Research Note

The effect of pigeon yolk sac fluid on the growth behavior of calcium carbonate crystals

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ABSTRACT Previous experiments have proved that thermodynamically unstable calcium carbonate vaterite can exist for long periods in the yolk sac of a pigeon embryo. The aim of this article was to demonstrate the effect of in vitro mineralization of yolk sac fluid on calcium carbonate by direct precipitation. Experiments were conducted using pigeon yolk sac fluid and using lecithin extracted from pigeon yolk sac fluid as a control to investigate the regulating effects of the organic components in the embryo on the formation of the calcium carbonate precipitate. Multiple characterization methods were employed to study the various morphological patterns, sizes, crystal growth, and crystal phase transformations of the calcium carbonate precipitates as regulated by the yolk sac fluid extracted at different stages of incubation. The experimental results demonstrate that as the incubation proceeds towards the later stages, the composition and environmental features of the yolk sac fluid become more favorable for the formation of relatively unstable calcium carbonate phases with high energies of the vaterite state. The experiments conducted with extracted lecithin as the template for crystal growth yielded similar results. A large amount of organic molecules with polar functional groups carried by the yolk sac fluid have strong effects and can both initially induce the crystallization and regulate the aggregation of calcium carbonate. Furthermore, this regulation process is found to be closely related to the lecithin contained in yolk sac fluid. These observations confirm the changes in yolk sac fluid composition during incubation have significant effects on the production of vaterite, which implicates the calcium transport during embryo growth.

Key words: pigeon, yolk sac, mineralization, calcium carbonate, lecithin

2015 Poultry Science 94:402–407
http://dx.doi.org/10.3382/ps/pev004

INTRODUCTION

Biomineralization involves the selective uptake of elements from the local environment and the incorporation of these elements into functional structures under strict biological control (Mann, 2001). In contrast to manually fabricated composite materials, natural biomaterials have specific hierarchical structures (Currey, 2005) and assembly methods (Yao et al., 2011). The presence of biomacromolecules changes the process of $CaCO_3$ deposition from homogeneous nucleation to heterogeneous nucleation and allows the kinetic limits of the reaction to be reached (Xu et al., 2008). For example, Qiao et al. (2008) found that fresh nacre surfaces from freshwater lustrous pearls (aragonite) could be used as templates for $CaCO_3$ deposition without additives. Furthermore, extensive studies have been conducted on the phase stabilization and habit modification in $CaCO_3$ crystal growth with natural biomaterial templates (Zhang et al., 2006; Natoli et al., 2010; Ren et al., 2010; Asenath-Smith et al., 2012; Xavier et al., 2012).

Previously, this research group demonstrated that the calcium carbonate in the yolk sac of incubating pigeon embryos exists as a mixture of two phases, of which the major component is unstable vaterite (Song et al., 2014). However, the reason why thermodynamically unstable vaterite can exist for long periods of time in the embryo remained unsolved. A reasonable assumption is that the composition of the yolk sac fluid itself significantly influences the mineralization of calcium carbonate.

In this experiment, the $CaCO_3$ precipitation reaction was carried out in pigeon yolk sac fluids (**YSF**) obtained from different stages of incubation to dynamically simulate the mineralization process that forms the spherocrystals in the yolk sac. This allowed us to observe the effects of the composition of the yolk sac fluid

Received June 30, 2014.
Accepted November 3, 2014.
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at different stages of incubation on the morphology, size, and crystal phase of the minerals. In particular, lecithin is a dispensable nutrient for embryo growth that accumulates in the yolk sac during incubation (Noble and Moorename, 1965). Yao et al. (1994) prepared calcium carbonate particles in compartmental water-lecithin systems and found that the sizes of the particles consisting of a mixture of vaterite and calcite decreased with increasing lecithin concentration. Because of its strong complexation with calcium ions (Grasdalen et al., 1977), for comparison, extracted egg-yolk lecithin (**EYL**) was also studied in terms of its ability to regulate the growth of inorganic calcium carbonate crystals. This experiment was conducted to gain better knowledge to determine which component in yolk sac fluid primarily regulates calcium carbonate deposition.

