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Co-Subsistence of Liquid Crystal Droplets and Calcium Carbonate Vaterite Crystals Reveals a Molecular Mechanism of Calcium Preservation in Embryogenesis

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The yolk sac equipped with vitelline fluid, plays a crucial role in supplying nourishment to the developing chicken embryo during embryonic and early post-natal development. The absorption and utilization of calcium in embryogenesis has been investigated for years. However, the preservation process of the calcium as it is transported through the chorioallantois membrane during embryogenesis remains largely unknown. In this study, we demonstrated that abundant liquid crystal droplets (LCLD) subsist with calcium carbonate vaterite crystals (CCVC) in the yolk sac, revealing a possible function of LCDL in CCVC crystallization.

Keywords: biological crystallization; calcium carbonate vaterite crystals (CCVC); calcium preservation; embryogenesis; liquid crystal droplet (LCDL); yolk sac

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I. INTRODUCTION

During avian and reptilian development, the yolk sac equipped with vitelline fluid is responsible for supplying nourishment to the whole embryo during embryonic development. This function continues within early postnatal development until the yolk sac, located in the abdomen, is completely absorbed. The process of how nourishment in vitelline fluid circulates into the embryo through the yolk sac membrane or extra-embryonic fetal membrane has been well studied. Vitelline circulation and mineral metabolism including iron, zinc, copper, iodine, manganese, selenium, chromium, nickel, cobalt, and calcium has been well studied previously [1–7].

In higher mammals, the yolk sac is normally considered an evolutionary vestige. However, as the first site of blood formation responsible for generating primitive macrophages and erythrocytes, this remnant structure serves important functions vital for embryonic development. Recently, research has begun to address its function in the cell proliferation and regeneration, the origination of hematopoietic stem cell (HSCs) in the yolk sac [8] and differentiation of HSCs in target organs in embryo [9]. Through wide-meshed capillary plexus by vitelline-tubular heart circulation, the yolk sac plays a crucial role in self-renewing proliferation by providing efficient microenvironment for the differentiation of these progenitor cells. Because there are strong-potential applications of HSCs for treatment of congenital human disease, studies on the yolk sac stem cells of both bird and mouse have been receiving tremendous attention [8–11]. This approach has promising prospects because HSC collection from extra embryonic tissues can be easily carried out without any damage to embryo at all.

HSCs generated in the yolk sac blood islands have an important function in ontogeny and organogenesis. HSCs are responsible for activation of genesis in embryonic tissues and organs including the liver, thymus, spleen and bone marrow [8,9]. Many minerals have proven irreplaceable for this biological architectural progress. Among numerous processes, the absorption and utilization of calcium plays a vital part during embryonic development and has been studied for years. Although its transportation through the yolk sac membrane and chorioallantois membrane has been well study, the preservation process of calcium in vitelline fluid still remains largely unclear. We have proved that the abundant stones found in the yolk sac of domestic fowl [12] are calcium carbonate particles in the form of vaterite crystal [13]. In this study, we demonstrate that abundant liquid crystal droplets (LCDLs) subsist with calcium carbonate vaterite in the yolk

sac during chicken development and reveal a prospective function of LCDL in calcium carbonate crystallization during biological processes. Together with our previous reports on the existence of LCLDs during chicken development [14,15], we hypothesized that the accumulation of calcium carbonate on lipid lamina of LCLDs is the major mechanism for calcium preservation in yolk sac during avian embryogenesis.

II. EXPERIMENTAL MATERIALS AND METHODS

Animal and Terminology

All animals we used in the experiments are covered under the regulation of University Institutional Animal Care and Use Committee (IACUC). The maintenance, use and other activities of vertebrate animals were overseen by IACUC. All experimental procedures carried out in this project were reviewed and approved prior to its initiation. The chickens used in our experiments were hosted mainly in the University Avian Animal Facility located at Luojia Domestic Animal Farm, Wuhan University School of Life Sciences. Fertilized White Leghorn eggs (*Gallus domesticus* L.) were incubated at a temperature of 37°C with relative air humidity of 60%. Experiments were conducted on certain days according to experimental procedure. The age of embryos was documented as day (D) and postnatal age of chicks was documented as Postnatal (P).

