Cells and Materials

Volume 7 | Number 1 Article 4

1997

A Novel In Vitro Model to Study the Calcification of Biomaterials

Q. Liu
University of Leiden

J. Weng
University of Leiden

J. G. C. Wolke
University of Leiden

J. R. de Wijn
University of Leiden

C. A. van Blitterswijk
University of Leiden

Cells and Materials Vol. 7, No. 1, 1997 (pages 41-51) 1051-6794/97$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

A NOVEL *IN VITRO* MODEL TO STUDY THE CALCIFICATION OF BIOMATERIALS

Q. Liu*, J. Weng, J.G.C. Wolke, J.R. de Wijn and C.A. van Blitterswijk

Biomaterials Research Group, University of Leiden, Prof. Bronkhorstlaan 10, Building 57, 3723 MB Bilthoven, The Netherlands

(Received September 19, 1996 and in revised form September 9, 1997)

Abstract

A novel *in vitro* model based on a solution mainly composed of sodium, calcium, chloride and phosphate ions, was developed to study the calcification of biomaterials at near physiological conditions. This model, due to its ability to quickly calcify the tested materials, is called Accelerated Calcification Solution (ACS). Polyactive™ 30/70, Polyactive™ 70/30 and its composites with nano-apatite were used as testing materials because of their known calcification behaviour. The results showed that Polyactive™ 70/30 and its composites could calcify in ACS in a relatively short period, while the polymer without filler failed to induce calcium phosphate precipitation in more conventional Simulated Body Fluid (1.5 SBF) in 9 days in this study. Polyactive™ 30/70 did not calcify in ACS and 1.5 SBF. As these results agreed well with the known *in vitro* and *in vivo* calcification behaviour of Polyactive™, we conclude that the ACS solution is a suitable model to study the calcification behaviour of biomaterials. The Ca-P mineral layer induced from ACS on Polyactive™ 70/30 and its composites was composed of octacalcium phosphate (OCP) and calcium hydroxyapatite. It is likely that the OCP first nucleated and grew on Polyactive™ 70/30 and its composites, then gradually transformed to calcium hydroxyapatite via hydrolysis. Also, the results showed that nano-apatite has the ability to promote calcification because nano-apatite/Polyactive™ 70/30 composites could quickly calcify in ACS as well as in 1.5 SBF.

Key Words: Calcification, polymers, Polyactive™, nano-apatite, composites, *in vitro*, calcium phosphate, octacalcium phosphate.

*Present address and address for correspondence:
Q. Liu
Cox Laboratory for Biomedical Engineering
Rice University, Houston, TX 77005, USA
Telephone Number: (713)-527-8101x3025
FAX Number: (713)-285-5353
E-mail: Qing@ruf.rice.edu

Introduction

Calcification or formation of a calcium phosphate phase in biomaterials is of great importance in biomedical science. Studies have demonstrated that calcification plays an important role in the bonding process of bone prosthetic materials such as Polyactive™ (a block copolymer of polyethylene glycol and poly (butylene terephthalate)) [2, 46, 47], hydroxyapatite [12, 13, 31, 48], Bioglass [26], glass ceramics [27, 28], and silica gel [33, 34]. On the other hand, calcification is not desirable in other applications of biomaterials, such as in bioprosthetic heart valves (bovine pericardium and porcine aortic valves) [18, 38], polyurethane heart valves and blood pump blades [8, 49]. *In vivo* calcification is a complicated biological process which involves many factors like calcium binding proteins, and cellular response of the host to the implants [42], and there is no doubt that intrinsic factors of the material play an important role in the process of calcification. Examples of intrinsic factors are calcium ion complexing ability of the material [45, 46], the dissolution rate of calcium phosphate from the implanted material [9-13, 31, 48], the hydroxyl groups formed on the surface of the material [33, 34] and the negative charges due to pre-treatment [38].

Methods to promote or to prevent the calcification process largely depend on the understanding of the mechanism of the calcification process. Also, in developing new materials for which the calcification is of great significance, it is important to know how to control the calcification process by engineering the structure or the surface of the material. A simple and rapid *in vitro* method is needed to examine the calcification properties of the material before cost and time consuming animal experiments are performed. Several *in vitro* tests have been developed such as the use of Simulated Body Fluid (SBF) [27] for studying the ability of the material to induce calcium apatite formation [33-36] and several other *in vitro* models for studying bioprosthetic heart valves and implantable polyurethane [23, 24, 50]. In practice, we found that those models are

Table 1. Ionic concentrations of SBF, 1.5 SBF and ACS

	Concentration (mM)							
	Na^+	K^+	Ca^{2+}	Mg^{2+}	HCO_3^-	Cl^-	HPO_4^{2-}	SO_4^{2-}
SBF	142	5	2.5	1.5	27	103	1	0.5
1.5SBF	213	7.5	3.8	2.3	6.3	223	1.5	0.75
ACS	136.8	4.64	3.87	-	-	144.5	2.32	-

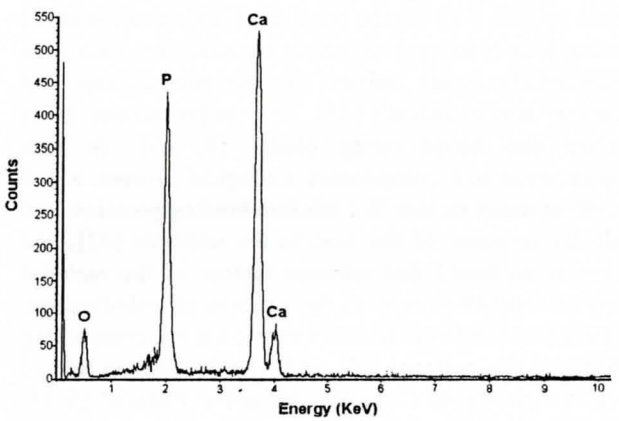


Figure 1. The EDX analysis results showed that the precipitation on the material was mainly composed of calcium and phosphorus.

---

not sensitive enough or take a long time to reveal differences between the ability of materials to induce calcification, or are unstable as a solution at room temperature. In this study, a new *in vitro* model using an "Accelerated Calcification Solution (ACS)" was developed. A series of materials with differing rates of calcification was used to evaluate the effectiveness of ACS.