MATERIALS AND METHODS

Egg and YSF Sampling

One hundred pigeon eggs used in these experiments were obtained from the pigeon farm at Central China Agriculture University. The eggs were incubated in batches in a tabletop incubator (with automatic turning) at 38.2 ± 0.1°C in air with a relative humidity of 60 ± 5%. The eggs were candled after 5 days of incubation, and the dead and infertile eggs were removed at this time. The YSF of fertilized eggs incubated for 5, 8, 12, 14, and 16 days were used for this study, each age had ten eggs.

Extraction of EYL

Ten raw eggs were carefully broken to separate the yolks from the embryos and whites, and the combined yolks were kept in a cold room (5°C) before use. Embryos at hatch were euthanized via CO_2 gas inhalation. The moisture content of the egg yolks was determined using a conventional oven-drying method at 100°C for 4 h (Warren et al., 1988). Ethanol (100%) was added to approximately 5 g of egg yolks to a final 5:1 ratio of solvent to egg yolks (wet weight). The mixture was stirred until the egg yolks were completely dispersed. The final concentration of ethanol was 91% after it was diluted with the water contained in the fresh yolks. The sample was centrifuged at 400 × g for 5 min. The lecithin-enriched fraction (supernatant) was transferred to a previously weighed round-bottomed flask, and the ethanol was removed by rotary evaporation. The EYL suspension was prepared by swelling lecithin-enriched fraction in de-ionized water.

$CaCO_3$ Precipitation Experiments

The YSF and EYL suspensions containing Ca^{2+} were prepared by YSF and EYL in 0.5 mol/L $CaCl_2$ solutions by sonication for 30 min, respectively. The precipitation was conducted by mixing a 0.5 mol/L Na_2CO_3 solution with a Ca^{2+}/YSF suspension and a Ca^{2+}/EYL suspension with stirring natural templates (25°C) (Yu et al., 2005). The solution was continuously stirred for 2 h, during which slow precipitation of milky white $CaCO_3$ occurred. The product was filtered, washed, and vacuum dried, and white calcium carbonate powder was obtained. To obtain a better understanding of the precipitation of the different carbonate polymorphs, control samples were created by conducting the experiments without the YSF or EYL.

Characterization

The white calcium carbonate powder was characterized by X-ray diffraction (XRD; X'Pert Powder, PANalytical, the Netherlands), scanning electron microscopy (SEM; Quanta FEG450, FEI, the Netherlands), and high-resolution transmission electron microscopy (HRTEM; JEM-2100, JEOL, Japan).

RESULTS AND DISCUSSION

The Effect of YSF and EYL on the Crystalline Phase of Calcium Carbonate

The small-angle X-ray diffraction spectrum of the EYL showed an interplanar distance d = 5.40 nm (Figure 1a), which proves that the EYL had a lamellar structure. When water was added (water: EYL = 50:100, w/w), the distance grew to d = 5.80 nm, and a secondary diffraction peak appeared (Figure 1b). The change in water content in the EYL caused this variation in the interplanar distance of EYL, which is consistent with the variation in the interlayer spacing of hydrous lecithin due to changes in water content (Dai, 1996). These results indicate that the EYL consisted of lecithin liposomes with concentric multilayered structures.

The X-ray powder diffraction patterns (Figure 2a, b) indicated that the product contained vaterite (JCPDS No. 72-0506) when YSF or EYL was used as template, whereas the product obtained in the control experiments consisted only of calcite (JCPDS No. 1650) (Figure 2c). Under natural environmental conditions, calcite is the thermodynamically stable form of calcium carbonate, whereas vaterite is extremely unstable (Cölfen, 2003). Vaterite appeared in the product after 5 days of incubation (Figure 2a). The intensity of the vaterite diffraction peak increased with incubation time, while the intensity of the calcite diffraction peak gradually decreased. In order to measure the ratio of vaterite to calcite in the product mixture, the integrated intensity of the vaterite peak (110) was used to represent the vaterite content in the mixture, and the integrated intensity of the calcite peak (104) was used for the calcite content. The formula $I_{104C}/I_{110V} = 7.691 \times X_C/X_V$ was obtained by linear fitting, in which I_{104C}/I_{110V} is the ratio of the vaterite peak intensity to calcium peak intensity and X_v/X_C is the molar ratio of vaterite to calcite