Sample Preparation and Frozen Section

Two procedures were used to prepare samples: smear-slide preparation and cryosection. For smear-slide preparation, samples were taken from the embryos at different stages. Each sample was smeared on a slide with PBS (PH 7.4) and mounted with a cover slip. Polarization microscopy observations were conducted immediately following the sample smear preparations. For cryosection, samples from the embrys were set into cryomatrix embedding agent (OCT) and placed in an aluminum foil basket. The samples were then frozen by dipping the foil basket into liquid nitrogen. Frozen tissue within the OCT block were placed on the cryostat microtome and sections of samples were cut at a thickness of 10∼30 μm for polarization microscopy. The sections for H&E staining were cut at a thickness of 5 μm. The samples collected using these two procedures were mounted with 20% of glycerol in PBS (PH 7.2) before proceeding to further analysis.

Histology Analysis

Hematoxylin and Eosin (H&E) staining were carried out on frozen section as previous described [16,17]. After Hematoxylin and Eosin staining, dehydration was performed in gradient ethanol. Xylene based Permount was used for permanent preservation with glass coverslips. Nikon conventional light microscope (Jnoec Ltd, Jiangnan, assembled in Nanjing, PRC) was used to perform histology analysis.

Polarization Microscopic Analysis

Samples obtained from the chick were prepared with smear-slide and cryosection preparations. Firstly, conventional observations were carried out under non-crossed polarizer and analyzer to identify localization of samples. The preliminary observation helped to evaluate distribution of LCDLs and calcium particle crystal that will be further categorize by histological analysis. Secondly, optical activity of the samples was observed between crossed polarizer and analyzer. Birefringence activities generated by various samples were documented for further analysis. The observation on optical activity proceeded with XS-213A-P Polarization Microscope (Jnoec Ltd, Jiangnan, Nanjing, PRC).

Measurement of Phase Transition

The measurements were performed on a combination of inverted microscope PE120 peltier system (Linkam Scientific Instruments, UK) and XS-213A-P polarization microscope. PE120 peltier system is set to work with 5 mm aperture at heating/cooling rate 0.1 to 20°C/min ranged. Its temperature stability is ± 0.1°C and controlled with RS232 on the temperature apparatus. Temperatures of phase transition between anisotropic and isotropic phases were recorded according to the observation of birefringence activities of the samples between two crossed polarizing prisms.

Image Analysis

The images from conventional and polarization microscopy were captured with Nikon or Olympus camera. The quantification analysis of birefringent intensity of the LCDLs and Calcium vaterite particles from yolk sac was measured and constructed at different developmental stages with the image analysis software *ImageJ 1.38* (NIH, Bethesda, MD).

