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ANALYSIS OF THE BONY INTERFACE WITH VARIOUS TYPES OF HYDROXYAPATITE IN VITRO

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(Received for publication May 11, 1993, and in revised form July 30, 1993)

Abstract

Rat bone marrow cells, capable of forming bone-like tissue, were cultured on three types of plasma sprayed hydroxyapatite that differed in degree of crystallinity from 15%, 43% to 69%. The interface between the mineralized extracellular matrix and the hydroxyapatite was studied with scanning and transmission electron microscopy. At the onset of bone formation, calcium and phosphorous-rich afibrillar globules, with a diameter varying from 0.2 to 0.8 µm, were produced and deposited onto the different substrata. These globules subsequently fused to form a homogeneous layer to which collagen fibres became anchored. Individual globules could be distinguished on the non-degrading 69% crystalline hydroxyapatite, but were partially fused with the degrading surfaces of 15% and 43% crystalline materials. Transmission electron microscopy revealed distinctly different interfacial structures with the various types of hydroxyapatite. A 20 to 60 nm thick, glycosaminoglycan containing electron dense layer was clearly visible on 69% crystalline hydroxyapatite, but was less well defined on the more amorphous materials. On the latter, a 0.4 to 1 µm wide amorphous zone was regularly seen to which both parallel and oblique aligned collagen fibres were attached. The results of this study show that the crystallinity of plasma sprayed hydroxyapatite is an important parameter which influences the establishment of the bony interface and may, as a result, have an effect on the bone formation rate and bonding strength between hydroxyapatite and bone tissue.

Key Words: Calcium phosphate, hydroxyapatite, interface, crystallinity, bone formation, mineralization, in vitro, osteoblast, plasma spray.

Introduction

The ability of bone to bond to certain calcium phosphate ceramics has been the basis of extensive research to elucidate the mechanisms underlying this phenomenon. Ultrastructural differences have been reported at the bony interface with hydroxyapatite and tricalcium phosphate in vitro. The former frequently showed an electron dense layer at the interface whereas this was only occasionally present at the latter material (van Blitterswijk et al., 1985). Additionally, higher push-out values from bone tissue have been found for hydroxyapatite compared to tricalcium phosphate (Klein et al., 1991), suggesting a relationship between interface structure and bonding strength. Since we have recently observed various interfacial structures between bone tissue and partially amorphous hydroxyapatite, including electron dense layers and amorphous zones (de Bruijn et al., 1992a), there are two major questions to be addressed: a) are differences in interfacial structures related to the bioactivity of a material, and b) what mechanisms underlie the formation of these interfacial structures?

To answer these questions, the bioactivity of a material should first be defined. According to the second consensus conference on definitions in biomaterials (Williams et al., 1992), a bioactive material is "a material that is designed to elicit or modulate biological activity". This is, however, a general definition, concerning both hard and soft tissue replacement materials. With regard to bone-biomaterial interactions, bioactive materials are thought to be able to generate a surface apatite layer with which bone tissue becomes incorporated (de Groot, 1981; Jarcho, 1981). The bond is therefore the result of biological and physico-chemical processes that have taken place at the interface between bone tissue and an implant material. For calcium phosphates, the main physico-chemical events occurring at the bone-biomaterial interface during this process are suggested to be dissolution of the ceramic surface, followed by re-precipitation or epitaxial crystal growth (Daculsi et al., 1990a,b; LeGeros et al., 1991). Subsequently, an incorporation of extracellular matrix biomolecules will eventually give rise to the interfacial bond (Hench, 1991; Kasemo and Lausmaa 1991; LeGeros et al., 1991).
The solubility of calcium phosphate ceramics is controlled by numerous factors which include crystal structure, microporosity, neck geometry, grain size and crystallinity (Klein et al., 1983; de Groot et al., 1985). With regard to the latter, it is known that the more crystalline a material is, the lower its dissolution rate (LeGeros et al., 1991). Since one of the first steps in the interfacial bond formation involves dissolution of the ceramic surface, one could hypothesize that variation in hydroxyapatite crystallinity may influence its bioactivity. The objective of this study, therefore, is to examine whether variations in crystallinity of hydroxyapatite will influence the establishment and ultrastructural morphology of the interface. The experimental system used is an osteogenic rat bone marrow cell culture that has previously been described as a suitable model to study bone-biomaterial interactions (Davies et al., 1992a).