Polyactive™ (from HC Implants, Leiden, The Netherlands), a block copolymer from polyethylene glycol and poly(butylene terephthalate), was chosen as test material because its ability to calcify has been well characterized [2, 39, 40, 46, 47]. By changing the weight ratio of PEG/PBT, a series of copolymers with

Figure 2 *(on facing page).* SEM micrographs showing the calcification developed on the materials. (**a**) The incomplete coverage of Poly-active™ 70/30 by the mineral layer after a 3 day immersion in ACS. (**b**) The complete coverage of 10% nanoapatite-polymer composites by fluorescent mineral crystals after a 3 day immersion in ACS. (**c**) The cross section of 10% nanoapatite composites showing calcification developed from the top (right) of the material towards the bottom (left) within the matrix. (**d**) Crystals on top of Polyactive™ 70/30 after 6 days immersion in ACS. (**e**) Very dense crystal plates on 50% nanoapatite-polymer composites after a 6 day immersion in ACS. (**f**) Morphological changes of the mineral layer. The bottom part of the mineral (★) is dense while the crystal plates (arrow) still exist on top of the dense mineral layer. This suggests that the crystal plates gradually transformed to a dense mineral layer, possibly accompanied by phase structure transformation.

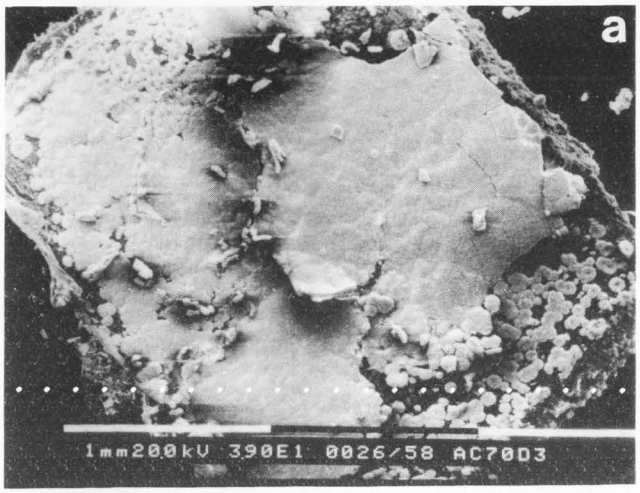
---

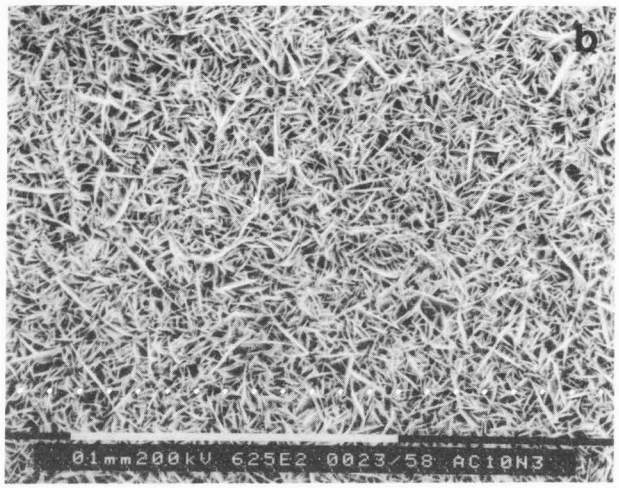
different rates of calcification can be obtained. In this study, we chose Polyactive™ 30/70 (which will not calcify *in vivo*), Polyactive™ 70/30 (which can be calcified *in vivo* and has bone-bonding ability) and a series of Polyactive™ 70/30 apatite composites with 10%, 25%, and 50% weight percent of a nano-apatite mineral powder.

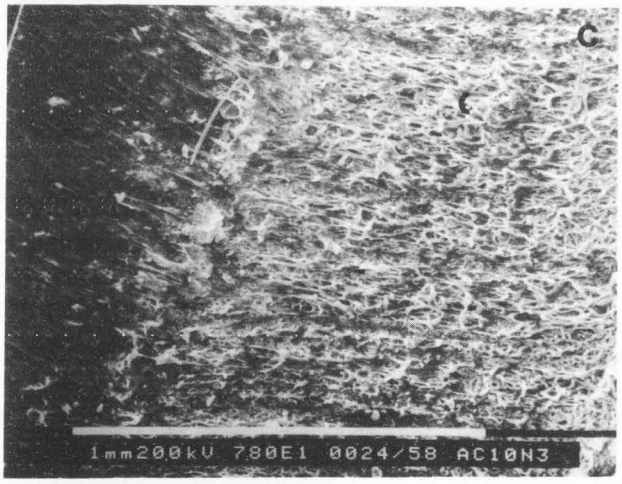
Materials and Methods

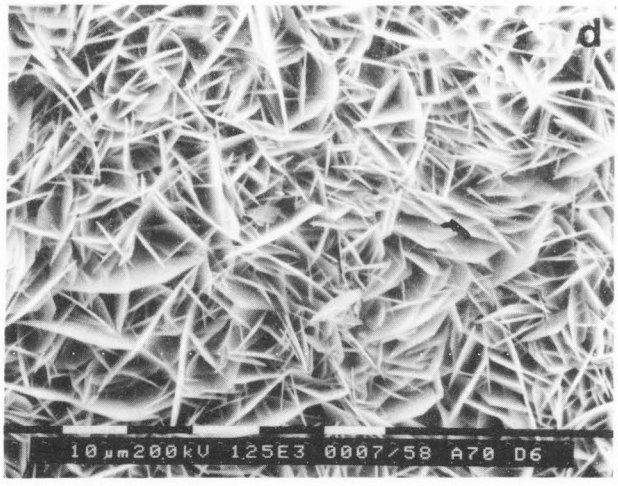
Accelerated Calcification Solution (ACS)

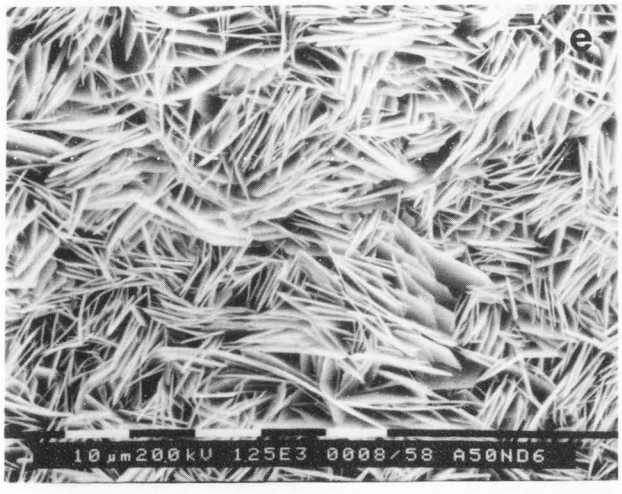
ACS, which has a [Ca]/[P] ratio of 1.67, was prepared by dissolving analytical grade NaCl, $CaCl_2$ and K_2HPO_4 to the following ion concentrations: [Na^+] =

