Micron 60 (2014) 39–48

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Characterization of calcium carbonate crystals in pigeon yolk sacs with different incubation times

Juan Song[a,b], Haixia Cheng[a], Xinyu Shen[a], Jingxiao Hu[a], Hua Tong[a,*]

[a] Key Laboratory of Analytical Chemistry for Biology and Medicine (Wuhan University), Ministry of Education, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, People's Republic of China
[b] Analytical and Testing Center, Hubei University of Technology, Wuhan 430068, People's Republic of China

ARTICLE INFO

Article history:
Received 29 May 2013
Received in revised form 8 January 2014
Accepted 14 January 2014
Available online 30 January 2014

Keywords:
Biomineralization
Pigeon
Yolk sac
Calcium carbonate
Vaterite

ABSTRACT

Calcium carbonate crystals are known to form in the yolk sacs of fertile pigeon eggs at late stages of incubation. The composition and structure of these crystals were investigated, the crystallization environment was inspected, and the physical chemistry constants of the yolk fluid were determined through the incubation period. Polarized light microscopy was used to observe the generation and distribution of calcium carbonate crystals in the yolk sac. In addition, X-ray diffraction was employed to analyze the composition and crystal phase of the yolk sac. A decalcification and deproteination method was established to analyze the ultrastructure and composition of the crystals, as well as the internal relationship between inorganic and organic phases of the crystals. Additionally, scanning electron microscopy, transmission electron microscopy, X-ray energy dispersive spectroscopy, and Fourier transform infrared spectroscopy were used to evaluate the characteristics of the crystals. Our results demonstrated that the calcium carbonate crystals were mainly composed of vaterite and calcite, with vaterite being the major component. Vaterite, a type of biomaterial generated by an organic template control, presented as a concentric hierarchical spherical structure. The organic nature of the biomaterial prevented vaterite from transforming into calcite, which is more thermodynamically stable than vaterite. Additionally, the configuration, size, and aggregation of vaterite were also mediated by the organic template. This bio-vaterite was found during the incubation period and is valuable in calcium transport during embryonic development.

1. Introduction

The study of biomineralization encompasses the science of the formation, structures, and properties of inorganic solids deposited in biological systems. This process involves the selective extraction and uptake of elements from the local environment and their incorporation into functional structures under strict biological control (Mann, 2001). A wide range of structures, minerals, and macromolecules make up these biomineralized tissues (Lowenstam and Weiner, 1989; Arias and Fernández, 2007; Behrens et al., 2007). It is well known that crystals nucleate and grow from saturated solutions. And so they do in vitro, but not necessarily in vivo. Biology has chosen another pathway; crystals are grown from an unstable solid colloidal phase, almost devoid of water (Weiner, 2008). The identification of this unexpected strategy has its origins in a much overlooked paper by Towe and Lowenstam (1967) showed for the first time that in the mineralized teeth of the chiton, a segmented mollusk, the initially formed mineral phase is not the same as the mature form, but transforms into the more stable mature phase. Biomineralization is a widespread phenomenon among living systems, e.g. egg and mollusk and crustacean shells (Arias and Fernández, 2003, crustacean carapaces (Luquet and Marin, 2004), echinoderm exoskeleton and spines (Killian and Wilt, 2008), sponge spicules (Sethmann and Wörheide, 2008), pearls (Soldati et al., 2008), bones and teeth.

The egg yolk calcium carbonate crystal is a type of biomaterial discovered several years ago in the yolk sacs of precocial birds during incubation (Cheville and Coignoul, 1984; Juurlink and Gibson, 1973). As one of the most common biological minerals, calcium carbonate ($CaCO_3$) has been found to show six different phase: calcite, aragonite, vaterite, calcium carbonate monohydrate, calcium carbonate hexahydrate, and amorphous form (Meldrum and Cölfen, 2008). In organisms, these crystals have a wide range of naturally occurring crystal habits and are normally found assembled into hierarchical structures which result in a difference of intriguing properties (Yu et al., 2004). Many researchers have focused on the mechanism of calcium transport within the yolk sac (Corradino, 1973; Rubin et al., 2006; Shepherd et al., 2007). Measurement of calcium in the yolks of pigeon eggs incubated from day 0 to day 17 suggested that the mobilization of calcium from shell to embryo

* Corresponding author. Tel.: +86 27 68752136; fax: +86 27 68752136.
E-mail address: huatong09@126.com (H. Tong).

0968-4328/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.micron.2014.01.003

commenced at approximately day 11 of the incubation period, accumulating both in the embryo and yolk sac. While the general patterns of calcium accumulation during embryogenesis in altricial birds closely resemble those in precocial birds, calcium mobilization from the shell begins later, proceeds at a slower rate, and results in a less mineralized hatchling than the calcium patterns observed in precocial birds (Hart et al., 1992). However, the compound state of calcium and the structural relationship between the organic and inorganic matrices of yolk calcium carbonate crystals are not clear.

The purpose of our study was to determine the characteristics of calcium carbonate crystals forming in the pigeon yolk sac and the organic/inorganic structures of these crystals. To do this, we examined bio-vaterite appearing in the yolk sac endoderm and yolk fluid throughout the incubation period. We also examined the complex microstructures and environmental conditions of the yolk sac and found that these characteristics may result in the enhanced calcium transport induced by bio-vaterite.

