates a diminution of the X-ray beam reaching cholesterol by layers of calcium phosphate. However, another region ($30 < {}^\circ 2\Theta < 55$) is more important for the discussion. The specific peaks of hydroxyapatite were found in the

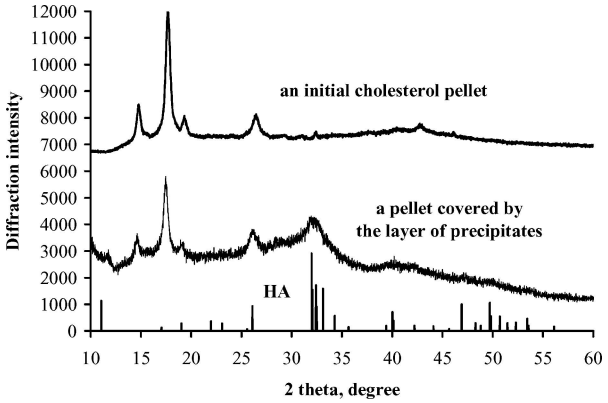


Figure 7 X-ray diffraction patterns of the precipitates from 4 times rSBF solutions on cholesterol. The diffraction patterns of pure cholesterol and well-crystallized hydroxyapatite are given for comparison.

diffraction pattern (best seen at $2\Theta = 32$–33° and 40°). However, those peaks were broad, while the peaks of the well-crystallized hydroxyapatite were sharp. This confirms the apatitic structure of the precipitates but shows that the precipitates were poorly crystalline (almost amorphous), while the level of crystallinity for the precipitates found in cardiovascular calcifications usually were a little bit better [3, 5]. Besides the obvious explanation by great differences between the chemical composition of human blood plasma and 4 times rSBF + BSA + glucose, this minor difference is likely due to the differences in the crystallization times: while amorphous calcium phosphate is itself unstable and rapidly transforms to a crystalline apatite, the rate at which this occurs can be significantly slowed down by addition of other ions such as magnesium and bicarbonate [3, 7]. In other words, unlike a long-term process of vascular calcification (months and years), the duration of our experiments (1 week) was not enough for such transformation to occur.

As shown in Fig. 4, after drying the surface layer of the precipitates might be easily peeled off. This was done under a binocular (magnification $\times 20$) using small tweezers. The fragments or chunks of the coating were collected to perform IR study. A typical spectrum of the precipitates is shown in Fig. 8. Strong absorption bands of phosphates, carbonates and water were found, which provided an extra confirmation that the precipitated consisted of water-containing carbonateapatite, similar to the chemical formula of biological apatite (see introduction). In addition, the IR bands were smooth and poorly resolved. A lack of any splitting of the principle phosphate bands at $\sim$1060 and $\sim$560 cm^{-1} is a strong indication that the mineral formed is poorly crystalline. This corresponds well with the aforementioned XRD data.

4. Conclusions

Thus, the crystallization experiments from condensed solutions of rSBF on the surface of porous pellets made of pure cholesterol have been performed. In order to simplify the study and accelerate the precipitation, some serious differences between our experimental

conditions and *in vivo* ones (4 times the ionic concentration of rSBF [27], mixing of calcium and phosphate containing solutions only inside the pores of the cholesterol pellets, as well as the duration of each experiment) have been made. In addition, most arterial calcifications occur in association with the elastin layer, rather than the cholesterol deposits, which actually are in the form of cholesterol fatty acid esters, not free cholesterol [1, 2, 5]. The aforementioned drawbacks certainly reduce applicability of our data to the real conditions of vascular calcification. On the other hand, strictly controlled experimental conditions (closely matching physiological ones), as well as addition of glucose and BSA to the solutions of rSBF undoubtedly were a big step forward in coming up to *in vivo* conditions. Therefore, we believe that the experimental approach discussed in this paper eventually will result in a good simulation model of vascular calcification.

Acknowledgements

This study was performed at University of Bochum (Germany). A valuable assistance of former colleagues is gratefully acknowledged. The study was supported by the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany, and the Fonds der Chemischen Industrie, Frankfurt/Main, Germany.