III. RESULTS

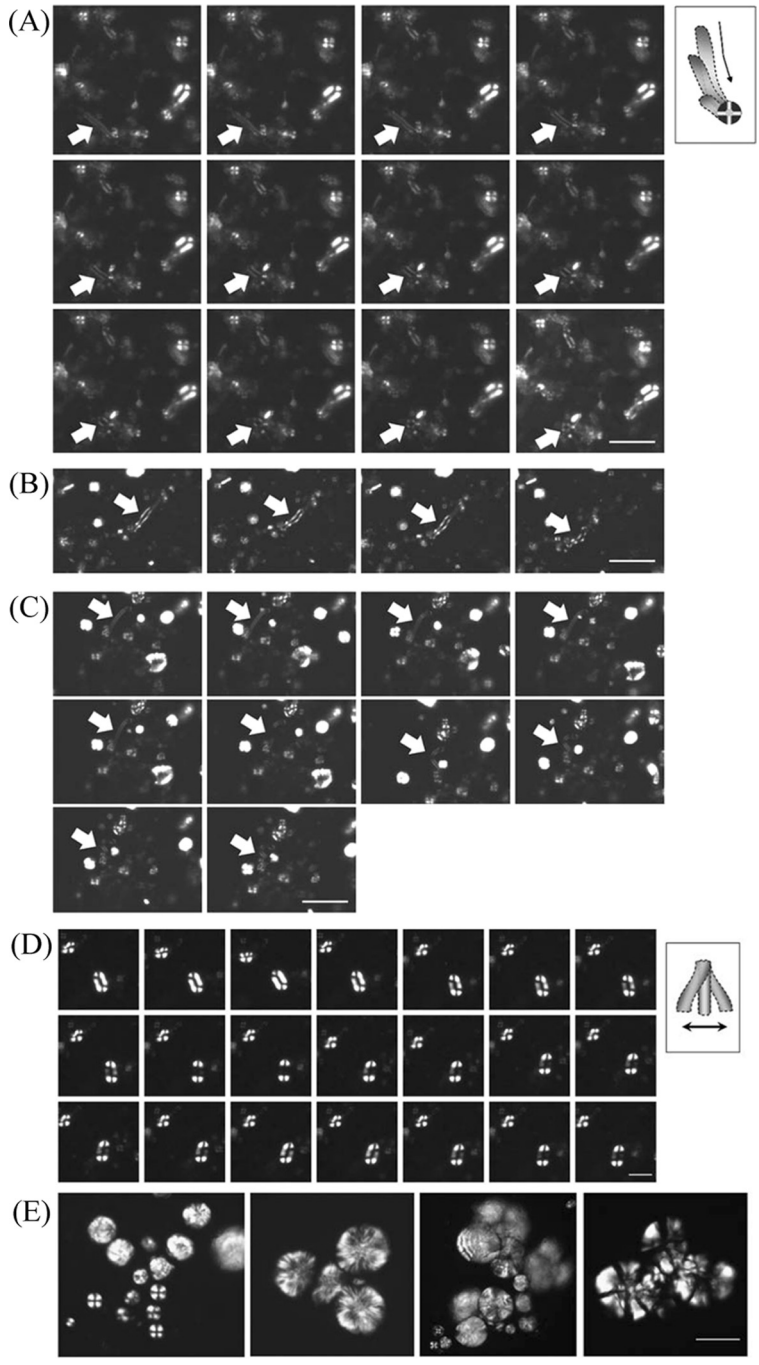
In the chicken embryonic yolk sac, there is an abundance of birefringent particles observable under polarization microscope ranging in diameter from 1 μm to over 0.1 mm. In our experiments, we demonstrated that some of these birefringent particles are liquid crystal droplets (LCDLs) in Maltese Cross or tubule formation. The rest are calcium carbonate vaterite crystal (CCVC) as previously reported [12,13]. Interestingly, some birefringent particles appear to have structural and thermal characteristics of both the liquid crystal and crystal states. From this initial observation, we have attempted to provide direct evidence that CCVCs develop from LCDLs in the yolk sac. We hypothesize that calcium preservation is completed through this biological process responsible for transitioning liquid crystals to calcium vaterite crystals by precipitating calcium on the lamina of the LCDL (see Discussion).

Fluidity of the Liquid Crystal Droplet in the Embryonic Yolk Sac

To characterize the fluidity of LCDLs in yolk sac, a pressure-recovery experiment was conducted on the yolk sac smear samples. The sample was mounted in the medium we described in Materials and Methods and protected with a cover-glass. Pressure was applied evenly on the cover-glass with rubber pressure applicator and observations of the effect of pressure on LCDLs were made on the sample between two crossed polarizing prisms.