Materials and Methods

Hydroxyapatite ceramics

Three types of hydroxyapatite coating, varying in crystallinity from 15%, 43% to 69%, were applied onto 13 mm round coverslips (Thermanox®) using the plasma spray method. Both 15% and 43% crystalline coatings, with a thickness of 20-50 µm, were produced by varying plasma spray parameters such as gun-substratum distance, powder flow and gas composition. The 69% crystalline coating was generated by thermal-treatment of a 240 µm thick 15% crystalline hydroxyapatite coating. It was gradually heated to 600°C (100°C/h) and after one hour at 600°C, was cooled to room temperature with a thermal drop of 100°C per hour. Due to the loss of the carrier polymer during this treatment, strength was gained by the higher coating thickness.

Prior to cell culture, the hydroxyapatite plasma sprayed coverslips were sterilized by 60Co gamma-irradiation (2.5 MRad) and placed in a 24-well tissue culture plate (Costar).

Rat bone marrow cell isolation and culture

Rat bone marrow cells were obtained from 100-120 g young adult male Wistar rats according to the method described by Maniatopoulos et al. (1988). In short, femora were excised and washed in α-minimal essential medium (α-MEM) containing ten times the normal concentration of antibiotics (see below for concentration). Epiphyses were removed and each diaphysis was flushed out with 15 ml supplemented α-MEM that contained 15% fetal bovine serum (FBS), 0.1 mg/ml penicillin G, 50 µg/ml gentamycin, 0.3 µg/ml fungizone and freshly added 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10-8 M dexamethasone. Marrow cells of both diaphyses were collected in 50 ml tubes (Costar) and resuspended by careful aspiration in a 25 ml syringe with a 20 G needle.

From this cell suspension, 150 µl droplets were placed onto five specimens of each coating, and onto uncoated Thermanox® coverslips. As a control, and to measure calcium release with atomic absorption spectrometry, three specimens of the various coatings were incubated with supplemented a-MEM in the absence of cells. All cultures were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO₂ and after 4 hours, 1 ml of medium was added to each well. At day 4, cells were refed with freshly prepared medium and then refed every other day. At day 18, cells were fixed and processed for scanning and transmission electron microscopy (SEM and TEM).

Scanning electron microscopy

Prior to fixation, cells were washed three times in α-MEM at 37°C without serum added and then twice with 0.1 M Na-cacodylate buffer (pH 7.4, 37°C). Fixation was carried out for 45 minutes at room temperature in 1.5% glutaraldehyde in the same buffer. This was followed by dehydration in a graded series of ethanol and critical point drying from carbon dioxide (Ladd Research Industries Inc., Burlington, VT). Overlying cell layers were removed with compressed air to facilitate examination of the elaborated mineralized extracellular matrix. In addition, some samples were placed in liquid nitrogen and subsequently fractured (by a three point bending method) to study the bone-hydroxyapatite interface. All specimens were subsequently sputter coated with gold (Polaron Instruments Inc., Doylestown, PA) and examined in a Hitachi (model 2500) SEM or a Philips SS25 SEM equipped with an X-ray microanalyzer (Voyager XRXMA system, Noran Instruments), at an accelerating voltage of 10 and 15 kV respectively.

Transmission electron microscopy

Cells were washed as described above, and fixed in Karnovsky's fixative containing 1.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, for 2 hours at 4°C. After washing in the same buffer, cells were post-fixed in a freshly prepared aqueous solution of 1.5% potassium ferrocyanide and 1% OsO₄, for 16 hours at 4°C, to enhance membrane contrast. Cultures that were stained for glycosaminoglycans, were pre- and post-fixed in the presence of ruthenium red, according to the method described by Luft (1971).