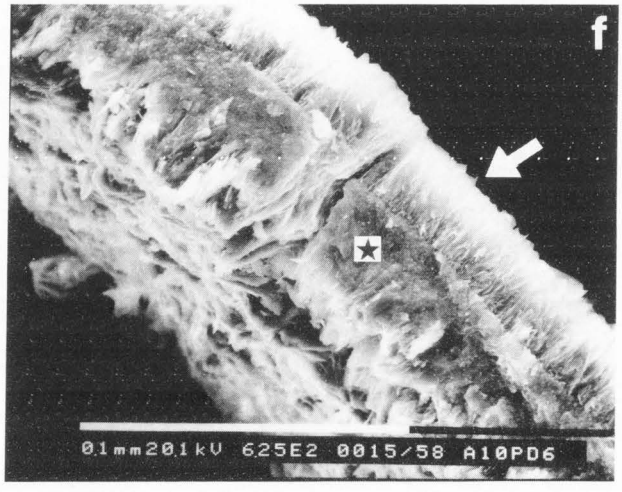

a
1mm20.0kV 390E1 0026/58 AC70D3


b
0.1mm20.0kV 6.25E2 0023/58 AC10N3


c
1mm20.0kV 7.80E1 0024/58 AC10N3


d
10µm20.0kV 1.25E3 0007/58 A70 D6


e
10µm20.0kV 1.25E3 0008/58 A50ND6


f
0.1mm20.1kV 6.25E2 0015/58 A10PD6

136.8 mM, $[Cl^-]$ = 144.5 mM, $[K^+]$ = 4.64 mM, $[Ca^{2+}]$ = 3.87 mM, and $[HPO_4^{3-}]$ = 2.32 mM. The solution was buffered with 50 mM Tris buffer at pH 7.4 at room temperature.

1.5 SBF, which has a ionic concentration of 1.5 times that of normal SBF, was also used and compared with ACS. Table 1 gives the ionic concentrations of SBF, 1.5 SBF and ACS.

Materials for ACS testing

Polyactive™ 30/70 and 70/30 (the figures represent the PEG/PBT ratio) were obtained from HC Implants, Leiden, The Netherlands. The molecular weight of PEG segments is 1000 Dalton. Nano-sized hydroxyapatite (nano-apatite) was synthesized by a hydrothermal process described elsewhere [37]. Composites with 10%, 25%, 50% weight percen-tage nano-apatite were made. Nano-apatite was added to a Polyactive™ 70/30 chloroform solution. After mixing by vigorously stirring, the mixture was precipitated in a large amount of ether. After drying, the precipitates were used for hot press moulding at 20 ton of pressure and 195°C. Polyactive™ 30/70 and 70/30 granulates were directly used for hot press moulding. The pressed plates were 2 mm thick. The samples with a size of 10 x 10 x 2 mm for ACS immersion testing were cut from the hot press plates.

ACS and 1.5SBF immersion experiment

Each sample was put into either 30 ml of ACS or 30 ml 1.5 SBF in a polystyrene disposable beaker, sealed and put in a water bath shaker at 37°C. At day 3, all the medium was exchanged at room temperature. At 3 and 6 days, the samples were taken out. After careful rinsing in distilled water and subsequent drying, samples were carbon coated for scanning electron microscopy (SEM) (Philips 525; Philips Electron Optics, Eindhoven, The Netherlands) and energy-dispersive X-ray analysis (EDX) (Voyager; NORAN, Middleton, WI) analysis. A small amount of precipitate was scraped off from the top of the samples from stored in the ACS solution for infra-red (Perkin Elmer 783, KBr tablets; Perkin Elmer, Norwalk, CT) analysis and powder X-ray diffraction analysis (XRD, Philips PW 1050, CuKα, 40 kV/35 mA).

A stability test for ACS and 1.5 SBF was also carried out under the same conditions without the presence of samples in the solution. All immersion tests were performed in triplicate.

Results

SEM and EDX results

ACS immersion. The stability test showed that the ACS solution was quite clear without precipitation during the test period, indicating that the solution is fairly stable at 37°C. On day 3, all the samples were covered by a layer of mineral except for Polyactive™ 30/70. This layer was composed of calcium and phosphate as confirmed by EDX (Fig. 1), and crystal plates were found to have grown from the surface of the tested materials (Fig. 2). For different materials, the coverage of the mineral was different: Polyactive™ 70/30 was incompletely covered by the calcium phosphate crystals (Fig. 2a, 2d). Composites with nano-apatite filler were all completely covered by the crystal plates (Fig. 2b, 2e). The thickness of covered mineral layer was proportionate to the filler content. At day 6, except for Polyactive™ 30/70, all the materials were completely covered by a thick calcium phosphate layer. On some

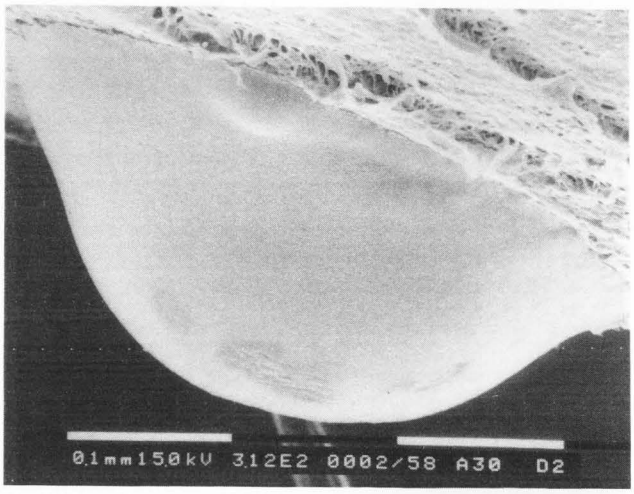


Figure 3. SEM micrograph showing the segregated globular mineral precipitated on Polyactive™ 30/70

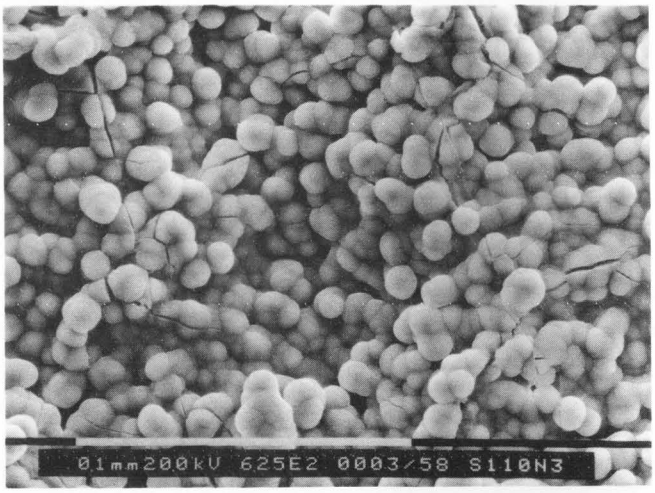


Figure 4. SEM micrograph showing the induced calcium phosphate layer on 10% nanoapatite/Polyactive™ 70/30 composites after a 3 day immersion in 1.5 SBF.