After the pressure was applied, some Maltese Cross LCDL spheres distorted into birefringent tubules. Upon the removal of pressure these tubular structures resumed its original shape, the LCDL spheres. The process of shape changing from birefringent tubule back to that of the Maltese Cross was captured in Figure 1A under a polarization microscope. The entire recovery process required approximately twenty to thirty minutes depending on the size of the birefringent tubule. The flexibility of birefringent tubules leads to a variety of recovery shapes and processes including twists (Figure 1B) and helical complexes (Figure 1C). Figure 1D documents a twisting pendulum movement of a birefringent tubular structure. From the movement and characteristics documented above, we conclude that LCDLs in yolk sac have typical fluidity and birefringence activity.

As crystal particles the CCVCs exhibit intense multicolored birefringence activity under the microscope. In the pressure-recovery experiment, the CCVC spheres could not endure the pressure applied on glass cover were broken into small fragments (Figure 1E).



Phase Transition of the Liquid Crystal Droplet

When observed on smear slides, LCDLs found in the chicken embryonic yolk sac exhibit Maltese Cross birefringence between polarizing prisms. The Maltese Crosses can be black-white or multicolored displaying the full range of the spectrum. Our experiment demonstrated that the birefringence of these Maltese Crosses can change with temperature alternation generated by thermo-stage indicating that it is a temperature-sensitive phase transition.

In our experiment, we also proved that the phase transition from liquid crystal Maltese Crosses to isotropic liquid droplets is reversible with temperature change. In Figure 2, we documented the phase transition of LCDLs extracted from a embryonic yolk sac; panels A to G show the transition from liquid crystal to isotropic state and panels H to T show the transition back from isotropic back to liquid crystal.

From observations of temperature-sensitive phase transitions, the different behaviors of black-white LCDLs and multicolored LCDLs are distinctly noticeable. In the phase transition from liquid crystal to isotropic state induced by temperature increase experiment, the black-white LCDLs lost their birefringence earlier than the multicolored LCDLs. Additionally, when this phase transition occurred to multicolored LCDLs, they initially change into a black-white birefringence state before transitioning into the isotropic state (see Figure 3 panel A to F).

When LCDLs transitioned from the isotropic to the liquid crystal state with temperature decrease, the black-white LCDLs resumed their birefringence significantly later than the multicolored LCDLs. Interestingly, during this phase transition, of the multicolored LCDLs, black-white birefringence recovered earlier than the multicolored LCDLs (see Figure 3 panel H to T). Our data implicates that the black-white LCDLs have a lower transition temperature than the multicolored LCDLs (about 1 to 2°C difference). Overall the phase

FIGURE 1 LCDLs taken from a chicken embryonic yolk sac display the fluidity characteristics of liquid crystal. (A) The tubular structure generated by external pressure shown resuming its original shape, the Maltese Cross is indicated by arrows. (B and C) Birefringent tubular structures can change their shape from straight to twisted tubules as indicated by arrows. A more extreme, coiled tubule structure is indicated by arrows in C. (D) A single liquid crystal tubule exhibits its fluidity with twisting pendulum movement. (E) After external pressure was applied, calcium carbonate vaterite crystals (left two panels) were fractured into small fragments (right two panels). The sketchs of fluidity movements are shown on the right of panel A and D. Scale bars for A-C = 5 μm; D = 4 μm; E = 5 μm.

