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# Amorphous calcium carbonate biomineralization in the earthworm's calciferous gland: Pathways to the formation of crystalline phases

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ABSTRACT

In this study, we investigated the microstructural transformations that take place during carbonate formation in the earthworm's calciferous gland by analysing the evolution from the precursor fluid of the solid phases (spherulites) to the final carbonate concretions released by the gland. Results from HREM and electron diffraction showed that the spherulithic deposits merely consisted of ACC partially transformed to vaterite. Furthermore, comparisons of the diffraction spectra and microstructural analyses allowed the identification of the transition sequences to more stable carbonates. And thus, transformations of ACC to calcite were observed on the surfaces of these amorphous globular aggregates as their smooth characteristic surface became rougher with time. This transition path was not unique, and the presence of aragonite, as an intermediate phase, has also been found. In this particular case, the transition process followed a completely different pathway with the crystallization starting in the centre of the sphere and progressively extending to the periphery, leading to the formation of radial aggregates. *In situ* experiments performed on the freshly extracted precursor fluid and analysed by FT-IR spectroscopy showed that ACC is the main constituent and is probably stabilised by macromolecules such as proteins and sugars. Furthermore, the Debye–Scherrer diffraction experiments showed that the carbonate phase present in this fluid remains stable as ACC for more than a week. All these features are indicative of this entire process being biologically controlled by the earthworms. The analysis of the amorphous structure factor of this ACC indicates that these transformations are preceded by short-range order modifications of the amorphous precursor phase.

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## 1. Introduction

Calcium carbonate formation in the earthworms' calciferous glands is a remarkable case of biomineralization. Furthermore, although the particular mechanism involved in the production of calcium carbonate by earthworms (Annelida, Oligochaeta) remains unknown, the catalytic role of carbonic anhydrase has been anticipated (Clark, 1957). The biological functionality of these glands in the family Lumbricidae is also intriguing. From the early work by Darwin (1881), several hypotheses have been proposed, namely pH buffering of the blood and the ingested plant material, respiratory functions, egg formation or simply spurious mineralization (for example, see reviews by Robertson (1936) and Piearce (1972). More recently, studies have mainly focused on the morphological characterisation of the solid calcareous concretions (see also Canti and Piearce, 2003). However, little is known about the crystallization process itself and, in particular, in relation to those aspects concerning the nucleation mechanism and the sequence of the polymorphic carbonate phases.

The formation of the stable crystalline polymorphs of calcium carbonate, aragonite and calcite is a common process in biological systems, but the less stable crystalline phases, such as vaterite and hydrocalcite (Gauldie et al., 1997; Estroff et al., 2003), can also be present in some extent. More recently, biomineralization studies (Raz et al., 2002, 2003; Weiss et al., 2002; Addadi et al., 2003; Politi et al., 2004) have shown that several organisms from different phyla produce amorphous calcium carbonate (ACC). Despite the fact of this phase being thermodynamically unstable and occurring relatively infrequently in inorganic systems, it seems to play a crucial role in biological mineralization by acting as a precursor phase of more stable polymorphs. Its high solubility also provides a source of calcium carbonate, which can be promptly stabilised or disestablished when necessary and according to the metabolic and functional requirements of the organisms. As a general rule, ACC is only stable during the lifetime of the animal, and its

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characterisation is difficult because it usually requires performing *in situ* experiments. Its amorphous nature also makes the structural characterisation difficult, and the application of more complex analytical techniques are necessary to provide enough information on the short-range order. Recent studies reporting variations in the pair distribution function of ACC (Becker et al., 2003; Levi-Kalisman et al., 2000; Lam et al., 2007) suggest that this phase can rapidly modify its local order through changes in the number of atoms surrounding the calcium ion, and consequently, can play an important role in the formation of a particular crystalline phase.

In this study, we investigated the microstructural transformations that take place during the carbonate formation inside the calciferous gland of the earthworm species *Lumbricus friendi* Cognetti. First, we identified the presence of ACC by FT-IR, and then we carefully followed the different evolution stages of calcium carbonate in the precursor fluid prior to the formation of the crystalline phases by performing *in situ* XRD experiments. This was achieved by using a goniometer with Debye–Scherrer geometry and quantifying the changes in the amorphous structure factor with time, as an indication of the modifications occurring in the local ordering. Finally, we analysed the solid concretions from both morphological and microstructural points of view by means of FE-SEM and HR-TEM.

To our knowledge, these are the first direct structural investigations of the different phases involved in the biomineralization process occurring in the earthworm's calciferous gland at the atomic level. This information is of great relevance, in particular in the early stages where the recognition of different phases based on purely morphological considerations is very complicated. This is due to the fact that a great number of carbonate polymorphs happen to grow in such a way under determined conditions so that identical forms are produced.