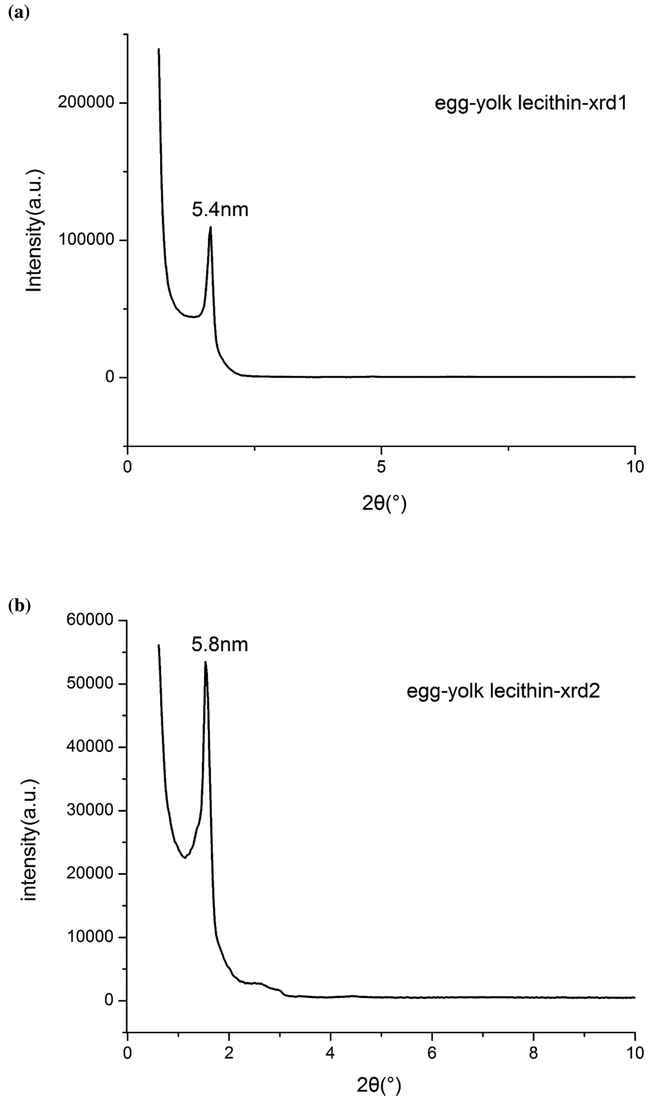

Figure 1. Small-angle X-ray diffraction patterns of (a) egg-yolk lecithin and (b) egg-yolk lecithin with some added water.