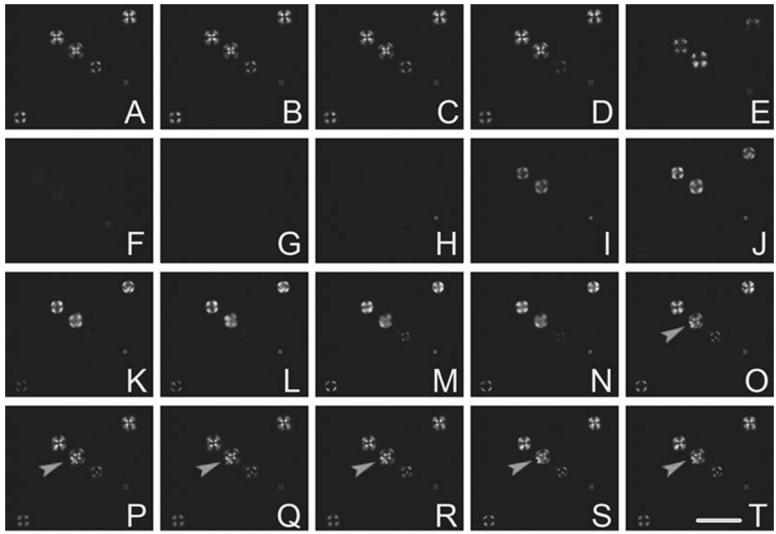


FIGURE 2 The phase transition of the LCDLs taken from a chicken embryonic yolk sac with increase and decrease of temperature. The transformation from liquid crystal Maltese Cross to isotropic state: Birefringence of Maltese Cross LCDLs disappeared with temperature increase and changed to isotropic droplets (panel A to G) at about 39°C. When sampling temperature decreased naturally at room temperature, the LCDLs resumed liquid crystal state in Maltese Cross. At the early stages, the recovering LCDLs retain some defects as indicated by arrows in panels O to T. These defects recover completely after an extended amount of time (20–30 minutes). Scale bar is 5 μm.

transition temperature is 39 to 40°C. Implications of these differences in temperature for biological crystallization will be discussed lately.

Interestingly, some recovered LCDLs have defects, which we documented in Figure 2 panel O to T. However as we have documented, these defects generally recovered naturally within a twenty to thirty minute recovery period. According to our experiments, the rate of defect incidence depends on the rate at which temperature is decreased. The more rapidly temperature is decreased, the less chance of generating defects during LCDLs recovery. This phenomenon is similar to our observations on the hepatic liquid crystal in chicken liver development [18].

Characteristics of the Chimera Liquid Crystal and Crystal Structure

Chimera birefringent particles composed of two parts, the birefringent cortex and the interior Maltese Cross, were found in the chicken

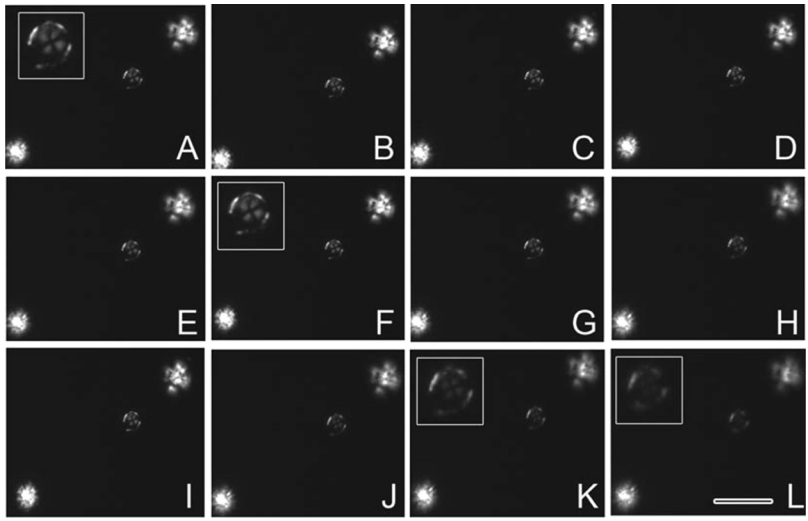


FIGURE 3 Partial phase transition of the birefringent particles in chicken embryonic yolk sac with temperature increase. Three particles are showed in each panel. The calcium carbonate vaterite crystals (CCVCs) are located on the top right and bottom left corner of each panel. The birefringent particle located between the two noted CCVCs exhibited partial phase transition. This particle is composed of two parts, the interior Maltese Cross portion and the birefringent cortex (insets in panels A and F). The interior Maltese Cross is generally not located in the direct center of the particle since the cortex region leaves homogeneous space, creating space for calcium crystallization. The temperature increase phase transition experiment showed that the interior Maltese Cross could change to an isotropic state while the cortex displayed no significant change (insets in panels K and L). The phenomenon suggested a partial phase transition happened at in the interior Maltese Cross but not at the cortex. Scale bar is 5 μm.

embryonic yolk sac. These chimera particles display the structural and birefringence characteristics of both liquid crystalline and crystalline. In the cortex, birefringence is usually not even. When the sample is observed from different angles, the uneven birefringence can be easily detected. The thicknesses of the cortex in each chimera vary dramatically ranging from less than 17 μm to more than 1 mm.