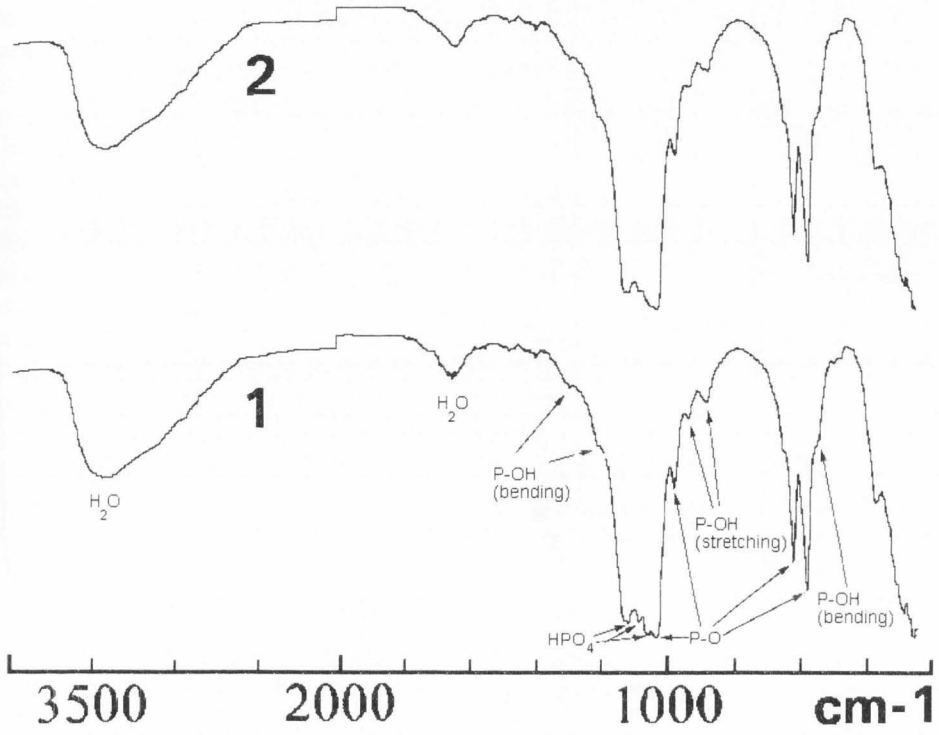


Figure 5. The infra-red spectra of minerals induced from ACS on different materials: (1) on Polyactive™ 70/30, (2) On 50% nanoapatite composites. Note the HPO_4 absorption bands in the spectra.

---

occasions, some isolated larger globular calcium phosphate mineral spots (confirmed by EDX) were found on top of Polyactive™ 30/70 and on the walls of the polystyrene disposable beaker with sizes exceeding 100 mm (Fig. 3).

Calcification of the bulk material was also observed. This calcification had developed from the top of the material towards the bottom of the sample as seen from the cross section of the material (Fig. 2c).

1.5 SBF immersion. The stability test for 1.5 SBF showed that the solution is also a stable solution during the testing period. Polyactive™ 70/30 and Polyactive™ 30/70 both failed to induce calcium phosphate precipitation after 9 days. However, the composites of nano-apatite/Polyactive™ 70/30 had induced calcium phosphate precipitation when immersed for 3 days (Fig. 4)

Infra-red and XRD spectra of the Ca/P mineral layer induced in ACS

The IR spectra of the mineral layer were basically similar regardless of the composition of the materials used in this study (Fig. 5). The broad bands at 3800 - 3000 and 1615 cm^{-1} are H_2O absorptions. The peaks at 1080, 1030, 964 cm^{-1} are absorptions from PO_4^{-3} groups. HPO_4^{-2} bands can be seen at 1280, 1200, 923, 868 and 530 cm^{-1}, and those are typical for octacalcium phosphate (OCP, $Ca_8H_2(PO_3)_6.5H_2O$) [3, 4, 17, 19, 20, 30, 43].

The XRD Spectra and the computer data analysis suggested that the calcium phosphate layer formed on the materials has both the octacalcium phosphate structure and calcium hydroxyapatite structure (Fig. 6).

Discussion

An ideal *in vitro* model to evaluate the calcification ability of biomaterials should be able to produce a mineral layer on the surface of the material within a short period of time, and the results should be predictive for the *in vivo* results. Polyactive™ 70/30, as has been demonstrated, can be calcified both *in vitro* and *in vivo* while Polyactive™ 30/70 does not have the ability to become calcified [2, 46, 47]. In the present study, the results of the ACS model agreed very well with these *in vivo* tests, because Polyactive™ 70/30 became calcified, in contrast to Polyactive™ 30/70. On the other hand, 1.5 SBF, although capable of inducing a Ca-P layer on nano-apatite composites, failed to produce a Ca/P layer on Polyactive™ 70/30 not even after 9 days immersion. In these respects, ACS is a preferable solution for *in vitro* testing, because of its ability to produce Ca-P layer on the biomaterials within short periods, and the results correlate very well with *in vivo* experimental results.

