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Discrimination between biologically relevant calcium phosphate phases by surface-analytical techniques

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ABSTRACT

The spatially resolved phase identification of biologically relevant calcium phosphate phases (CPPs) in bone tissue is essential for the elucidation of bone remodeling mechanisms and for the diagnosis of bone diseases. Analytical methods with high spatial resolution for the discrimination between chemically quite close phases are rare. Therefore the applicability of state-of-the-art ToF-SIMS, XPS and EDX as chemically specific techniques was investigated. The eight CPPs hydroxyapatite (HAP), β-tricalcium phosphate (β-TCP), α-tricalcium phosphate (α-TCP), octacalcium phosphate (OCP), dicalcium phosphate dihydrate (DCPD), dicalcium phosphate (DCP), monocalcium phosphate (MCP) and amorphous calcium phosphate (ACP) were either commercial materials in high purity or synthesized by ourselves. The phase purity was proven by XRD analysis. All of the eight CPPs show different mass spectra and the phases can be discriminated by applying the principal component analysis method to the mass spectrometric data. The Ca/P ratios of all phosphates were determined by XPS and EDX. With both methods some CPPs can be distinguished, but the obtained Ca/P ratios deviate systematically from their theoretical values. It is necessary in any case to determine a calibration curve, respectively the ZAF values, from appropriate standards. In XPS also the O(1s)-satellite signals are correlated to the CPPs composition. Angle resolved and long-term XPS measurements of HAP clearly prove that there is no phosphate excess at the surface. Decomposition due to X-ray irradiation has not been observed.

1. Introduction

Calcium phosphate phases (CPPs) form a group of solid compounds that are highly relevant in biology and medicine as main constituents of human and animal hard tissue, i.e. of bone and teeth. They also play a major role in quite different fields and technologies, e.g. as fertilizer in agriculture or food additive in nutrition. Numerous reviews on the chemistry and use of CPPs in medical applications have therefore been published [1–3]. Hydroxyapatite (HAP), octacalcium phosphate (OCP) and amorphous calcium phosphate (ACP) are the natural CPPs occurring in bone [4]. Therefore the phase assignment of these CPPs is pivotal for the elucidation of mechanisms occurring during bone remodeling and bone diseases.

Small differences in local composition and atom density cause the structural and chemical variability of CPPs and are key challenges for a qualitative and quantitative analysis. Whereas this problem can be well solved for macroscopic amounts of crystalline material by X-ray diffraction (XRD), the analysis of thin films, surfaces and nanoparticles remains difficult, due to weak or missing diffraction intensity. Nevertheless the investigation of bone cross sections has become possible by using μXRD at a synchrotron facility [5,6]. Campi et al. showed by μXRD with a lateral resolution of 1 μm that ACP is formed as an intermediate phase during the bone formation process [4]. To our knowledge only Chusuei et al. [7] and Lu et al. [8] published systematic analytical studies of CPPs using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and X-ray Photoelectron Spectroscopy (XPS) as surface sensitive techniques to date. In fact, XPS as the elder technique was already used earlier, but did not offer enough information for the unequivocal distinction of different CPPs. Chusuei et al. combined XPS with ToF-SIMS and compared the results for six CPPs, namely HAP, α-tri-Calcium phosphate (α-TCP), β-tri-Calcium phosphate (β-TCP), OCP, ACP and dicalciumphosphate dehydrate (DCPD). The authors demonstrated that Cs^+ as well as $(CsI)_nCs^+$-cluster primary ions (PI)

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provide useful information in the SI spectra, and they determined the PO_3^-/PO_2^- SI intensity ratio for the different CPPs [7]. Lu et al. extended the approach of Chusuei et al. and applied the Principal Component Analysis (PCA) method to improve the data analysis and distinguish the different CPPs better. They used an almost similar set of seven CPPs, namely HAP, β-TCP, OCP, dicalcium phosphate (DCP), DCPD, ACP and monocalcium phosphate (MCP), but concluded that an unambiguous identification of the phases is difficult by applying ToF-SIMS [8], due to the similar fragmentation by primary ion bombardment.

Due to the improvement of the lateral resolution of modern surface analytical methods like ToF-SIMS or XPS within the last decade, the assessment of the local bone quality of bone cross sections by these techniques comes into reach. With ToF-SIMS actually a lateral resolution up to 100 nm can be achieved in optimal cases. As was shown by Henss et al. in a previous study [9], ToF SIMS is indeed a suitable method to assess bone quality, and the Ca content can be determined with appropriate standards [10]. However, for a deeper insight the unequivocal identification of the different CPPs plays a key role. CPP assignment by mass images and local spectra of bone cross sections would clearly help to identify ongoing bone remodeling sequences for bone diseases. Therefore, the analysis of calcium phosphate phases by surface analytical techniques as reported by Lu et al. and Chusuei et al. is reconsidered and extended in the present paper. Firstly, a modern 25 keV Bi-cluster PI source was used for the ToF-SIMS measurements. Also all biologically relevant CPPs were included into the analysis in order to improve the quality of the PCA analysis carried out with the ToF-SIMS data. Furthermore, EDX and XPS measurements were applied to all eight CPPs. EDX is widely used in the field of bio analytical CPP analysis as well as in other fields, for example, in order to determine the Ca/P-ratio in bone. Often the Ca/P ratio obtained by EDX measurements is used for phase assignment [11–13]. Analyzing the pure CPPs, we experienced problems using EDX, which causes serious concerns as EDX is used in many works in the field of biomaterials. EDX is inherently only a semi-quantitative method. To become quantitative, so called ZAF values must be calculated from suitable standards and applied to the EDX data. The ZAF values consist of three factors. Here *Z* is the correction factor accounting for the effect of the matrix on the primary electron beam, *A* corresponds to the absorption of X-rays by the matrix and *F* takes the fluorescence of X-rays by other atoms in the matrix into account. Modern instruments have implemented databases for these ZAF values. Landis et al. reported already in 1978 about a linear correlation between the peak intensity ratio of I_{Ca}/I_P and the atomic Ca/P ratio of eight investigated CPPs [14]. Nicholson and Dempster give an overview on energy dispersive microprobe analysis of bone tissue [15].

In the following the results of our systematic analytical study of eight CPPs obtained by XPS, EDX and ToF-SIMS are reported. It is demonstrated that the different CPP samples could be distinguished from each other by state-of-the-art analytical techniques. It is further shown, that it is possible to discriminate between all CPPs, making use of modern Bi-cluster PI source, as well as with a single XPS detail spectra measurement of the O(1s) signal and its shake-up/off features.

2. Materials and methods

2.1. Materials

All CPP powders, except OCP, were obtained commercially in high purity from Sigma–Aldrich. Their purity was confirmed by analysis certificates from the distributors, as well as by our own measurements (XRD (S1), ATR-IR (S2), XPS, EDX and ToF-SIMS measurements are given in this paper, AAS and XRF results are unpublished). OCP was synthesized following the route given by LeGeros [16]. In brief, 250 mL of 0.04 M CaAc-(Fluka, p.a.) and NaH_2PO_4-solutions (Sigma–Aldrich, puriss. p.a.) were mixed dropwise over a period of 4 h into a three-necked flask equipped with a Dimroth condenser. The flask was heated with an oil-bath up to 62 °C. The white precipitate was filtered and washed three times with ethanol and high purity water. Table 1 gives an overview of the investigated calcium phosphate phases.

The crystal structures of all powders were verified by XRD analysis prior to the surface analysis. Measurements were carried out with an PANanalytical X'Pert Pro X-ray diffractometer with Cu $K\alpha_{1,2}$ anode without monochromator, operated at 40 kV and 40 mA, Cu Kα radiation wavelength of 1.54060 Å and Cu Kβ wavelength of 1.54443 Å. The Cu Kβ contribution was subtracted mathematically from the diffraction patterns.

For EDX, XPS and ToF-SIMS measurements powders were compacted into pellets. To assure that the CPPs were not affected by the high pressures (up to 3500 bar) during pressing, the XRD analysis was repeated for the resulting CPP pellets. It was found that two of the phases were affected through compaction in their grain orientation perpendicular to the pellet surface, namely MCP and DCPD. The diffraction patterns show a slightly preferred orientation in these cases. It is known from studies on other materials that the orientation of single crystals can in rare cases influence the results of EDX and XPS measurements. As we are not investigating single crystals with perfectly orientated surfaces, we assume that there is no influence of the analysis data of compressed powders. In addition, the sampling depth of both applied methods is higher than the size of a unit cell. Another possibility might be that the ionization probability in ToF-SIMS is influenced by the crystal orientation. This could be in principle the case for single crystals but was to our knowledge never shown for matrix signals of polycrystalline samples in literature. Finally, the results for MCP and DCPD are in good agreement with those of all other phases within our work. As the results of the EDX, XPS and ToF-SIMS measurements show that the results for MCP and DCPD do not deviate from what one would expect in the context of the measurements on all other samples, we assume that there is no significant effect. Beyond those slight changes of MCP and DCPD discussed above none of the investigated CPPs changed its orientation or crystal structure because of the pressures applied to the samples during compacting. The diffraction patterns for the powders and pellets, as well as the powder patterns along with the respective ICDD stick patterns are shown in the electronic supplement S1.

As a further quality control ATR-IR spectra were collected with a Bruker Alpha spectrometer equipped with a platinum ATR module. All of the spectra are depicted in the electronic supplement S2. They show the characteristic pattern for the respective CPP. No impurities were detected.

Table 1
Overview of the analyzed calcium phosphate phases (CPP) in this work. O_{PO} is the number of phosphate-bound oxygen atoms, while O_{bulk} is the sum of all oxygen atoms. See Section 3.1.2 for further explanations.

Phase	Composition	Atomic Ca/P-ratio	O_{PO}/O_{bulk}-ratio
MCP	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.50	0.440
DCP	$CaHPO_4$	1.00	0.750
DCPD	$CaHPO_4{\cdot}2H_2O$	1.00	0.500
ACP	$Ca_2P_2O_7{\cdot}H_2O$	1.00	0.875
OCP	$Ca_8H_2(PO_4)_6{\cdot}H_2O$	1.33	0.760
α-TCP	$Ca_3(PO_4)_2$	1.50	1.000
β-TCP	$Ca_3(PO_4)_2$	1.50	1.000
HAP	$Ca_{10}(OH)_2(PO_4)_6$	1.67	0.923