All samples were dehydrated through a graded series of ethanol and embedded in Epon. Ultra-thin sections were processed on a LKB ultramicrotome, with a sectioning orientation almost parallel to the plasma sprayed surface. Such an orientation, as opposed to transverse sectioning, facilitated the sectioning procedure, since placement of the pyramid could be made such that first the cellular material was cut, followed by the plasma sprayed hydroxyapatite. Specimens were routinely stained with uranyl acetate and lead citrate to enhance contrast, and examined in a Philips EM 201 or EM 400 TEM operated at an acceleration voltage of 80 kV.
Bony interface with various types of hydroxyapatite

Figure 1. (A-C) Powder X-ray diffraction patterns of the three types of hydroxyapatite coating, showing a crystallinity of 15% (A), 43% (B) and 69% (C). The increase in crystallinity is shown by an increased peak size. No other phases than hydroxyapatite are detected. (D-F) Scanning electron micrographs of the coatings prior to the experiments. The 15% crystalline coating (D) is smoother than the 43% crystalline coating (E), which shows numerous partially melted particles (large arrow head) and cracks (small arrows). (F) The underside of the 69% crystalline coating, which had been adjacent to the plastic substrate. It is relatively smooth in surface topography and contains remnants of the plastic that was burned off during the heat treatment. (G-I) Scanning electron micrographs of the same coatings as in Figs. 1D-F, after submersion in supplemented culture medium, without cells. Both 15% (G) and 43% (H) crystalline coatings show a distinct surface alteration. The cracks (small arrows) are more abundant and wider, while the surface of the coatings reveal numerous submicron cavities (small arrow heads). Besides the disappearance of the plastic remains, the 69% crystalline coating (I) does not show any morphological alterations. Field widths: (D-I) 35.1 µm.

Atomic absorption spectrometry

Medium removed from the different hydroxyapatite coatings and the control samples was stored at -20°C and was later used undiluted for the assessment of released calcium with a Varian SpectrAA 300/400 atomic absorption spectrometer. Data were normalized to the amount of calcium measured in supplemented medium alone.

Results

Coating characterization

X-ray diffraction patterns of the three plasma sprayed coatings show a gradation of crystallinity, from 15% to 69% crystalline hydroxyapatite, indicated by higher and sharper peaks (Figs. 1A-C). The surface morphology, prior to the experiments, of the 15% and 69% crystalline plasma sprayed coatings was fairly smooth, whereas the surface of 43% crystalline hydroxyapatite contained numerous small, not fully melted hydroxyapatite particles (Figs. 1D-F). Small cracks were present in all coatings. Figures 1G-I shows the control materials after incubation in supplemented culture medium without cells for 18 days. The medium had been refreshed three times weekly. The surfaces of both 15% and 43% crystalline hydroxyapatite contained numerous large cracks and submicron cavities as opposed to the highly crystalline, heat-treated coating which remained unaltered. The partially melted hydroxyapatite fragments on the 43% crystalline hydroxyapatite are more prominent. A blast of compressed air resulted in detachment of an approximately 1 µm thick shell of the former.
Figure 2. (A) The submicron cavities (arrow heads), as observed in Figures 1g and 1h, are part of an approximately 1 µm wide coating surface that is porous in appearance and that undergoes degradation. Underneath this degrading surface, the hydroxyapatite shows a pitted structure on which fibrillar, proteinaceous material was occasionally observed (b). Field widths: (A) 3.6 µm; (B) 11.4 µm.

Figure 3. Atomic absorption data showing accumulative release from the different hydroxyapatite coatings with time. The amount of released calcium is normalized to calcium measured in supplemented culture medium alone.

Cellular responses
Following inoculation, the bone marrow cells adhered and spread over all materials and mineralized cell nodules were formed. Since the nodules gradually increased in size with time, mineralization was in a later stage of development in the centre of the nodules as opposed to the periphery. The steps of early bone-like tissue formation could therefore be examined in the transition zone between these regions. Ultrastructurally, these stages were observed on 69% crystalline hydroxyapatite as follows. The initial formation of a granular layer, that comprised individual mineralized globules, was seen with SEM at the periphery of the nodules. Towards the centre, these globules fused and formed a continuous layer of mineralized material to which collagen fibres became attached and incorporated (Fig. 4A). The transition zone was different in appearance on both 15% and 43% crystalline coatings. Here, no distinct a fibrillar mineralized globules, as described above, were observed at the periphery of the nodules, since they seemed to have partially fused with the degrading ceramic surface (Fig. 4B). Towards the centre of the nodule however, the surface was more granular in appearance and collagen fibres were integrated in a similar way to that observed on the 69% crystalline coating. The coverage with mineralized globules on 69% crystalline hydroxyapatite seemed to be less extensive than on both 15% and 43% crystalline coatings, although we do not have quantitative data to support this observation. Following
Bony interface with various types of hydroxyapatite