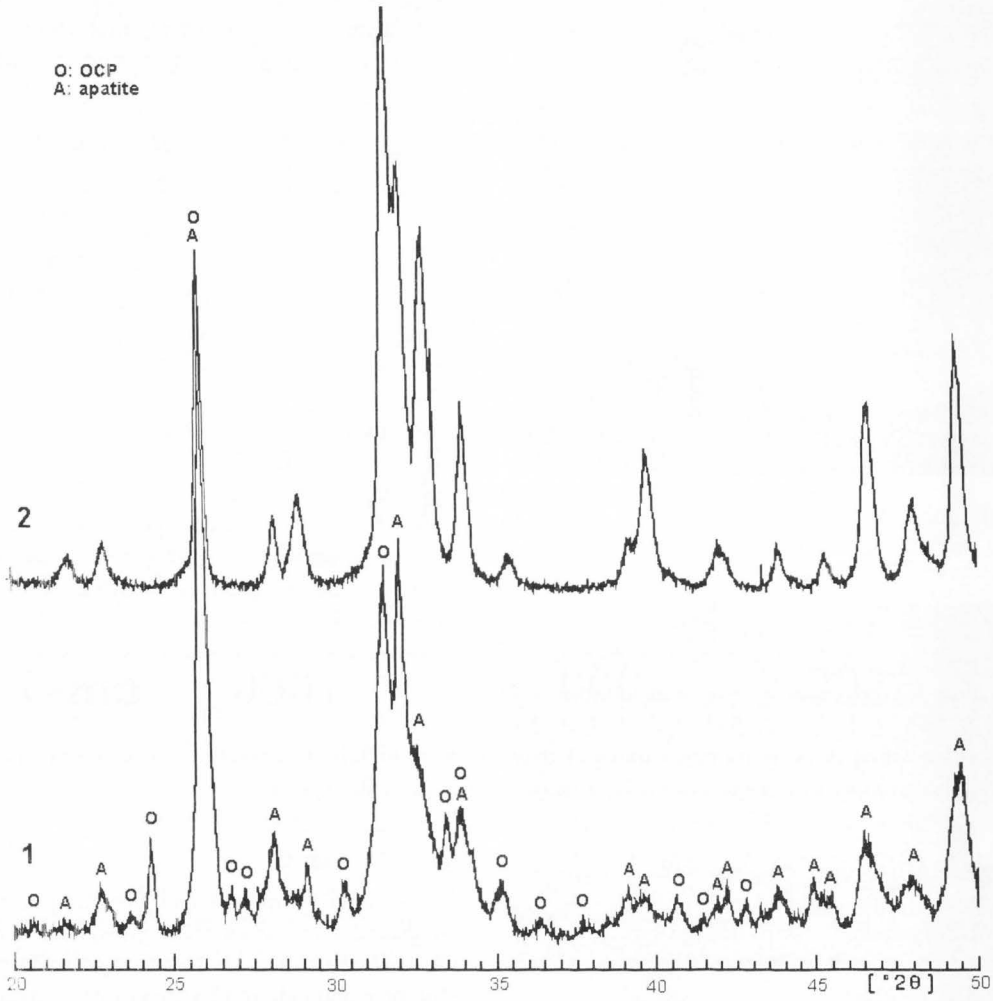


Figure 6. The XRD spectrum of the minerals induced from ACS after a 6 day immersion (1). (2) is an apatite spectrum. Note the OCP peaks (O) and the apatite peaks (A) in spectrum 1.

The ACS in this study is a highly supersaturated calcium and phosphate solution similar to the solution used by Golomb *et al.* [24]. The $[Ca^{2+}]$ $[HPO_4^{2-}]$ = 9 mM^2, and [Ca]/[P] was kept at 1.67, which is the same ratio as in hydroxyapatite. The existence of relatively large amounts of Na^+ and Cl^-, from NaCl, actually decreases the ion activity product (IAP) of Ca^{2+} and HPO_4^{2-}, resulting in IAP < $[Ca^{2+}][HPO_4^{2-}]$ = 9 mM^2. Therefore the ACS solution is quite stable as compared to the solution used by Golomb and Wagner [24].

The first step in calcification, as in any crystal precipitation, is nucleation from the supersaturated solution. However, supersaturation alone does not mean that nucleation will occur. In order for nucleation to occur, a certain amount of energy is needed for the system to overcome the activation energy barrier. The value of the activation energy is the critical factor in determining the rate of nucleation in a supersaturated system. Generally speaking, there are two ways to lower the activation energy: (1) increasing the degree of supersaturation, or (2) decreasing, at a given supersaturation, the interfacial energy [16]; the latter can be achieved by introducing a solid, where the solid/ion cluster interfacial energy can be lower than the surface/solution and ion cluster/solution interface energies. Under those conditions nucleation can occur at the solid surface, followed by crystal growth.

It has been demonstrated that Polyactive™ 70/30 has the ability to absorb Ca^{2+} from calcium containing media probably through a Ca^{2+} complexing mechanism of PEG segments [2, 45, 46]. Therefore, when Polyactive™ 70/30 samples are put in ACS solution, Ca^{2+} and HPO_4^{2-} ions will diffuse into the polymer matrix. Because of the calcium ion complexing ability of the PEG segment, the

Ca^{2+} concentration within the polymer matrix and near the surface can reach high values locally. Therefore, the activation energy of nucleation for calcium phosphate is decreased both by the introduction of the Polyactive™ surface and the increased degree of supersaturation. Once the nucleation of the mineral occurs at the surface of polymer, it will be easily followed by the mineral crystal growth on these surface nucleation sites. Calcification can also develop towards the centre of the sample due to the relatively high concentrations of Ca^{2+} and HPO_4^{2-} in the permeable polymer matrix. On the other hand, the availability of space for crystal growth will favour calcium phosphate precipitation on the surface of the already formed Ca-P layer.

According to the discussion above, in order to decrease the activation energy for nucleation, the supersaturation of the solution should be as high as possible, but should not exceed the critical supersaturation above which homogenous nucleation will occur and which renders the solution unstable. Use of both IR and XRD is a reliable way to determine the structure of Ca-P mineral [30, 32]. The Ca-P layer induced from ACS is mainly composed of OCP and calcium hydroxyapatite according to IR and XRD Spectra. The IR absorption bands for OCP and apatite have been extensively studied and reviewed, and are readily available [3, 4, 17, 19, 20, 30, 43]. The IR spectrum of the mineral obtained in this experiment is quite similar to that of OCP, including some typical absorption bands for OCP: P-OH bending modes at 1280 cm^{-1} and 1200 cm^{-1}; P-OH stretching mode of HPO_4 groups at 920 cm^{-1} and 869 cm^{-1}; and HO-PO_3 bending mode in HPO_4 at 530 cm^{-1}. However, IR spectra can not reveal whether the mineral is only OCP or a mixture of OCP and hydroxyapatite. XRD results clearly showed the mineral had the structure of OCP and hydroxyapatite (Fig. 6). SEM observation showed that the original plate-like crystals (typical OCP crystal morphology) were gradually transformed into a dense crystal layer with the plate-like crystal on top. Therefore we conclude that the calcium phosphate layer was composed of OCP and calcium hydroxyapatite. The calcium hydroxyapatite was likely transformed from OCP. The transformation of OCP to apatite is possible. The transformation mechanisms have been extensively described by other authors [6, 7, 15, 16, 29, 30].