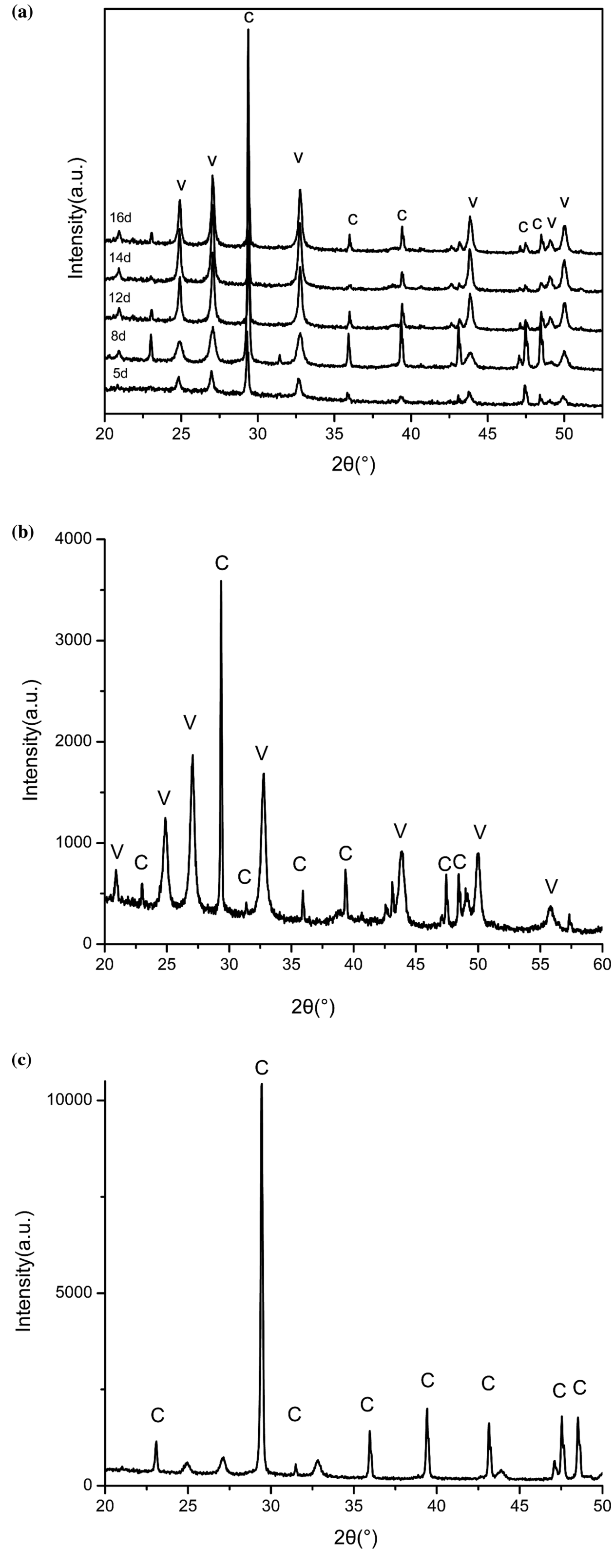

Figure 2. XRD patterns of samples prepared from Na_2CO_3 solutions with (a) yolk sac fluid from different stages of incubation, (b) egg-yolk lecithin, and (c) neither (control). C denotes calcite and V denotes vaterite.

within the mixture (Kontoyannis and Vagenas, 2000). According to the analytical results, the vaterite contents in the minerals produced using yolk sac fluid obtained on the fifth, eighth, 12th, 14th, and 16th days of incubation were 13%, 20%, 25%, 90%, and 95%, respectively. The morphology and crystal phase of the calcium carbonate were both affected by the YSF. In particular, spherocrystal vaterite was obtained when the YSF was used, and the vaterite content increased with the incubation time. Furthermore, YSF and EYL are both capable of inducing heterogeneous nucleation of calcium carbonate and could lead to the formation of spherical aggregated vaterite particles. Thus, the formation of vaterite may be related to the lecithin itself.

Morphology of the Calcium Carbonate Deposited in YSF and EYL

The SEM image (Figure 3a) of the control sample deposited under the same conditions but without yolk