Figure 4. Scanning electron micrograph showing distinct mineralization globules (small arrows), to which collagen fibres are incorporated (large arrows), on 69% crystalline hydroxyapatite (A). The globules are partially fused with the surface of 15% crystalline hydroxyapatite (B). Field widths: (A) 12.2 µm; (B) 5.9 µm.

Figure 5. Freeze-fractured, transverse section of a 69% crystalline coating. Individual mineralization globules (arrows) are deposited on the surface of the hydroxyapatite (HA). Field width: 3.5 µm.

Figure 6. Freeze-fractured transverse section of a 15% crystalline coating. (A) Mineralized extracellular matrix (ecm) is deposited onto the hydroxyapatite (HA). (B) At higher magnification, no individual mineralization globules can be discerned as they are fused in the interface zone (iz) with the degrading hydroxyapatite surface as seen in Fig. 2A, to which mineralized collagen fibres are closely attached. Field widths: (A) 34.1 µm; (B) 8.3 µm.
freeze-fracturing, transverse sections of the interfaces between the three types of hydroxyapatite and the mineralized extracellular matrix showed distinctly different structures. At the surface and in microcracks of the 69% crystalline coating, afibrillar mineralized globules with a diameter ranging from 0.2 to 0.8 µm were observed, and a distinct boundary was seen between the hydroxyapatite surface and the mineralized globules (Fig. 5). In contrast to this, the degrading outer shells of both 15% and 43% crystalline hydroxyapatite coatings were intimately integrated with the mineralized extracellular matrix (Figs. 6A,B). Union of this matrix with the ceramic surfaces therefore could not be distinguished for both 15% and 43% crystalline coatings as compared to 69% crystalline hydroxyapatite, although a clear transition between the mineralized extracellular matrix incorporated with the degrading shell, and the underlying bulk coating was still present.

Transmission electron microscopy revealed two distinctly different interfacial structures, that varied in the presence or absence of a degrading shell. An intimate contact was observed between the mineralized extracellular matrix and the 69% crystalline coating (Figs. 7A,B), while a degrading shell was frequently present between this matrix and the crystalline parts of both 15% and 43% crystalline hydroxyapatite (Figs. 7C,D). Since the contour of the original plasma sprayed surface was maintained, this shell represented the degrading outer surface of the hydroxyapatite. An interesting finding was the apparent relation between crystalline orientations in the plasma-sprayed hydroxyapatite surface and the resulting alignment of needle-shaped crystals from

Figure 7. (A,B) Transmission electron micrograph of an undecalcified interface between mineralized extracellular matrix (ecm) and 69% crystalline hydroxyapatite (HA). In (A), the crystallites of the ceramic are aligned almost parallel to the surface, resulting in a smooth interface. Contrarily, in (B), the hydroxyapatite crystallites are aligned more oblique to the outer surface which results in a more irregular interface, with which needle-shaped crystals of the extracellular matrix are closely associated (arrows). (C) The interface with 43% crystalline hydroxyapatite is shown. The original contours of individual plasma sprayed particles can still be distinguished (arrows heads), which are part of the degrading shell. (D) Interface between mineralized extracellular matrix and the 15% crystalline hydroxyapatite coating that contains few crystalline domains. All sections are unstained. Field widths: (A) 3.1 µm; (B) 2.2 µm; (C) 4.6 µm; (D) 10 µm.
Bony interface with various types of hydroxyapatite

Figure 8 (A,B). Sections which are partially decalcified by staining with uranyl acetate and lead citrate. (A) A thin, collagen free electron dense layer is present (arrows) between 69% crystalline hydroxyapatite (HA) and the surrounding mineralized extracellular matrix (ecm). (B) With 15% crystalline hydroxyapatite, this layer cannot be observed, while collagen fibres with their characteristic banding pattern are aligned perpendicularly to the amorphous hydroxyapatite surface (large arrows). Field widths: (A) 3.8 µm; (B) 3.5 µm.