In the chimera structure, a homogeneous space between the interior Maltese Cross and cortex observed. This homogeneous space could be generated by calcium precipitation from outside towards the inside of the LCDL laminas.

The two portions of chimera birefringent particles exhibit different phase transition responses to temperature increase. When heat is

applied, the birefringence of the cortex shows no detectable or exceedingly little changes. However, the interior Maltese Cross progressively lost birefringence as heat was applied which was documented in Figure 3. In these series of panels, the two CCVCs on top right and left bottom corner display no obvious change on their birefringence after temperature increase. Between these two CCVCs is a chimera particle, whose phase transition documented with the CCVCs as reference to progress. The insets in panel A, F, K and L, distinctly exhibit the phase transition of the interior Maltese Cross.

Distribution of Liquid Crystal Droplets and Calcium Carbonate Vaterite Crystals

Using polarization microscopy, the distribution of LCDLs and CCVCs in the embryonic yolk sac was inspected. Our data revealed that total birefringence generated by LCDLs and CCVCs displays an interesting distribution pattern in the yolk sac. We found that, observing from the cortex to the central area of the yolk sac, the birefringence generated by crystal and liquid crystal increases steadily (Figure 4A and K). This birefringence pattern is revealed by its intensity and gray value as shown in Figure 4H and K.

When the birefringence generated by LCDLs was removed by temperature increase, the total intensity and gray value composition was dramatically changed. When birefringent LCDLs were transited into isotropic droplets that generated no birefringence (see panels L and M). At this stage, all intensity and gray value distribution are attributed by the CCVCs birefringence, which do not fade with temperature increase. From plot-profile analysis, we found that birefringence is dispersed in a pattern that increases significantly as we inspected from the cortex to the central portion of the yolk sac (see panels G, I and K). Based on this analysis, we conclude that the formation of CCVC takes place more frequently in the center than in the outer cortex of the yolk sac. This phenomenon indicates that the crystallization of LCDLs into CCVCs is initiated from the center and extends outwards towards the cortex of the yolk sac. Our observations support this hypothesis i.e., CCVC crystallizes from calcium precipitation on the laminas of LCDLs that we will discuss more detail.

Histological analysis reveals that the existence of CCVC in the yolk sac is always accompanied by stromal cells. Correspondingly, more CCVCs are surrounded by more stromal cells (panels C and F) and fewer CCVCs by fewer stromal cells (panels B and D). LCDLs in the yolk sac show no significant distribution patterns and can be detected starting from very early on in embryonic development at early stage,