## 2. Materials and methods

### 2.1. Collection of the 'milky fluid' and solid concretions

Earthworm specimens obtained from our laboratory cultures were dissected in deionised water to obtain both the 'milky fluid' (precursor fluid) (MF) and the mineral concretions secreted by the glands. The precursor fluid was collected using a plastic syringe and placed in sealed vials to perform X-ray diffraction (XRD) and Fourier Transform-Infrared spectroscopy (FT-IR) determinations. Two different fluid samples were obtained on the basis of their distinct density and their location: (i) low density milky fluid (LDMF) collected from the oesophageal pouches and (ii) high density milky fluid (HMDF) from the oesophagus lumen. In addition, the solid concretions (SC) stored in the oesophageal pouches were also carefully picked up using tweezers. Both liquid and solid samples were analysed separately using different technical procedures.

### 2.2. In situ analyses of the MF by FT-IR and XRD

The aggregation state of the calcium carbonate present in the two types of MF samples was characterised by means of two different techniques: X-ray scattering and FT-IR. In the case of XRD, the absence of Bragg reflections in the scattering patterns was considered to be diagnostic of an amorphous state of the samples (Klug and Alexander, 1954; Cullity, 1976). In this case, the diffraction profile is characterised by only the presence of a diffuse scattering arising from the short-range ordering in the sample.

FT-IR was performed on a Nicolet 6700 with a resolution of 0.5 $cm^{-1}$. Prior to FT-IR analyses, samples were pressed into KBr pellets. The FT-IR vibration spectra were used as a precise fingerprint of the amorphous aggregation state of the samples based on the specific variations in the vibration spectra of the ACC compared to those of the crystalline carbonate phases (Beniash et al., 1997; Politi et al., 2004). The presence of a broad band at 1084 $cm^{-1}$ corresponding to the $\nu_1$ mode of the carbonate oxy-anion is of particular importance since this vibration mode is connected to the orientation disorder of the $CO_3^=$ and is not FT-IR active in the crystalline phases of calcium carbonate. The simultaneous presence of the $\nu_1$ (1084 $cm^{-1}$), $\nu_2$ (866 $cm^{-1}$) and $\nu_3$ (1420–1470 $cm^{-1}$) bands together with the absence of the $\nu_4$ mode (714 $cm^{-1}$) are, therefore, the diagnostic features employed in the identification of ACC. FT-IR analyses were also applied to identify the presence of organic macromolecules in both the MF and SC concretions by analysing the decarbonated samples. In this case, the presence of the 1655 $cm^{-1}$ band characteristic of amide groups was attributed to proteins, whereas the bands at 1100, 1560 and 1630 $cm^{-1}$ are indicative of the presence of sugars and carboxyl groups.

Furthermore, in order to investigate the stability of the amorphous aggregation state of the MF we performed time-elapsed XRD experiments. Samples of freshly extracted MF were injected into Lindemann capillaries, hermetically sealed and immediately placed in the goniometer. A Debye–Scherrer diffractometer equipped with a Coupled Charge Device (CCD) detector (Bruker SMART-CCD 1000) and Mo K$\alpha$ radiation ($\lambda$ = 0.071073 nm) was employed to obtain the diffraction patterns. Experiments were carried out at $2\theta = 7^\circ$–$90^\circ$ to give a maximum value of the momentum transfer $Q_{max}$ of 1.2 $nm^{-1}$. Readings were taken at the start of the experiment and after 1 h intervals during the first day (24 h) and then daily for a week, with 10 min counting time for each individual spectrum. Raw data from the CCD detector were converted to lineal spectra by radial averaging of the Debye–Scherrer rings derived from the CCD frames. The GADDS software package from Socabim was used for this purpose. Additionally, the spectrum of the empty sample container (i.e. the Lindemann capillary) was obtained separately under the same conditions and its contribution to the scattering (corrected for absorption) was subtracted from the sample.

An application of the Rietveld general procedure, specifically developed for microstructural analysis of the amorphous and disordered phases, was used to model the profile broadening. This method allows for the determination of the maximum correlation length of the scattering domains by approximating the amorphous phase to a nano-crystalline solid (i.e. when the long-range order is lost). Modelling the amorphous structure factor was achieved under the assumption that its structure would be approximated from that of its crystalline polymorphs (when available). Initially, fits were obtained using vaterite, aragonite and calcite as structural models. The best result, in the least square sense, was obtained by refining calcite in the *R*-3*m* super-group instead of the usual *R*-3*c* and thus, reducing the *c*-axis length by half. With this procedure, the $CO_3^=$ groups were allowed to rotate freely and to become equivalent. However, although this model is useful in characterising the maximum length of the coherent diffracting domains, it is limited when trying to describe the local atomic ordering of the amorphous structure. This is the consequence of the fitting model being constrained to crystallographic structural parameters (i.e. only crystallographic positions are permitted). For this reason, the modelling of the amorphous stages is only based on the coherence losses, which are attributable to microstructural effects (microstrain and very small 'crystalline' sizes). Therefore, the Reverse Monte Carlo Method (RMC) was employed to characterise the diffuse scattering of both the completely amorphous samples and the partially crystallized ones. In RMC, the atomic coordinates for a certain set of ions are non-periodically arranged in a simulation box and varied randomly. The optimal configuration is achieved when the total calculated powder structure factor $S(Q)$

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