Application of physical methods in the investigations of crystal-related arthropathies

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Introduction

The application of physical methods to understanding the role of crystals in pathogenesis of various arthropathies can help in two ways. Firstly, in exploration of the nature of crystalline deposits—for example, identification of crystals—and, secondly, in understanding the very processes of crystal growth (and dissolution).

The assessment of the crystals mainly comprises:

(1) Determination of chemical composition and defects such as non-stoichiometry and foreign phase precipitation.

(2) Identification of crystal structure and determination of crystallographic parameters.

(3) Visualisation of structural imperfections.

(4) Examination of morphology for determination of conditions of crystal growth. (Finer structural details on a crystalline surface are often associated with the growth history of the crystal.)

Physical methods available for crystal characterisation can be broadly divided into two categories.

(1) **Methods based on the interaction of crystals with electromagnetic radiation**—for example, visible light, infrared radiation, x-rays. These are: (a) polarisation light microscopy; (b) infrared absorption photometry; (c) x-ray diffraction methods, including topographic method; and (d) x-ray extended absorption free structure (EXAFS).

(2) **Methods based on material-electron interaction:**

(a) transmission electron microscopy (TEM); (b) scanning transmission electron microscopy (STEM); (c) analytical electron microscopy—energy dispersive x-ray analysis (EDX) and wavelength dispersive analysis (WDX); and (d) electron diffraction.

Detailed description of each of the methods is beyond the scope of this paper. Here I intend to deal briefly with the subjects of infrared absorption spectrometry, x-ray diffraction, EXAFS, and electron microscopies, and show how they have been applied to investigate arthropathic crystallisation. Where appropriate, examples of other biological and non-biological crystals will be cited.

Methods

INFRARED SPECTROMETRY

Vibrations of molecular bonds in a material cause characteristic absorption of the infrared region of the electromagnetic spectrum. The absorption for each bond occurs at specific frequencies, which may reveal the nature of vibrations and stereochemistry of the molecular groups in question. The characteristic peaks may be used like fingerprints to determine the presence of different molecular groups in a substance.

Absorption spectra of apatite and calcium pyrophosphate crystals are different. They may be used to detect the presence or otherwise of the compounds in arthropathic deposits.

In the case of hydroxyapatite infrared spectra may yield additional information on crystallite size. Fig. 1 shows absorption spectra of apatite from calcific periarthritis. The apatite is finely divided. Its crystallite size from line broadening measurements in x-ray powder diffraction line was about 50 nm.¹ Fig. 1a shows that the peaks at 3572 wavelengths/cm are missing in the range of the human deposit but are present in that of synthetic crystals of about 1 μ m.³ The absence of the above peaks in the spectrum of the finely divided apatite is due to the fact that the stretching and bending modes of OH bonds are

Fig. 1 Infrared spectra of (a) finely divided apatite from periarthritic deposits and (b) Durango hydroxyapatite. Note that peaks at 3572 cm and 620 cm are absent in spectrum of human deposits.

perturbed by the H bonding of water to surface OH ions of the apatite.²

Infrared absorption spectrometry has also been used to show the presence of CO₃²⁻ ions in human deposits but I shall refer to this in conjunction with the x-ray diffraction results.

X-RAY DIFFRACTION

Whenever an x-ray beam is incident on a crystal it is scattered by the atoms in the crystal. The atoms in the crystals are arranged in a regular manner with a definite periodicity, allowing identification of a series of rows of planes with a constant interplanar spacing. Due to this geometry of the atoms, scattered x-rays, when superimposed on each other, are

annulled except in certain directions where there is a constructive reinforcement. This phenomena is called diffraction. The direction of a diffracted beam can be found by Bragg's law:

$$n\lambda = 2d \sin \theta,$$

where n = order of diffraction (n = integer) λ = wavelength of x-rays d = interplanar spacing (characteristic of the crystal lattice) and θ = angle of diffraction.

Experimentally, one can use x-rays of known wavelength λ , measure angle θ , and hence determine the interplanar spacing d . Because biological crystals are very small one uses the powder diffraction method. In practice, powdered specimen is filled in a thin tube or coated on the surface of a very thin glass rod (about 1 mm in diameter) and mounted in a Debye-Scherrer camera. A film is then placed in the camera. This surrounds the specimen to record diffracted beams as arcs. Such a set of lines is unique to the crystalline substance in question and may therefore be used as the 'fingerprint' of the substance. From interplanar spacings one can evaluate lattice constants of the crystal structure with a great accuracy.

Both hydroxyapatite and calcium pyrophosphate have an immensely complex growth system.¹⁻⁴ Many different phosphate salts can grow from the same reaction system. Powder diffraction methods, therefore, are immensely useful in determining crystal growth conditions in *in vitro* systems. Fig. 2 shows diffractographs of (pyrophosphate) crystals grown from solution and gel and that of a deposit from articular cartilage. From these patterns it was deduced that other compounds such as calcium pyrophosphate tetrahydrate (CPPT) can grow in *in vitro* systems. Pathological deposits have been shown to contain only the mixture of monoclinic and triclinic phases of calcium pyrophosphate dihydrate (CPPD). Because of the fingerprint nature of diffraction lines it is possible to detect as little as 0.1% weight of the calcium phosphate in another.

Careful determination of lattice parameters allows detection of foreign atoms (impurity) incorporated into the host lattice. Several ions such as F^- , Cl^- , CO_3^{2-} may be accommodated substitutionally, in hydroxyapatite

Fig. 2 X-ray powder diffractographs of pyrophosphates: (a) grown from solution (b) grown in gelatin gel, and (c) found on cartilage surface.

Table 1 Lattice parameters of apatite from particular deposits from three patients

Sources of apatite	Lattice parameters (Å)	
	'a'	'c'
Case 1	9.37	6.88
Case 2	9.38	6.87
Calcey (at one week)	9.38	6.88
Durango hydroxyapatite	9.42	6.88
Fluorapatite	9.37	6.88
Carbon apatite (7% weight CO ₃)	9.38	6.89

Accuracy of lattice parameters is $\pm 0.01\%$.

lattice. Such substitution normally alters the lattice parameters of a crystal by small amounts. Table 1 lists the lattice parameters of the apatite from particular deposits of three patients, simple calcey at one week¹ (Harries *et al.*, p. 102) Durango, fluorapatite and carbon apatite. The 'a' lattice parameter of the human and calcey deposits is consistently smaller than that of hydroxyapatite, and similar to those of both carbon apatite and fluorapatite. F⁻¹ and/or CO₃²⁻ ions could therefore be present in the above crystals, but no fluorine was found by modified Diggins and Rosse method (1981). Infrared spectra (Fig. 3) of all the deposits show peaks at 1460 cm, 1415 cm, and 869 cm. These bands appear in the spectra of synthetic carbon apatites.^{5,6} It is therefore apparent that the in vivo deposits contain carbon apatite. The amount of CO₃²⁻ content can in fact be estimated from the lattice parameter measurements. LeGeros has shown that 'a' parameter of the hydroxyapatite cell decreases linearly with increasing CO₃²⁻ concentration in the range 0–24% weight.⁷ On this basis human deposits we examined

contain CO₃²⁻ in the range 8–9% weight. This finding raises question regarding the source of CO₃²⁻ incorporation in in vivo mechanisms of arthropathic apatite growth.

The mode of carbonate substitution into biological apatite remains controversial.⁵ The controversy centres around the site of CO₃²⁻ substitution in the hydroxyapatite lattice. From electron spin resonance and the infrared studies of ¹³C-enriched apatites Doi *et al.* concluded that CO₃²⁻ ions are substituted at PO₄³⁻ sites.⁶ LeGeros also reached this conclusion because carbonate substitution causes shrinkage in 'a' parameter of the CO₃²⁻ group.⁷ Matalon and DeBenyacer, however, claim that carbonate content of calcified deposits from propositus of an idiopathic chondrocalcinosis show changes in carbonate content on heating.⁸ This change was variable from specimen to specimen. From this it was surmised that in the above deposits CO₃²⁻ replaces both PO₄³⁻ and OH⁻ groups but that the ratio replacing PO₄³⁻ and OH group does not remain constant. The exact site of CO₃²⁻ substitution in arthropathic

deposits cannot be easily determined by the existing data on infrared absorption and lattice parameter.