References

1. A. BOSKEY and E. P. PASCHALIS, in "Bone Engineering," edited by J. E. Davies (EM Squared, Toronto, 2000) p. 75.
2. J. JANSEN and P. N. VUONG, *Z. Kardiol.* **90** (2001) III/6.
3. R. Z. LEGEROS, "Calcium Phosphates in Oral Biology and Medicine" (Karger, Basel, 1991).
4. H. A. LOWENSTAM and S. WEINER, "On Biomineralization" (Oxford University Press, Oxford, 1989).
5. B. B. TOMAZIC, *Z. Kardiol.* **90** (2001) III/68.
6. G. GACULSI, P. PILET, M. COTTREL and G. GUICHEUX, *J. Biomed. Mater. Res.* **47** (1999) 228.
7. J. C. ELLIOT, "Structure and Chemistry of the Apatites and other Calcium Orthophosphates" (Elsevier, Amsterdam, 1994).
8. E. D. EANES, *Calcif. Tiss. Res.* **20** (1976) 75.
9. E. D. EANES and A. W. HAILER, *ibid.* **66** (2000) 449.
10. E. C. MORENO, T. M. GREGORY and W. E. BROWN, *J. Res. Natl. Bur. Stand.* **72A** (1968) 773.
11. M. B. TOMSON and G. H. NANCOLLAS, *Science* **200** (1978) 1059.
12. P. KOUTSOUKOS, Z. AMJAD, M. B. TOMSON and G. H. NANCOLLAS, *J. Am. Chem. Soc.* **102** (1980) 1553.
13. A. EBRAHIMPOUR, J. ZHANG and G. H. NANCOLLAS, *J. Cryst. Growth* **113** (1991) 83.
14. E. P. PASCHALIS, K. WIKIEL and G. H. NANCOLLAS, *J. Biomed. Mater. Res.* **28** (1994) 1411.
15. W. ACHILLES, *Contrib. Nephrol.* **58** (1987) 59.
16. W. ACHILLES, U. JOCKEL, A. SCHAPER, M. BURK and H. RIEDMILLER, *Scanning Microsc.* **9** (1995) 577.
17. P. T. CHENG, *Rheum. Dis. Clin. North Am.* **14** (1988) 341.
18. G. K. HUNTER, S. C. NYBURG and K. P. PRITZKER, *Coll. Relat. Res.* **6** (1986) 229.
19. H. J. UDICH, H. HOFT and H. BORNIG, *Biomed. Biochim. Acta* **44** (1985) 547.
20. S. BUSCH, H. DOLHAINE, A. DUCHESNE, S. HEINZ, O. HOCHREIN, F. LAERI, O. PODEBRAD, U. VIETZE, T. WEILAND and R. KNIEP, *Eur. J. Inorg. Chem.* (1999) 1643.
21. R. KNIEP and S. BUSCH, *Angew. Chem.* **108** (1996) 2788; *Angew. Chem. Int. Ed. Engl.* **35** (1996) 2624.
22. K. SCHWARZ and M. EPPLE, *Chem. Eur. J.* **4** (1998) 1898.
23. S. V. DOROZHKIN, E. I. DOROZHKINA and M. EPPLE, *Crystal Growth & Design* **4** (2004) 389.
24. S. V. DOROZHKIN and E. I. DOROZHKINA, *Colloid and Surfaces A: Physiochem. Eng. Asp.* **215** (2003) 191.
25. S. V. DOROZHKIN, E. I. DOROZHKINA and M. EPPLE, *J. Applied Biomaterials Biomechanics* **1** (2003) 200.
26. H. M. KIM, T. MIYAZAKI, T. KOKUBO and T. NAKAMURA, in "Bioceramics 13," edited by S. Giannini and A. Moroni (Trans Tech Publ., Switzerland, 2001) p. 47.
27. E. I. DOROZHKINA and S. V. DOROZHKIN, *J. Biomed. Mater. Res.* **67A** (2003) 578.

Received 1 February
and accepted 9 May 2005