Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl20

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Version of record first published: 16 Jun 2011

To cite this article: Xuehong Xu, MengMeng Xu, Odell D. Jones, Xunzhang Chen, Li Yanfei, Guifang Yan, Yuexing Pan, Harry G. Davis, Yi Xu, Joseph L. Bryant, Shangen Zheng & Donald D. Anthony (2011): Liquid Crystal in Lung Development and Chicken Embryogenesis, Molecular Crystals and Liquid Crystals, 547:1, 164/[1854]-172/[1862]

To link to this article: http://dx.doi.org/10.1080/15421406.2011.572042

Mol. Cryst. