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In situ time-resolved X-ray diffraction study of evolution of nanohydroxyapatite particles in physiological solution

J.V. Rau [a,*], A. Generosi [a], D. Ferro [b], F. Minozzi [c], B. Paci [a], V. Rossi Albertini [a], G. Dolci [c], S.M. Barinov [d]

[a] *Istituto di Struttura della Materia, CNR, Via del Fosso del Cavaliere, 100-00133 Rome, Italy*
[b] *Istituto per lo Studio dei Materiali Nanostrutturati, CNR, c/o Dipartimento di Chimica, "Sapienza" Università di Roma, Piazzale Aldo Moro, 5-00185 Rome, Italy*
[c] *"Sapienza" Universitá di Roma, Dipartimento di Scienze Odontostomatologiche, Viale Regina Elena, 287/a-00161 Rome, Italy*
[d] *Baikov Institute of Metallurgy and Materials Science, Russian Academy of Sciences, Leninskij prospect 49, Moscow 119991, Russia*

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ABSTRACT

Nanosized hydroxyapatite (nano-HA) is known to be of enhanced biological efficacy, being used in medical events as a mix with physiological solution, saline or patient's blood before the application. This study is aimed at the investigation of the time evolution of both phase composition and particle size of nano-HA in aqueous (isotonic 0.9% NaCl) solution. An energy-dispersive X-ray diffraction method, allowing the real time rapid data collection was employed. The X-ray amorphous component of initial powder was shown to convert fully into the crystalline hydroxyapatite (HA), the characteristic crystallization time being of approximately 25 min. The initial crystallite average size (approximately 35 nm) was enlarged by a factor of about 4 within the first 100 min after mixing the powder with the physiological solution and no more structural changes were detected during the following period. The sigmoidal kinetics of the HA crystal growth was evidenced.

1. Introduction

The human bone and teeth are complex biological nanocomposites consisting of a nanocrystalline apatite minerals (calcium phosphates: 60–70% in bone and 97% in dental enamel) embedded in the template of protein collagen fibrils three-dimensional matrix [1,2]. Bones and teeth have complex hierarchical structures over several length scale level [3–5]. The main bone mineral calcium phosphate is carbonate-substituted hydroxyapatite containing between 2.3 and 8 wt.% of carbonate [6]. Calcium phosphate based biomaterials have been in use in medicine and dentistry for more that two decades [7,8]. Belonging to the group of calcium phosphates, the hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, has a chemical composition similar to the natural bone mineral and, being stable, biocompatible, biodegradable and osteoconductive [9–11], it is widely applied as bone graft material. Through osteoconductive mechanism HA forms chemical bonds with living bond tissue [12].

The biologically oriented research on materials to promote the bone growth is in continuous evolution. In order to produce an excellent bone graft material it is necessary to follow compositional and structural properties of a natural bone, whose mineral part is characterized by low crystallinity, preferred crystallographic orientation and nanometer size scale [3–5]. It is also known that with age, a slow transformation of a poorly crystallized biological apatite into a better crystallized HA takes place in bone and dentin [2]. The studies in the biotechnology research area are aimed at the design of artificial nanostructures that can interact with and replace natural bone material. In bone and teeth biocomposites, the carbonated apatite crystals are among the smallest biologically produced crystals known (with average size in bone about $50\times25\times2$–4 nm and in tooth enamel about $100\times50\times50$ nm) [1,13]. Nanocrystalline HA particles are similar to natural bone apatite in composition, structure and size [14]. Compared to micron-size particles, nano-HA possess improved mechanical properties and superior bioactivity for promoting bone growth and regeneration [15]. Indeed, coatings, ceramics, polymers, scaffolds and composites of hydroxyapatite nanoparticles stimulating the osteoblast activity are promising materials in various tissue engineering applications for bone growth [9,10,16–19].

The X-ray diffraction has always been the main technique to study the phase development in powder samples. However, the application of the conventional laboratory angular dispersive X-ray diffraction method (ADXD) to monitor rapid phase transformations has an important limitation due to the long acquisition time required. To resolve this kind of problems, an alternative method, the energy dispersive X-ray diffraction (EDXD) has been developed [20]. The EDXD technique applied in this work allows the real time rapid collection of diffraction patterns *in situ* at selected time intervals, being therefore a powerful tool to characterize the biomaterials structural transformations.

The present study was aimed at the application of the EDXD technique to investigate *in situ* the phase development and the

* Corresponding author. Tel.: +39 06 4991 3641; fax: +39 06 4991 3951.
E-mail address: giulietta.rau@uniroma1.it (J.V. Rau).

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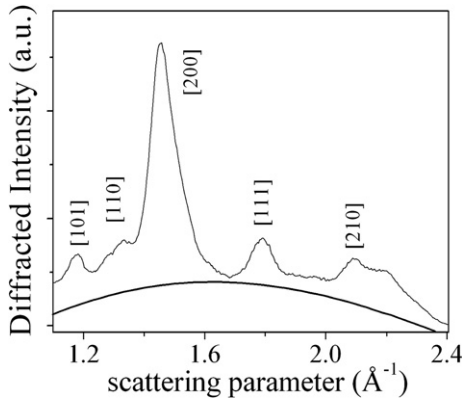

Fig. 1. EDX diffraction pattern collected as a function of the scattering parameter q from the initial nano-HA powder. The most intense Bragg peaks are labelled. The bump below the crystalline signals is associated to a disordered portion of the crystalline powder.

particle size evolution taking place in the nano-HA powder, when mixed with the physiological solution.

2. Experimental methods and materials

The nano-HA powder was supplied by Ghimas S.p.a. Company (Bologna, Italy). After mixing of nano-HA powder with a physiological solution (aqueous isotonic 0.9% NaCl solution for injections) (0.2 g: 0.1 ml, respectively), it turns into a cream-like paste that sets to a firm mass at physiological temperature.

Scanning Electron Microscopy technique (SEM) (a LEO 1450 VP apparatus) with a tungsten emitter and resolution of 4 nm was used to observe in secondary electron mode the morphology of the nano-HA powder and the prepared cream-like mixture of the nano-HA and physiological solution. The SEM apparatus is coupled with a system for microanalysis (Energy Dispersive X-ray Spectroscopy—EDXS) INCA 300 for qualitative/quantitative analysis of the elements. The samples were sputter coated with gold.

An X-ray diffraction pattern represents the intensity of the X-ray radiation elastically scattered by a sample as a function of the momentum transfer Δq. The momentum transfer amplitude takes the name of scattering parameter: $q\ (E, \vartheta) = a\ E \sin \vartheta$, E being the energy of the electromagnetic radiation, 2ϑ the scattering angle and $a =$ constant $= 1.014$ Å^{-1}/keV. To perform the reciprocal space scan, namely to collect a diffraction pattern, two modes are thus available. The first, most conventional, consists of using a monochromatic X-ray radiation (selecting a single energy component), carrying out an angular scan by the mechanical movement of the arms of the diffractometer (Angular Dispersive mode). Alternatively, an X-ray white beam can be used, fixing the scattering angle 2ϑ (Energy Dispersive mode) and executing the reciprocal space scan electronically. The diffraction pattern is collected through the measurement of the energy spectrum of such polychromatic beam after it is scattered by the sample. In this case, a Solid State Detector (SSD) is utilized. The SSD is able to detect not only the number of photons diffracted by the sample, but also the energy of each of them, too. The advantage of EDXD is that no movement is needed during the data collection, which makes the procedure of analysis much more reliable than that for the classical ADXD. Indeed, since the diffraction geometry (i.e. irradiated volume and surface) remains unchanged during the measurement, no problems coming from renormalization or from irradiation of different parts of the sample at different angles occur. Such problems are particularly serious when inhomogeneous samples are studied and/or when time resolved measurements, which require the collections of many diffraction patterns, are carried out. Furthermore, since a parallel collection of the experimental points at the various q-values takes place in the ED mode, the acquisition time is much reduced. EDXD makes use of the instruments similar to those used in the ordinary X-ray fluorescence spectroscopy, the difference being that the spectrum provides structural information rather than spectroscopic ones. The ED diffractometer is a non commercial apparatus [20], very simple from the mechanical point of view. It consists of two arms pivoting around the optical centre of the instrument, where the sample holder is located. A white X-ray radiation is produced by a commercial W-anode X-ray tube (15–55 keV) and is collimated upstream and downhill the sample by four W slits. The detection is accomplished by an EG&G high purity germanium SSD. The detector is connected to a PC via ADCAM hardware and the signal is processed by a Maestro software, which performs the necessary analogue to digital conversions. Neither monochromator nor goniometer are required in the ED mode.

3. Results and discussion

In-situ time resolved EDXD measurements were performed to characterize the nano-HA powder and, subsequently, to monitor the structural evolution after mixing it with the physiological solution. First, the optimal experimental conditions were chosen in order to explore a q-range containing the most intense first order crystalline reflections attributable to the precursor material. The nano-HA initial powder was characterized collecting a diffraction pattern in the following conditions: scattering angle $2\vartheta = 7.0°$, X-ray beam energy $E = 50$ keV. Indeed, the nano-HA powder proved to be polycrystalline, the diffracted signals (labelled in Fig. 1) corresponding to a hexagonal lattice, the space group being $P6_3/m$. However, the initial powder exhibited also a consistent amorphous contribution, which is clearly visible as a bump deforming the EDXD pattern base line.

After this preliminary study, the nano-HA powder was mixed with the physiological solution onto an appositely designed sample holder positioned in the optical centre of the diffractometer. The experimental conditions were kept as previously described, and the phase evolution was monitored in real time collecting diffraction patterns, in reflection mode, every 5 min during the first hour. Subsequently, the patterns were acquired every 20 min for another 48 h. The obtained sequence of diffraction patterns is shown in Fig. 2 as a function of the scattering parameter q and of time. The arrow connecting the maxima

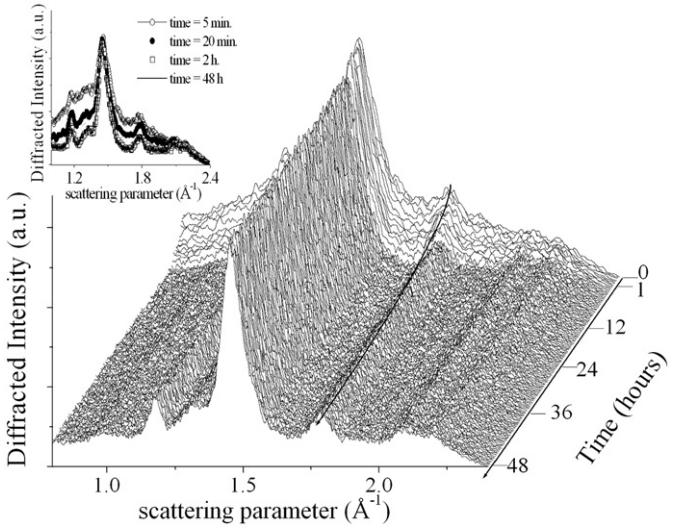

Fig. 2. Sequence of EDX diffraction patterns collected as a function of the scattering parameter q and of time during the powder/solution interaction. The arrow connecting the [111] reflection evidences the loss of the overall diffracted intensity. In the inset this effect is evidenced by the comparison of a few patterns collected at 5 min, 20 min, 2 h and 48 h after mixing of the initial nano-HA powder with the physiological solution.