It is not surprising to find that the Ca-P layer is composed of OCP because OCP nucleates and grows more easily than hydroxyapatite. OCP often occurs as a transient intermediate in the precipitation of thermodynamically more stable hydroxyapatite and biological apatite [17]. It is widely believed that OCP is the bone mineral precursor [6, 7, 14, 17, 30] and the dentin precursor [14] due the strong similarity beween their structures. The close structural relationship between OCP and hydroxyapatite has been used to explain the incorporation (via hydrolysis) of impurities, particularly CO_3^{2-}, Mg^{2+} and Na^+. Therefore, this ACS solution might also be useful for studying the transition process of OCP to hydroxyapatite and biological apatite.

Nano-apatite was suggested to have better osteoconductivity than sintered hydroxyapatite particles, due to its similarity to bone mineral in morphology, crystal structure, composition and crystallinity [37]. However, no direct evidence to prove this has been provided. In the present and previous experiments, it has been found that addition of nano-apatite to Polyactive™ indeed improves the ability of the polymer to calcify both in SBF and ACS. The possible mechanisms of the improvement are: (1) the nano-apatite dispersed in Polyactive™ may act as a nucleation site for the OCP-like phase, (2) incorporation of nano-apatite to the polymer may increase the Ca^{2+} and HPO_4^{2-} concentrations within the polymer. Both mechanisms will decrease the interfacial energy of the solid surface. Thus, a calcium phosphate mineral layer can be formed on the surface of polymer within a shorter period of time. It has also been found that addition of calcium phosphate to Polyactive™ promoted calcification both *in vitro* and *in vivo* [21, 22].

Our experimental results suggest that the supersaturation of ACS is beneficial for the speed of testing the calcification ability of materials. 1.5 SBF failed to induce a layer of mineral on Polyactive™ 70/30 within 9 days immersion period. Two factors may contribute to this. First, IAP, the product of $[Ca^{2+}][HPO_4^{2-}]$, in 1.5 SBF is less than IAP in ACS, or the interfacial energy of Polyactive™ 70/30 is higher in 1.5 SBF than in ACS. The second factor is the presence of Mg^{2+} and other ions in SBF. It is known that Mg^{2+} can inhibit the nucleation of apatite, as has been shown for the induction period of apatite nucleation on silica gel [5, 35]. Hence, as compared to ACS, the induction time of the nucleation in 1.5 SBF may be quite long, and therefore no Ca-P precipitation occurs within a short period of time, unless the material has a very strong ability to calcify.

In Polyactive™ 30/70 materials, some isolated, large Ca-P globular deposits could be observed on the surface (Fig. 3). Those globules have a very different morphology from the Ca-P layer on Polyactive™ 70/30 (Fig. 2), and do not necessarily indicate an ability of the material to calcify. In our experience, a confluent mineral layer formation is the indication of such ability.

SBF solution has been extensively used due to the fact that its ion concentrations are similar to that of body fluid, and it can produce carbonated apatite on biomaterials [27, 33-36]. Although the ion concentrations

in ACS solution are different from those in body fluid, and ACS does not give rise to carbonated apatite formation, it does show the nucleation ability of calcium phosphate on the materials as discussed above. Therefore, ACS is a suitable model for the study of the ability of biomaterials to induce Ca-P nucleation and growth, whereas SBF would be a more suitable solution to produce carbonated apatite coatings on biomaterial surfaces and indeed has been successfully used for such purposes [1, 25, 44].

From our results, we conclude that ACS is a suitable model solution for the examination of the calcification ability of materials *in vitro*. Although SBF has proven to be capable of inducing precipitation of a carbonated calcium phosphate layer on certain materials, even concentrated solutions failed to induce precipitation on Polyactive™ 70/30 after 9 days immersion in our experiment. Since this polymer has been shown to become rapidly calcified *in vivo*, SBF does not seem to be a proper model for fast screening of the differences in the calcification ability of materials. ACS, however, forms a thick layer of calcium phosphate on the 70/30 polymer and its composites. Therefore, ACS may be used for more rapid screening of materials regarding their ability to calcify *in vivo*.

Conclusion

A novel and simple *in vitro* model to examine the calcification of biomaterials has been developed in the present study. In combination with the known calcification behaviour of Polyactive™, this model solution has proved to be fast and effective for a comparison of the calcification rate of biomaterials. This study also showed that by incorporating nano-apatite into Polyactive™, the calcification rate of the resulting materials can be significantly enhanced.

References

[1] Abe Y, Kokubo T, Yamamuro T (1990) Apatite coating on ceramics, metals and polymers utilizing a biological process. J Mater Sci Mater Med **1**: 233-238.

[2] Bakker D, Grote JJ, Vrouenraets CMF, Hesseling SC, de Wijn JR, Van Blitterswijk CA (1990) Bone-bonding polymer (Polyactive™). In: Clinical Implant Materials. Heimke G, Stoltese U, Lee AJL (eds). Elsevier Science Publ, Amsterdam, pp 99-104.

[3] Berry EE, Baddiel CB (1967) Some assignments in the infra-red spectrum of octacalcium phosphate. Spectrochim Acta **23A**: 1781-1792.

[4] Berry EE (1967) The structure and composition of some calcium-deficient apatites. J Inorg Nucl Chem **29**: 317-327.

[5] Blumenthal NC (1989) Mechanisms of inhibition of calcification. Clin Orthopaed Rel Res **247**: 279-289.

[6] Brown WE (1962) Crystal structure of octacalcium phosphate. Nature (London) **196**: 1048-1055.

[7] Brown WE, Eidelman N, Tomazic B (1987) Octacalcium phosphate as a precursor in biomineral formation. Adv Dent Res **1**: 306-313.

[8] Coleman D (1981) Mineralization of blood pump bladders. Trans Am Soc Artif Intern Organs **27**: 708-713.

[9] Daculsi G, LeGeros RZ, Nery E, Lynch K, Kerebel B (1989) Transformation of biphasic calcium phosphate ceramics in vivo: ultrastructural and physicochemical characterization. J Biomed Mater Res **23**: 883-894.

[10] Daculsi G, LeGeros RZ, Mitre D (1989) Crystal dissolution of biological and ceramic apatites. Calcif Tissue Int **45**: 95-103.

[11] Daculsi G, Passuti N, Martin S, Deudon C, Legeros RZ, Raher S (1990) Macroporous calcium phosphate ceramic for long bone surgery in humans and dogs. Clinical and histological study. J Biomed Mater Res **24**: 379-396.

[12] Daculsi G, LeGeros RZ, Deudon C (1990) Scanning and transmission electron microscopy, and electron probe analysis of the interface between implants and host bone. Osseo-oalescence versus osseo-integration. Scanning Microsc **4**: 309-314.

[13] Daculsi G, LeGeros RZ, Heughebaert M, Barbieux I (1990) Formation of carbonate-apatite crystals after implantation of calcium phosphate ceramics. Calcif Tissue Int **46**: 20-27.