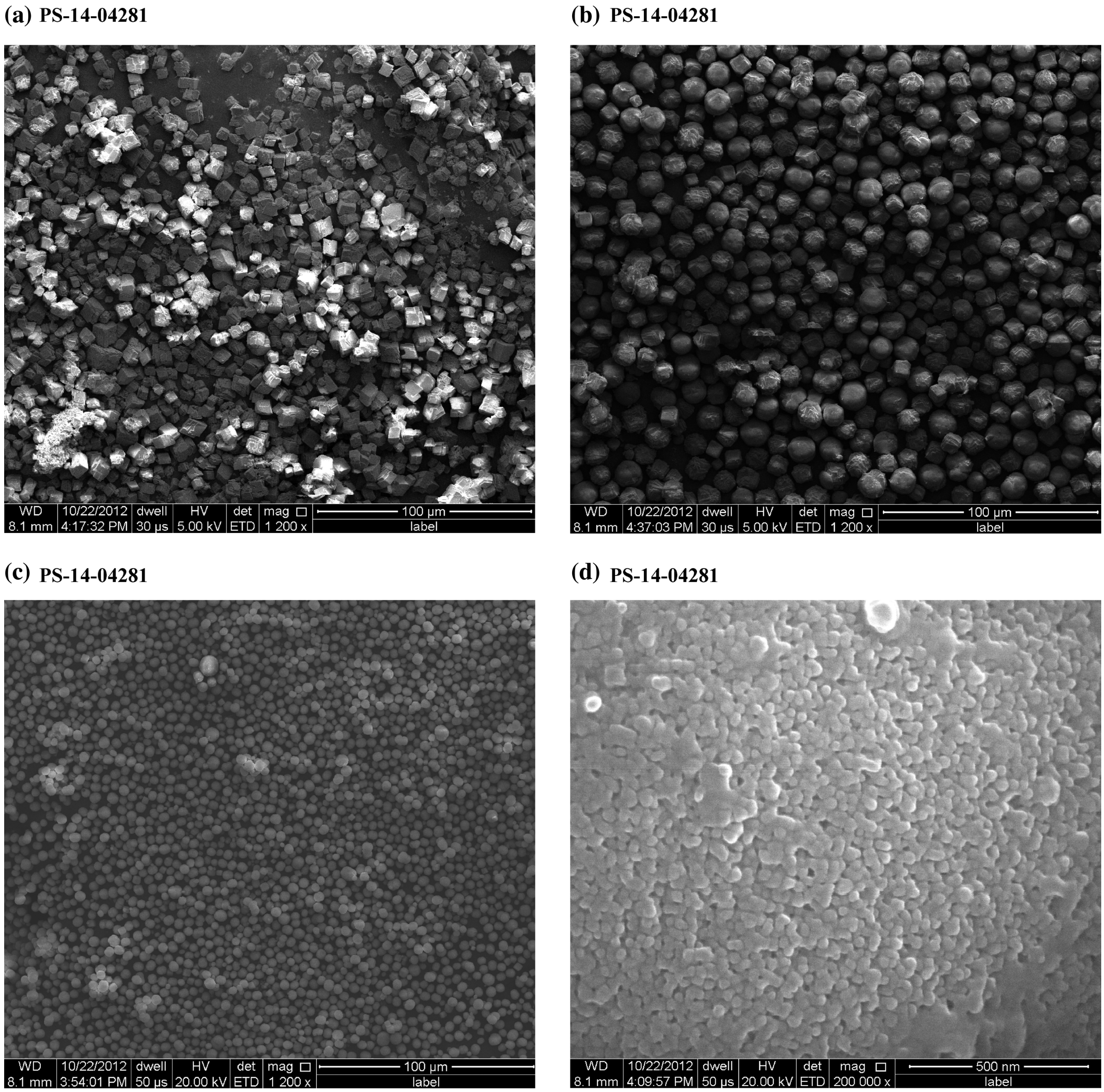

Figure 3. SEM images of calcium carbonate samples obtained from (a) the control experiment and experiments conducted with (b) egg-yolk lecithin and (c) yolk sac fluid. (d) Magnified image of (c).

sac fluid shows that pure calcite (rhombic hexahedron at 20μm) was obtained as the only product. Meanwhile, when the EYL (Figure 3b) or YSF (Figure 3c, d) was present, the synthesized calcium carbonate mineral particles gradually changed from diamond to cube shapes, and spherical aggregates of these particles formed. The shapes of the $CaCO_3$ particles correspond to the typical morphology for each polymorph. High-resolution field-emission SEM images of the synthesized vaterite show the sizes of these nanospherical particles (Figure 3d).