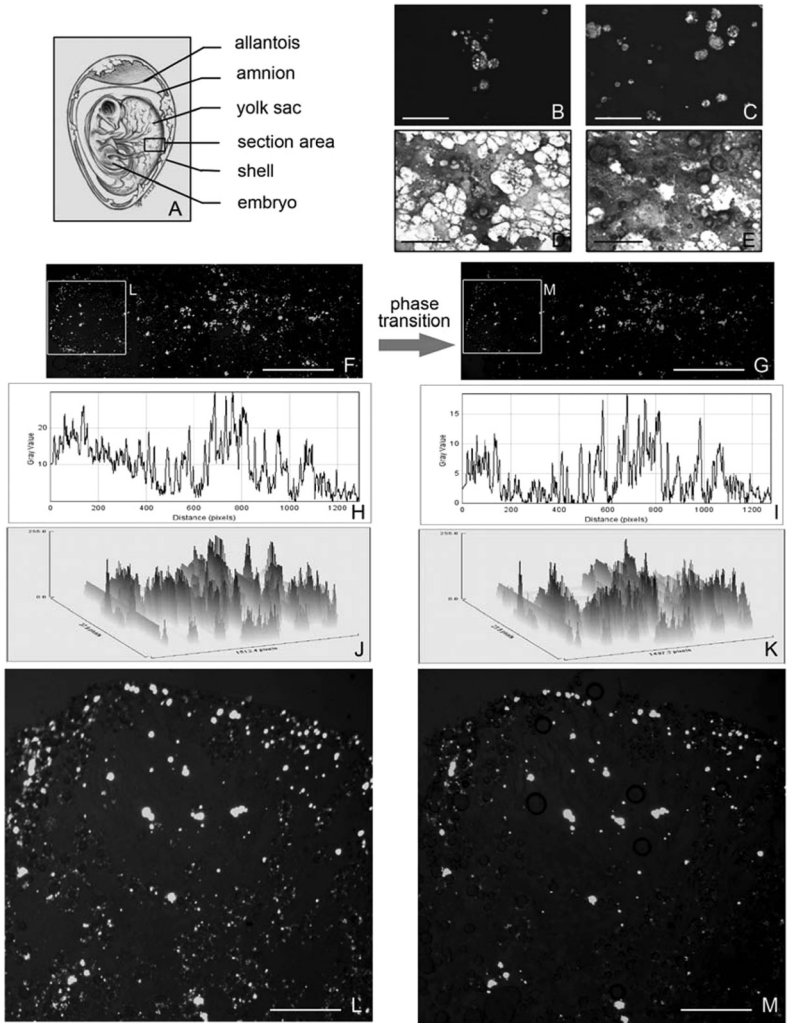


FIGURE 4 The distribution of LCDLs and CCVCs in the embryonic yolk sac. The sketch scheme of a whole chick embryo shows location of yolk sac (panel A). HE staining shows that the CCVCs are always surrounded by stromal cells. Numbers of stromal cells around CCVC particles in central (C and E) and cortex (B and D) region are significantly different in yolk sac. In panels F and G respectively, the birefringence patterns of before and after temperature increase phase transition in yolk sac are shown. Accordingly, the changes between the before and after birefringence intensities were analyzed and shown in both gray value line graphs (H and I) and 3-dimensional pixel graph (J and K). The excerpt views of the cortex region exhibit the birefringence change before (L) and after the transition (M). Scale bars are 5 μm in B-E; 100 μm in F-G; 20 μm in L and M.

starting day E2 [14,18]. However, as we have shown above, this even distribution pattern changes during calcium crystallization.

IV. DISCUSSION

Calcium carbonate vaterite crystal, present in 87% of all gallstones, is the major component of human gallstones topping the composition of both aragonite and calcite, which exist in 55% and 45% respectively [19]. The gallbladder, where gallstones are formed, is full of liquid crystals suspended in bile [20,21]. The co-existence of liquid crystals and calcium carbonate crystals is commonplace in several biological systems [22,23]. In this study, we propose that the co-existence of LCDL and CCVC in chicken embryonic yolk sac could be the link between liquid crystal and crystal. Our data revealed this link by discovering an intermediate chimera structure that displays both LCDL and CCVC characteristics. This finding reveals a biological process that may have implication in gallstone formation.

During chicken development, multiple organs and tissues exhibit liquid crystal droplets at different embryonic stages. These droplets are found in the mesonephros and the metanephros during kidney development, as well as in the liver, brain, and blood of the spleen and heart, etc. [13,18]. Among these organs, the yolk sac is the earliest that shows liquid crystal appearance [14]. The LCDLs are observable starting at E2 with the number of the LCDLs increases progressively as development continues. From days E12 to E16, the number of LCDL present reaches its maximum. At this peak, calcium carbonate vaterite crystals begin to appear. From this stage and onwards, LCDLs and calcium carbonate vaterite crystals co-subsist in the embryonic yolk sac with the ratio of crystals to LCDLs present increasing throughout the rest of development. By the time the chick emerges after 21 days of incubation, the yolk sac is full of the vaterite crystals with a few LCDLs remaining. This yolk sac rich with vaterite crystal will then be completely absorbed into the chick by the postnatal age of two to three weeks.