APPLICATION OF SYNCHROTRON X RADIATION

Intensities of diffracted beams are very small. In a laboratory, therefore, with normal x-ray tube sources it requires about 48 hours to obtain a powder diffraction pattern of a well-crystallised specimen—which contains a few tens of milligrams. It is therefore often impossible to obtain a diffraction pattern of minute deposits in situ—say on a cartilage surface. Recently, however, it has become possible to use synchrotron radiation source, which provides 'tunable' x-rays of very high intensity, the diffraction (Fig. 4) pattern of crystalline deposits on cartilage surface. The pattern covers the diffraction angle in the range $\theta = 1^\circ$ – 12° . This opens up exciting prospects for studying in situ arthropathic deposits. It is concluded (Fig. 4) that crystallites on cartilage show no preferred orientation as they do in bone and enamel.

EXAFS

This is really a form of x-ray absorption spectroscopy. An absorption spectrum of a substance is obtained by shining an intense x-ray beam—for example, that from synchrotron source—on it. The spectrum arises because x-ray absorption cross section for the excitation of an electron from a deep core of the atom exhibits oscillation as a function of x-ray (photon) energy.

Fig. 3 Infrared spectra of a typical periarticular deposit. Peaks at 1460 cm, 1415 cm, and 869 cm are additional to those in the pure hydroxyapatite spectrum.

Fig. 4 X-ray diffraction pattern of in situ deposits on articular cartilage surface taken with synchrotron radiation source.

Fig. 5 A schematic diagram of an EXAFS spectrum.

This manifests itself as a fine structure in the x-ray absorption spectrum just above the absorption edge (Fig. 5). In contrast to x-ray diffraction, EXAFS studies are restricted to the immediate vicinity of the absorber element. They therefore yield information on the local structure and chemical states of bonding sites. On interatomic distances co-ordinate number and geometry of an atom within a complex organic matrix can therefore be studied.

So far EXAFS has not been used to obtain information on arthropathic deposits. Miller *et al.* and Binstead *et al.* have applied EXAFS to bone mineral^{9,10} and have concluded that the calcium 'environment' in bone mineral is not the same as synthetic and geological hydroxyapatite. Instead the bone mineral is shown to have a structure that is intermediate between amorphous calcium phosphate and crystalline hydroxyapatite with high defect density. EXAFS has also shown that in development of bone the calcium environment changes in a similar manner to that of in vitro maturation of amorphous calcium phosphate to poorly crystallised hydroxyapatite.

METHODS BASED ON ELECTRON MICROSCOPY

When a focused—high energy—electron probe makes an impact with a material it generates a variety of different signals.

Low energy secondary electrons (energy <50 eV) generated from near the surface of a material gives information on topography and is used in scanning electron microscopy (SEM). Elastically transmitted electrons are capable of diffraction and give information on structure. The

Fig. 6 A sequence of events in the growth of CuAlS_2 crystals grown by chemical vapour deposition techniques.

collision of electrons within the material gives rise to the emission of characteristic x-ray radiation which are used in EDX and WDX to give information on chemical composition. With the aid of modern microscopes very high resolution (2Å in TEM and 50Å in SEM) images can be obtained. The values of these techniques hardly needs to be emphasised. The EDX technique allows construction composition maps with a resolution of 1 μm^2 and has shown that the calcified lesions in simple calcergy have layers and that the Ca:P ratio in each layer is different.¹

So far high resolution microscopy has thrown little light on growth mechanisms of crystals in human arthropathic deposits.

SEM, however, has been used to evaluate a sequence of events (Fig. 6) in the growth of tiny crystals of CuAlS_2 grown by chemical vapour deposition technique.¹¹

In conclusion, I should like to emphasise that an integrated programme of investigation combining the above methods is likely to contribute in the understanding of the crystal deposition processes in arthropathies.

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