$CaCO_3$ Crystal Growth in the Presence of YSF Obtained after Different Incubation Times

The SEM and HRTEM images were obtained to determine the effect of YSF at different stages of incubation on the growth of vaterite. The vaterite content in the product mixture obtained in the presence of yolk sac fluid increased with incubation time, and the morphology of the $CaCO_3$ consequently gradually became spherical (Figure 4). Very pure vaterite can be synthesized using YSF from the later stages of incubation, and the particle size of the $CaCO_3$ minerals is limited as well. Well-dispersed crystal particles of about 4 μm were synthesized using the YSF obtained in the later stages (Figure 4d, e). This proves the ability of YSF to control the nucleation of calcium carbonate and shows that YSF provides a favorable environment for the production of vaterite while preventing it from transforming into calcite, which is thermodynamically more stable. High-resolution field-emission SEM images show that the sizes of the synthesized nanospherical vaterite particles are from 30 to 60 nm (Figure 4f). This is significantly smaller than the particle size of the bulk

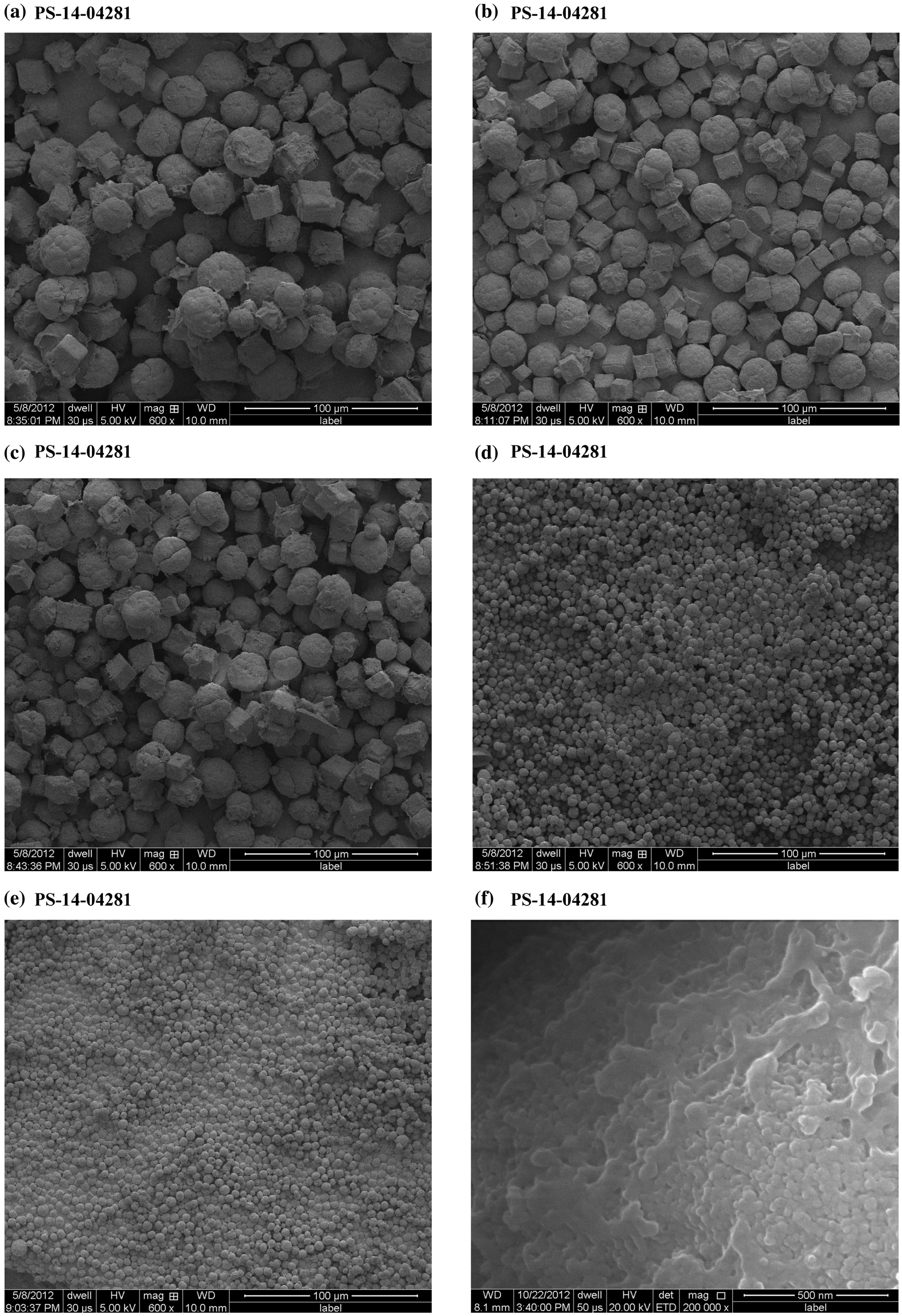

Figure 4. SEM images of calcium carbonate prepared in yolk sac fluid obtained after different periods of incubation: (a) 5 d, (b) 8 d, (c) 12 d, (d) 14 d, and (e) 16 d. (f) Magnified image of (e).