[14] Driessens FCM (1988) Physiology of hard tissues in comparison with the solubility of synthetic calcium phosphates. In: Bioceramics: Materials Characteristics Versus in Vivo Behavior. Ann New York Acad Sci **523**: 137-156.

[15] Eanes ED, Meyer JL (1977) The maturation of crystalline calcium phosphate in aqueous suspension at physiologic pH. Calcif Tissue Res **23**: 259-269.

[16] Eanes ED (1992) Dynamics of calcium phosphate precipitation. In: Calcification in Biological Systems. Bonucci E (ed). CRC Press, Boca Raton, FL, pp 1-17.

[17] Elliott JC (1994) General chemistry of the calcium orthophosphate. In: Structure and Chemistry of the Apatites and Other Calcium Orthophosphates. Elsevier Science Publ, Amsterdam, pp 13-23.

[18] Ferrens VJ, Boyce SW, Billingham ME, Jones M, Ishihara T, Roberts WC (1980) Calcific deposits in porcine bioprostheses: structure and pathogenesis. Am J Cardiol **46**: 721-734.

[19] Fowler BO, Moreno EC, Brown WE (1966) Infra-red spectra of hydroxyapatite, octacalcium

phosphate and pyrolysed octacalcium phosphate. Arch Oral Biol **11**: 432-444.

[20] Fowler BO, Markovic M, Brown WE (1993) Octacalcium phosphate. 3. Infrared and Raman vibrational spectra. Chem Mater **5**: 1417-1423.

[21] Gailard ML, Van den Brink J, Van Blitterswijk CA, Luklinska ZB (1994) Applying a calcium phosphate layer on PEO/PBT copolymers affects bone formation in vivo. J Mater Sci Mater Med **5**: 424-428.

[22] Gailard ML, Van Blitterswijk CA. (1994) Pre-operative addition of calcium ions or calcium phosphate to PEO/PBT copolymers (Polyactive™) stimulates bone mineralization in vitro. J Mater Sci Mater Med **5**: 695-701.

[23] Glasmacher B, Reul H, Rau G, Erckes C, Wieland J (1987) In vitro investigation of the calcification behaviour of polyurethane biomaterials. In: Polyurethane in Biomedical Engineering II. Elsevier Science Publ, Amsterdam, pp 151-168.

[24] Golomb G, Wagner D (1991) Development of a new in vitro model for studying implantable polyurethane calcification. Biomaterials **12**: 397-405.

[25] Hata K, Kokubo T, Nakamura T, Yamamuro T (1995) Growth of a bone-like apatite layer on a substrate by a biomimetic process. J Am Ceramic Soc **78**: 1049-1053.

[26] Hench L (1992) Bioactive bone substitutes. In: Bone Grafts and Graft Substitutes. Habal MB, Reddi AH Eds). W.B. Saunders, Philadelphia, PA, pp 263-275.

[27] Kokubo T, Kushitani H, Sakka S, Kitsugi T (1990) Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W. J Biomed Mater Res **24**: 721-734.

[28] Kokubo T (1992) Bioactivity of glasses and glass ceramics. In: Bone-Bonding Biomaterials. Ducheyne P, Kokubo T, Van Blitterswijk, CA (eds). Reed Healthcare Communications, Leiderdorp, pp. 31-46.

[29] LeGeros RZ (1984) Preparation of octacalcium phosphate, OCP: A direct fast method. Calcif Tissue Int **37**: 194-197.

[30] LeGeros RZ (1991) Biological relevant calcium phosphate: preparation and characterization In: Calcium Phosphates in Oral Biology and Medicine. Karger, Basel, pp 4-45.

[31] LeGeros RZ, Daculsi G, Orly I, Gregoire M, Heughebaert M, Gineste M, Kijkowska R (1992) Formation of carbonate apatite on calcium phosphate materials: dissolution/precipitation process. In: Bone-Bonding Biomaterials. Ducheyne P, Kokubo T, Van Blitterswijk CA (eds). Reed Healthcare Communications, Leiderdorp, The Netherlands, pp. 201-212.

[32] LeGeros RZ (1993) Biodegradation and bioresorption of calcium phosphate ceramics. Clin Mater **14**: 65-88.

[33] Li P, Ohtsuki C, Kokubo T, Nakanish K, Soga N, Nakamura T, Yamamuro T (1992) Apatite formation induced by silica gel in a simulated body fluid. J Am Ceram Soc **75**: 2094-2097.

[34] Li P, Ohtsuki C, Kokubo T, Nakanish K, Soga N, Nakamura T, Yamamuro T (1993) Process of formation of bone-like apatite layer on silica gel. J Mater Sci Mater Med **4**: 127-131.

[35] Li P, Ohtsuki C, Kokubo T, Nakanish K, Soga N, Nakamura T, Yamamuro T, (1993) Effect of ions in aqueous medium on apatite formation on silica gel and its relevance to bioactivity of bioactive glasses and glass ceramics. J Appl Biomater **4**: 221-229.

[36] Li P, Ohtsuki C, Kokubo T, Nakanish K, Soga N, de Groot K (1994) A role of hydrated silica, titania and alumina in forming biologically active bone-like apatite on implant. J Biomed Mater Res **28**: 7-15.

[37] Li Y, de Groot K, de Wijn JR, Klein CPAT, Van de Meer S (1994) Morphology and composition of nanograde calcium phosphate needle-like crystals formed by simple hydrothermal treatment. J Mater Sci Mater Med **5**: 326-331.

[38] Liu Q, Lai Q, Zhang W (1992) The calcification mechanism of bioprosthetic heart valve. Beijing Biomed Eng **11**: 35-38.

[39] Radder AM, Leenders H, Van Blitterswijk CA (1994) Interface reactions to PEO/PBT copolymers (Polyactive) after implantation in cortical bone. J Biomed Mater Res **28**: 141-151.

[40] Radder AM, Davies JE, Leenders H, Van Blitterswijk CA (1994) Interfacial behavior of PEO/PBT copolymers (Polyactive) in a calvarial system: an in vitro study. J Biomed Mater Res **28**: 269-277.

[41] Schoen FJ, Fernandez J, Gonzales-Lavin L, Cernaianu A (1987) Causes of failure and pathologic findings in surgically-removed Ionescu-Shiley standard bovine pericardial heart valve bioprostheses: Emphasis on progressive structural deterioration. Circulation **76**: 618-627.

[42] Schoen FJ, Harasaki H, Kim KM, Anderson HC, Levy RJ (1988) Biomaterial associated calcification: pathology, mechanism and strategies for prevention. J Biomed Mater Res **22**: 11-36.