2. Materials and methods

2.1. Materials and instruments

The pigeon eggs used in our experiments were obtained from the pigeon farm of Central China Agriculture University. They were incubated in batches in a tabletop incubator (with automatic turning) maintained at $38.2 \pm 0.1\,^{\circ}C$ with relative air humidity of $60 \pm 5\%$. Eggs were candled after 5 days; dead and infertile eggs were discarded. Polarized light microscopy (PLM; Sunny XP, China), X-ray diffraction (XRD; X'PertPowder, Panalytical, the Netherlands), pH meter (Sartorius PB-10, Germany), conductivity meter (ZhiGuang DDS-307, China), scanning electron microscopy (SEM; QUANTA FEG450, FEI, the Netherlands), transmission electron microscopy (TEM; JEM-2100, JOEL, Japan), X-ray energy dispersive analysis (EDX; Oxford, UK), and Fourier transform infrared spectroscopy (FT-IR; Nicolet 5700, Thermo, USA) were employed in this study.

2.2. Experimental process

2.2.1. Biomineral collection from yolk sac and PLM analysis

Eggshells were carefully broken, the yolk sac separated from the egg white and embryo. The needle and syringe were used to extract yolk fluid from yolk sac. The yolk fluid and yolk sac endoderm of fertilized eggs incubated for 0, 8, 13, or 18 days were subjected to PLM analysis. In order to maintain the native sample in the in vivo state, our methods were free from any biochemical and histochemical processes. All the samples were prepared on glass slides for direct observation. Then the sample was put under PLM for observation. The PLM image was taken by a digital camera.

2.2.2. pH and conductivity of bio-vaterite in a crystalline environment

The fertilized eggs were incubated for 0, 8, 13, or 18 days before analysis. The yolk fluid was collected on each of the above-mentioned days and was diluted in double distilled water. A pH meter was employed to determine the acidity of each sample, and a conductivity meter was used to measure the conductivity of each sample.

2.2.3. Enrichment

After 10, 13, and 16 days of incubation, the yolk fluid was extracted from the yolk sac and allowed to flow into a beaker. The yolk fluid was then mixed with an appropriate amount of distilled water and homogenized at 2500 rpm for 20 min. The homogenized solution was left undisturbed for 1 h, and sand-like particles at the bottom of the beaker were isolated. The precipitate was washed repeatedly until the solution was clear and free from suspended solids, and was then dried in a vacuum sealed stand. This precipitate was confirmed to be bio-vaterite by microscopic examination.

2.2.4. Purification

The surface of the isolated bio-vaterite appeared to be sticky, when observed through SEM (Fig. 1). Washing the samples in ethanol removed most of the surface impurities to reveal particles with significantly increased surface definition and porosity (Fig. 2).

2.2.5. Microscopic fracture

A small amount of bio-vaterite was placed on a slide and then covered with another slide. Under light microscope, the 2 slides were carefully pressed with appropriate force to ensure that most of the calcium carbonate crystals broke without affecting analysis. After being broken, the internal morphology and structure of the bio-vaterite were exposed and investigated by SEM, TEM, and X-ray energy dispersive spectroscopy (EDS).

2.2.6. Decalcification

The bio-vaterite was mixed with either a 5% HCl solution or 4 mol/L EDTA solution and decalcified for 5 min. After centrifugation, the residue was washed thrice with distilled water and dried in vacuum for subsequent analysis. Samples incubated with 5% HCl were fully decalcified, and those incubated with EDTA were partially decalcified.

2.2.7. Deproteinization

The sample was deproteinized by exposure to 5% NaClO solution. Samples were dried in a vacuum for subsequent analysis.

2.2.8. Structure and composition analysis

SEM, EDX, TEM, XRD, and FT-IR were used to determine the structure and composition of the bio-vaterite.

2.2.8.1. SEM morphology observation and EDS elemental analysis. Samples were sputtered with a 20-nm gold film for morphology analysis by SEM using an ion sputtering apparatus. Micro-element identification was then carried out by EDS.

2.2.8.2. TEM ultrastructure analysis. After fishing with a copper screen, the purified native samples, decalcified samples, and deproteinized samples were placed on copper microgrids covered with a thin carbon film and were examined by TEM at an accelerating voltage of 200 kV.

2.2.8.3. FT-IR organic functional group analysis. Organic functional groups of the native sample, as well as those of the decalcified or deproteinized samples were mixed with KBr and pressed into pellet, the samples were analyzed and characterized by FT-IR, with a resolution of $4\,cm^{-1}$, at a scanning speed of 30 times per second; the wave number ranged from 100 to $4000\,cm^{-1}$.

2.2.8.4. XRD crystal analysis. XRD was employed also to analyze the sample. The working conditions of the XRD included CuKα radiation via a rotating anode at 40 kV and 50 mA, and the data was collected in steps of 0.05° per second with the scattering angles (2θ) ranging from 20° to 70°.

3. Results and discussion

3.1. Appearance

After incubation for 0, 8, 13, or 18 days, yolk sacs were collected for PLM to observe the morphology and developmental process of