precipitate produced in the control experiments conducted without YSF, and the particles obtained with YSF are clearly self-assembled aggregates of $CaCO_3$ nanocrystals. This demonstrates the specific regulatory effects of YSF on the $CaCO_3$ deposition process.

The HRTEM (Figure 5) images reveal distinct nanoscale aggregate structure of the spherical vaterite particles, which are assembled from nanoparticles with sizes of less than 10 nm. The directed accretion of the nanocrystals that commonly occurs in aqueous

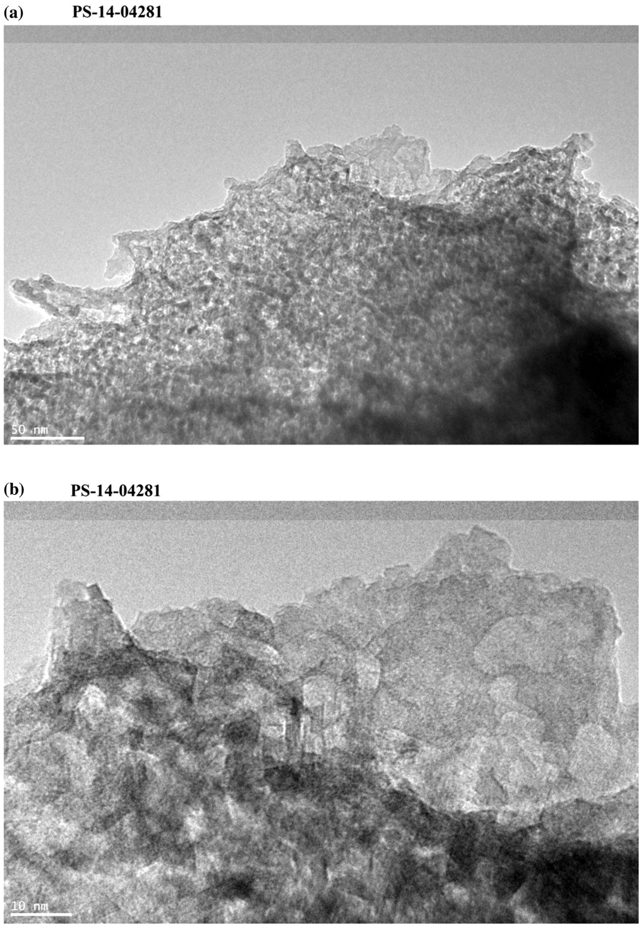

Figure 5. TEM images of the calcium carbonate particles obtained after $CaCO_3$ precipitation in the presence of yolk sac fluid obtained after 16 d of incubation.

solutions with additives replaces the typical dislocation pathway and 2-D pathway of crystal growth (Koch, 2007).

The simulated in vitro mineralization carried out in the presence of EYL was very similar to that observed in the presence of YSF, which indicates that lecithin might be one of the primary components in YSF acting as a template for calcium carbonate deposition. The characteristic interactions between lecithin molecules and calcium ions can induce the formation of massive local oversaturation zones that are specifically favorable for nucleation of calcium carbonate minerals with high energies like vaterite. Furthermore, the amphiphilic molecules attached to the microcrystal planes prevent the transformation into calcite. Thus, this investigation of the spherical, hierarchically structured aggregated vaterite particles and the regulation of their aggregation process by yolk sac fluid have direct implications for the study of calcium transport during embryo growth.

ACKNOWLEDGMENTS

Financial support for this research was provided by grant no. 31071265 (to H. Tong) from the National Natural Science Foundation of China.

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