Figure 9 (A,B). Ruthenium red stained specimen of 69% crystalline hydroxyapatite (HA). Electron-dense, glycosaminoglycan-rich material is present at the mineralized extracellular matrix (ecm)/hydroxyapatite interface (arrows) and collagen fibres are mainly aligned parallel to the hydroxyapatite surface. Note that the gap between the electron-dense layer and the hydroxyapatite is either the result of tissue shrinkage, or the decalcification step in the staining procedure. The region shown in Fig. 9B is an enlargement of an area similar to Fig. 9A. Field widths: (A) 14.5 µm; (B) 7.1 µm.

On partially decalcified TEM sections, a collagen free, 20 to 60 nm thick electron dense layer was frequently seen interposed between the mineralized extracellular matrix and the 69% crystalline hydroxyapatite surface (Fig. 8A). In undecalcified specimens, it could not easily be distinguished, due to the presence of needle-shaped crystals that were in direct contact with the hydroxyapatite surface. On both 15% and 43% crystalline coatings, the electron dense layer was less distinctly present and collagen fibres were often seen oblique to the degrading shell (Fig. 8B).

The periphery of mineralization globules in the extracellular matrix stained positively in ruthenium-red treated cultures. A positive stain was furthermore observed, in varying intensities, at the interface between the different hydroxyapatite coatings and the surrounding matrix.
J.D. de Bruijn et al.

Figure 10 (A,B). Ruthenium red stained specimen of 43% crystalline hydroxyapatite (HA). Several electron-dense, glycosaminoglycan containing layers can be observed (arrows), one of which is always in close proximity to the hydroxyapatite surface. The region shown in Fig. 10B is an enlargement of an area similar to Fig. 10A. Field widths: (A) 6.2 μm; (B) 2.8 μm.

Bone-like tissue. Ruthenium-red containing, or glycosaminoglycan-positive, material was observed both at the surface and in micro cracks of the plasma sprayed ceramics. The interface between 69% crystalline hydroxyapatite and bone-like tissue showed a distinct continuous glycosaminoglycan-positive layer, with a thickness of approximately 60 nm (Figs. 9A,B). The "gap" between the electron dense, glycosaminoglycan-positive layer and the hydroxyapatite is an artifact, either due to tissue shrinkage, or to the partial decalcification step during the ruthenium red staining procedure. At the surface of 43% crystalline hydroxyapatite, several glycosaminoglycan-positive layers were frequently observed (Figs. 10A,B). A more diffuse layer was seen at the interface between the most amorphous coating and bone-like tissue (Figs. 11A,B). Although collagen fibres were mostly seen parallel to the surface of 69% crystalline hydroxyapatite, they were frequently seen perpendicularly aligned, interdigitating with the surfaces of the amorphous hydroxyapatite coatings.

Discussion

The results of this study have shown that partially crystalline hydroxyapatite coatings, that have not been
subjected to a thermal-treatment, comprise a rapidly degrading outer shell which is incorporated with the mineralized extracellular matrix. This multi-phasic character of the coating corresponds well with observations by LeGeros et al. (1991), who reported differences between the outer and inner layers of plasma sprayed hydroxyapatite coatings. The thermal-treatment procedure of the most amorphous coating to produce the 69% crystalline hydroxyapatite has, however, resulted in recrystallization and crystal growth of the amorphous microcrystalline hydroxyapatite domains, and thereby increased coating stability and reduced the degradation rate.

Therefore, differences in the physico-chemical nature of the coatings may be the basis for the observed variety of interfacial structures at the bony interface with three types of hydroxyapatite. With SEM, a stable, non-degrading surface of 69% crystalline hydroxyapatite was observed on which the different stages of early bone formation could clearly be distinguished. This was morphologically similar to previous observations of early bone formation on tissue culture polystyrene in vitro (Davies et al., 1991a,b) and hydroxyapatite in vivo (Orr et al., 1992). TEM further revealed a direct apposition of bone tissue to the surface of 69% crystalline hydroxyapatite, without an intervening degrading shell. The distinct glycosaminoglycan-rich, electron dense layer can therefore be illustrated as an accumulation of afibrillar globules and proteins as a result of the stable surface, or low surface reactivity, of this highly crystalline hydroxyapatite. Conversely, a high surface reactivity was observed with more amorphous hydroxyapatite, indicated by the incorporation of afibrillar globules in the degrading surface of the material. As a consequence, individual globules could not be discerned with SEM (compare Figs. 4, 5 and 6), which could explain the diffuse glycosaminoglycan-rich layer in the degrading surface of amorphous hydroxyapatite, since glycosaminoglycans are mainly present in the periphery of afibrillar globules (de Bruijn et al., 1992a). Alternatively, the diffuse glycosaminoglycan-rich layer may be explained by the adsorption of these calcium binding proteins or other acidic sulphated proteins, stained by Ruthenium Red, to the porous hydroxyapatite surface.