[43] Soptrajanov B, Petrov I (1967) On the structure and symmetry of the phosphate ions in some calcium phosphates. Croatica Chem Acta **39**: 37-45.

[44] Tanahashi M, Yao T, Kokubo T, Minoda M, Miyamoto T, Nakamura T, Yamamuro T (1994) Apatite coating on organic polymers by a biomimetic process. J Am Ceramic Soc **77**: 2805-2808.

[45] Thoma RJ (1987) Poly(ether)urethane reactivity with metal-ion in calcification and environmental stress cracking. J Biomater Appl **1**: 449-486.

[46] Van Blitterswijk CA, Bakker D, Leenders H, Van den Brink J, Hesseling SC, Bovell Y, Radder AM, Sakker JR, Gallard M, Heinze PH, Beumer GJ (1992) Interfacial reactions leading to bone-bonding with PEO/PBT copolymer (Polyactive™). In: Bone-Bonding Biomaterials. Ducheyne P, Kokubo T, Van Blitterswijk CA (eds). Reed Healthcare Communications, Leiderdorp, The Netherlands, pp. 153-171.

[47] Van Blitterswijk CA, Van den Brink J, Leenders H, Bakker D (1993) The effect of PEO ratio on the degradation, calcification and bone bonding of PEO/PBT copolymer (Polyactive). Cells Mater **3**: 23-36.

[48] van Blitterswijk CA, Bovell YP, Flash JS, Leenders H, Van den Brink I, de Bruijn JD (1995) Variation in hydroxyapatite crystallinity: Effects on interface reactions. In: Hydroxyapatite Coated Hip and Knee Arthroplasty. Epinette JA, Geesink RGT (eds). Expansion Scientifique Francaise, Paris, pp 33-40.

[49] Wisman CB, Pierce WS, Donachy JH, Pae WE (1982) A polyurethane trileaflet cardiac valve prosthesis: In vitro and in vivo studies. Trans Am Soc Artif Intern Organs **28**: 164-168.

[50] Wouters LHG, Rousseau EPM, Van Steenhoven AA, German AL (1987) An experimental set-up for in vitro analysis of polyurethane calcification. In: Polyurethane in Biomedical Engineering II. Planck H, Syre I, Dauner M, Egbers G (eds). Elsevier Science Publ, Amsterdam, pp 169-181.

Discussion with Reviewers

K.M. Kim: As opposed to the authors' claim of the "near physiological conditions," the degree of supersaturation in ACS is excessive. The [Ca]x[P_i] product in ACS is several times greater than that of human body fluid. The results obtained from such a high degree of supersaturation are likely to be misleading. Any *in vitro* study should simulate in vivo phenomena as closely as possible. In a simple aqueous solution, spontaneous calcification takes place at much lower concentrations of Ca and P_i (e.g., Boskey and Posner [51]). It is not surprising that non-apatite is the predominant deposit formed *in vivo*. The authors ignore calcific deposits (?) on the surface of Polyactive™ 30/70 (Fig. 2c).

Authors: In our opinion the ACS solution is in a near physiological condition because of the pH (7-7.4), ionic strength and temperature (37°C) of the solution. Although the [Ca][P_i] product is several times higher than that of normal circulated human body fluid, it is very likely that in a local area where bone is in a process of healing, the [Ca][P_i] product is also several times higher than that of normal body fluid. We do agree with the reviewer that working with such highly supersaturated Ca-P solutions might lead to misleading results. Therefore, in our experiment both negative controls (Polyactive™ 30/70, polystyrene beaker itself) and positive control samples (Polyactive™ 70/30) were used. Although some isolated Ca-P spots were also found in negative control samples as stated in the manuscript, they were morphologically quite different from the Ca-P mineral layer on Polyactive™ 70/30 and its composites. Therefore, we stated that "those globules have a very different morphology from the Ca-P layer on Polyactive™ 70/30 (Fig. 2), and do not necessarily indicate an ability of the material to calcify. In our experience, a confluent mineral layer formation is the indication of such ability. Thus we feel that our results are unlikely to be misleading.

The ACS solution has been used in our laboratory for a long time and proved to be successful not only in the case of Polyactive™, but also in the case of plasma sprayed calcium phosphate coatings and fluoride calcium phosphate coating, titanium and its alloy [53], modified bamboo [52], etc.

G. Daculsi: The authors should determine the ionic concentrations (Na, Ca, Cl, P, K) in solution after incubation.

Authors: It would offer some additional advantages if the ionic concentrations of Na, Ca, P, Cl, K were determined. Since we have used EDX to identify all the precipitation which was visible under SEM, and the precipitation was further identified by IR and XRD, we feel that it is not absolutely necessary to further determine the ionic concentrations.

G. Daculsi: How is it possible with the high content of Cl and Na, compared to Ca and P, that you do not observe these ions by EDX on "precipitation"?

Authors: Although the Na^+ and Cl^- concentrations in ACS are high, the concentrations are just as high as that in a normal saline (0.8 % NaCl). The reason why Na^+ and Cl^- were not observed in the Ca-P precipitates is still unknown. We speculate that one of the reasons is probably the high crystallinity of the Ca-P precipitation.

G. Daculsi: In SBF the precipitation is a carbonated apatite similar to that obtained *in vivo* or in cellular culture. In your case you see OCP. You can not compare and consider that ACS is better than SBF for screening. If precipitation is not carbonated apatite, cell adhesion (osteoblasts, osteoclasts) and phenotypic expression cannot be similar.

Authors: Based on our experiment, we stated that ACS is a suitable model to study the calcification of biomaterials. One obvious reason is that Polyactive™ 70/30, which can be calcified both *in vivo* and *in vitro*,

can not be calcified in SBF. More precisely, ACS is a model to study the ability of biomaterials to induce Ca-P nucleation and precipitation, and this ability is generally believed to have a close correlation to the *in vivo* calcification behaviour of biomaterials. It is not our intention to say that the calcification layer induced from ACS is better than that from SBF in view of the biological properties.

Additional References

[51] Boskey AL, Posner AS (1976) Formation of hydroxyapatite at low supersaturation. J Phys Chem **80**: 40-45.

[52] Li SH, Liu Q, De Wijn JR, Zhou B, De Groot K (1997) In vitro calcium phosphate formation on natural composite materials: bamboo. Biomaterials **18**: 389-395.

[53] Wen HB, Liu Q, De Wijn JR, Cui FZ, De Groot K (1997) A simple way to prepare bioactive Ti-6Al-4V surface. Trans Soc Biomater 1997 Annual Meeting. Society for Biomaterials, New Orleans, LA, p 10.