The co-subsistence of LCDL and vaterite particles during chicken development indicates a possible connection between the two. According to our results, we hypothesis that LCDL is a progenitor structure of the vaterite crystal and that the vaterite crystal grows by precipitating calcium carbonate on the concentric laminas of the LCDL during embryonic development. The concentric lamination of LCDL provides the preferred matrix framework for calcium carbonate crystallization. When calcium is absorbed from the egg-shell and transported into the yolk sac through the trabeculae, it will continuously precipitate on the

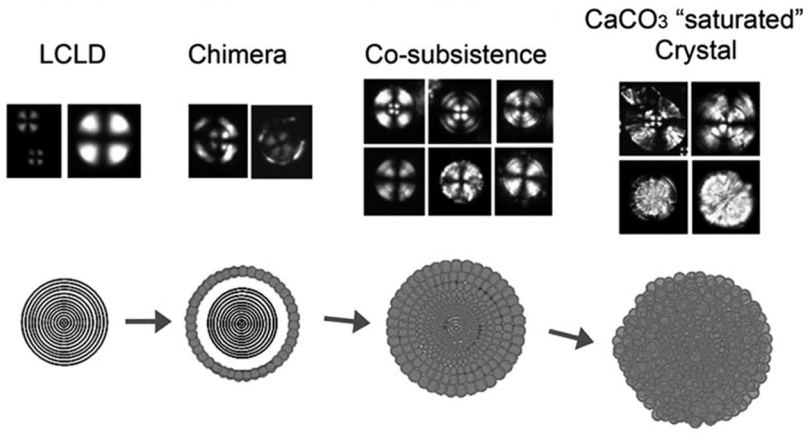


FIGURE 5 A hypothetical model for calcium carbonate vaterite crystal (CCVC) formation from liquid crystal droplet (LCDL). This model is based on the intermediary birefringent particle, the chimera particle, and the partial phase transition we found the chicken embryonic yolk sac.

outer lamina of LCDL and induce the growth of vaterite crystals. We have derived from our observations that the conversion from LCDL to crystal occurs in the following steps. Calcium precipitation initiates on the outer lamina. As crystal growth continues it expands outwards on the lamina, making the entire structure enlarge forming the chimera structure. This precipitation then continues on every lamina, moving inwards from the outermost non-crystal lamina. At the end of this process, the intermediate co-subsistence is formed. Once this transitional structure is formed, further calcium precipitation occurs and the crystal Maltese Cross is entirely over taken by the expanding crystal growth. At this last stage all lamination disappear and the Maltese Cross is eliminated, resulting in the final crystalline structure. This resultant vaterite crystal displays a stunning multicolored birefringence (see Figure 5).

V. CONCLUSION

Chimera birefringent particles composed of an interior Maltese Cross and a birefringent cortex provides a link between liquid crystal and calcium carbonate vaterite particles. Our hypothesis assumes that the progressive precipitation of calcium carbonate initiates on the outer lamella of LCDLs, which supplies an efficient three-dimension matrix for the crystallization to gradually infiltrate inwards on the

liquid crystal Maltese Cross. With the complete saturation of each lamella layer, crystals formed from that layer expands outwards and the process carries on at the next outer layer. Eventually this process forms the individual vaterite crystal particle from pre-existing LCDL in the chicken yolk sac.

The biological process of vaterite crystals developing from LCDLs in the chicken embryonic yolk sac from liquid crystalline to crystalline could be a model for other calcium stone formation in both animals and humans. This knowledge can be invaluable for pathological studies of gallstone formation, the formation of interesting growths such as the otolith in fish and avian inner ears, and the development of pearl in oysters.

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