The close association between needle-shaped crystals from the extracellular matrix and those of the hydroxyapatite in Fig. 7B, suggest the possibility of epitaxial crystal growth as described by Daculsi et al. (1990a,b), LeGeros (1988), and LeGeros et al. (1991), since a similar relationship is not shown in Fig. 7A where the hydroxyapatite crystal orientation is parallel to the bone. Another remarkable finding was the presence of oblique and sometimes perpendicularly aligned collagen fibres to the surface of the amorphous coatings. This has, until now, only been observed with Bioglass and glass-ceramics and was considered by Hench (Hench et al., 1971; Hench and Wilson, 1984) and others (Davies, 1990) to be of importance for the bone-bonding capacity of these materials. Since on sintered, low surface reactive hydroxyapatite mainly parallel aligned collagen fibres with the material surface have been described, collagen interdigitation may be merely the result of the surface reactivity of a material.

The results reported herein can give a possible explanation for the variety of interfacial structures that have been observed at the bony interface of hydroxyapatite in vivo, which included amorphous and/or electron dense layers. Jarcho (Jarcho et al., 1977; Jarcho, 1981) was the first to show an amorphous bonding zone, approximately 0.2 µm wide, at the interface between bone tissue and a dense hydroxyapatite ceramic, whereas Denissen et al. (1980) and Frank et al. (1991) demonstrated an amorphous or granular zone up to 1 µm wide, interposed between bone tissue and dense hydroxyapatite. Ganeles et al. (1985) observed a similar, but 0.3 to 0.5 µm thick, granular, amorphous, collagen-free electron dense layer, while van Blitterswijk et al. (1985, 1990) and de Lange et al. (1987, 1990) described a submicron thick electron dense layer at the bone-hydroxyapatite interface, which was continuous with the lamina limitans of the surrounding bone. However, before comparing the above described interfacial structures with the results presented in this paper, it should be mentioned that with the in vitro system used, only the very early interfacial phenomena are studied, which may be quite different from the long-term in vivo situation. Furthermore, the above mentioned electron dense and amorphous interfacial structures are most probably the result of combined biological and physico-chemical processes.

The crystallinity of plasma sprayed hydroxyapatite coatings has received more attention recently for its influence on bone-bonding and degradation properties. Most plasma spray companies strive for a high coating crystallinity because that is thought to increase coating stability and thus reduce its degradation rate. This attempt is supported by a study of Gabbi et al. (1992) who reported an adverse effect of amorphous coatings on the establishment of an interface with bone. Other studies, however, have suggested more amorphous coatings to be advantageous if coating longevity is desired (Dhert et al., 1991; Maxian et al., 1992, 1993). Maxian et al. (1992, 1993) reported a higher calcium dissolution in vitro for poorly crystalline hydroxyapatite coatings than for amorphous coatings and observed that these coatings displayed greater crystallographic alterations and greatest surface film formation. The percentage of implant surface in contact with cancellous bone was furthermore not significantly different between amorphous and poorly crystalline coatings for a period up to 12 weeks (Maxian et al., 1992), which led them to the conclusion that amorphous coatings may be more advantageous if coating longevity is desired. However, they did not use highly crystalline hydroxyapatite coatings which, in contrast to their statements, are generally thought to have a low surface reactivity, resulting in coating stability. We recently published the results of a study in which the same hydroxyapatite coatings, as those used in the present study, were investigated for their degradation characteristics (de Bruijn et al., 1992b). It was
observed that crystalline hydroxyapatite coatings, which had not been subjected to a thermal treatment, were composed of larger crystalline fragments surrounded by an amorphous phase. Dissolution of the amorphous phase caused the release of the crystalline fragments, which might have adverse effects on the surrounding tissue. Although a heat treatment of hydroxyapatite coatings was beneficial with respect to their stability and low surface reactivity, and thus bioactivity. Therefore, when discussing the effect of crystallinity of plasma sprayed hydroxyapatite coatings on their stability, the coating should be well characterized. For example, a high crystallinity gained by heat-treatment will result in more stable coatings, due to the absence of amorphous phases. Conversely, highly crystalline hydroxyapatite coatings which have been produced without a heat-treatment, will be composed of relatively large crystalline domains surrounded by an amorphous phase. Dissolution of this amorphous phase will release the crystalline domains and thus severe coating breakdown will occur.

As discussed previously, the degrading surface of more amorphous coatings most likely represents a dynamic zone in which dissolution and reprecipitation occurs. It may therefore be favourable for bone formation and bonding since interlocking between the altering hydroxyapatite surface and the mineralized extracellular matrix will occur. This is in contrast to the results of a study by Kitsugi et al. (1988) who suggested that bonding does not vary in hydroxyapatite with different sintering temperatures implanted in bone tissue. They, however, only used mechanical testing after 10 and 25 weeks and did not perform any histomorphometry at earlier time periods, which would be most interesting if differences were to be expected.

Summarizing, we have shown that the interfacial structural arrangements between hydroxyapatite and bone tissue are related to the crystallinity and degradation rate of the hydroxyapatite. This may, as a result have an effect on the bone formation rate and bonding strength between hydroxyapatite and bone tissue, the former of which is under investigation at present.

Acknowledgements

The authors acknowledge Jolanda de Blieck-Hogervorst for help with the atomic absorption spectrometer, Bob Chernecky and Lambert Verschragen for printing the photographic material, and Yvonne Bovell for technical support. Financial supports from the Medical Research Council (MRC), Canada and the Netherlands Organization for Scientific Research (NWO) are gratefully acknowledged.

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**Discussion with Reviewers**

**D.B. Jones:** In culture conditions cells are very sensitive to salt concentrations as they are living under conditions very close to their ultimate salt tolerance (270 mOsmolar). Dissolution of CaP from the biomaterial can cause reprecipitation near the cells. Only 2 mM phosphate is sometimes needed for precipitation to occur. Also high CaP levels can affect the cells due to osmotic effects. The addition of beta glycerophosphate may well cause an artifact in addition to this for the same two reasons: a) an increase in the osmolarity of the medium, and b) in conjunction with alkaline phosphatase activity a precipitation of phosphates. Have the authors considered these three effects together and separately?

**Authors:** Yes. With regard to the first effect, dissolution and/or (re)precipitation of calcium and phosphorous from the hydroxyapatite substrata will certainly be involved in the interface formation. There are indications that this physico-chemical process is needed for bone bonding to occur and may enhance the rate of bone apposition (van Blitterswijk et al., 1993). With regard to possible effects of β-glyceroephosphatase, we have previously grown human fibroblasts on similar plasma sprayed hydroxyapatite substrata in α-MEM supplemented with dexamethasone, ascorbic acid, and 10 mM β-glyceroephosphate. These cultures did not show any (re)precipitation of calcium phosphates on the cells or substrata, nor did the cells show any adverse effects due to the culture medium or test materials. Additionally, we have recently published the results of another study in which the same culture system was used on various types of calcium phosphates (de Bruijn et al., 1992c,d).
The results of these studies showed that mineralization occurred already after 2 weeks on hydroxyapatite, tricalcium phosphate and tetracalcium phosphate. Fluorapatite, however, showed the formation of a mineralized extracellular matrix only after 8 weeks of culture, while it will also occur (re)precipitation of the hydroxyapatite substrate which will also occur in vivo, cellular processes are the only reason for the observed mineralization and that precipitation of CaP derived from β-glycerophosphate does not occur.

S.B. Doty: Articles by Chung et al. (1992), Gronowicz et al. (1989) and Khouja et al. (1990) have indicated that the addition of 10 mM beta-glycerophosphate to culture media leads to artificial precipitation of calcium phosphate onto biological and non-biological substrates. Has the use of beta-glycerophosphate in your culture system influenced the results described in this paper?

Authors: No, please also see our answer to Dr. Jones above. Furthermore, since the results described herein, with regard to both the TEM and SEM observations, are identical to those observed in the in vivo environment [for a comparison regarding the SEM observations, see Orr et al. (1992)], the presence of β-glycerophosphate has not influenced the results described in this paper.

D.B. Jones: The authors consider the major factor of bone biocompatibility to be the biophysical-chemical interface, but calcium phosphates are well known to bind proteins at high capacity and also specifically, this depending on structure and chemical composition. Would they please comment on this in relation to their findings?

Authors: The adsorption of proteins may indeed play a role in the response of the osteogenic cells to the artificial substrata, by modulating the cellular activity as we have shown in other models (Shelton et al., 1988), and could thus also have (in)directly influenced the interface formation. In addition to adsorption of serum proteins, osteocalcin and osteopontin, for example, are produced and secreted by the osteogenic cells in this culture system and have a high affinity for hydroxyapatite, while the latter also contains a cell binding RGD-sequence. We have recently speculated that protein adsorption on calcium phosphates is of importance with regard to both bone formation onto, and cell mediated resorption of these materials (de Bruijn et al., 1993).

G. Daculsi: You have performed 5 tests for each crystallinity index? How have you determined the crystallinity index?

Authors: The percentage of crystallinity of plasma sprayed coatings was determined by scraping off the coatings and performing X-ray diffraction (XRD) on these powders. The data were then compared with a standard curve made from X-ray diffraction data of both sintered (at 1250°C) and unsintered starting powder (used for plasma spraying). More specifically this was done as follows: the peaks were cut-out using a pair of scissors and weighed. The total weight of the sintered starting-powder peaks was thought to represent 100% crystallinity. A standard curve was then made by preparing various ratios of sintered/unsintered powders (e.g., the "peak-weight"-crystallinity of 50% sintered and 50% unsintered powder was taken as 50% crystalline, while the "peak-weights" of XRD-data from a mixture of 60% sintered and 40% unsintered powder was taken as 60% crystalline, etc.). Next, the weight of the peaks after plasma spraying was measured (from which the amorphous background area was removed), and via the standard curve, the crystallinity was measured. This way of measuring crystallinity was shown to be reasonably accurate, compared to other methods, including: 1) use of an image analysis system to measure the total surface area (instead of weighing), 2) polishing a cross-section of the coating, slightly etching away the amorphous phases and measuring the ratio of crystalline/amorphous phases, only measuring the surface area of certain peaks [e.g., between 30 and 35 degrees (2Θ)], showed similar crystallinity values. It should be mentioned that these methods of measuring crystallinity are semi-quantitative, as the produced XRD-data are dependent on the type of XRD-machine used. However, because of the lack of standards for measuring crystallinities, we believe it is a good and representative method.

G. Daculsi: Have you determined the other calcium phosphate phases present in your three coatings (α or β tricalcium phosphate, tetracalcium phosphate, etc.)?

Authors: From the X-ray diffractograms of our plasma sprayed coatings, it was concluded that only a very small percentage of other phases (such as CaO, tricalcium phosphate and tetracalcium phosphate) was present (< 3%). Thus, the amorphous phase mainly represented amorphous hydroxyapatite.

G. Daculsi: Based on the similarity of early events involving deposition of extracellular matrix by bone cells on tissue culture polystyrene and the 69% crystalline hydroxyapatite used in your study, do you believe that these events are independent of the substrate, providing it is stable?

Authors: The initial formation of afibrillar mineralized globular accretions has indeed been shown to be independent of the substratum used as it has been described on a variety of artificial substrata, including various types of polystyrene, titanium and titanium-alloy (Davies et al., 1991b) as well as hydroxyapatite (e.g., this paper). However, the accretions are also visible on the
Bony interface with various types of hydroxyapatite relatively unstable 15% crystalline hydroxyapatite (Fig. 4), although they have partially fused with the material surface. Therefore, we believe the early events of bone formation to be the same for all types of material, provided they are biocompatible and do not adversely affect mineralization.

Additional References


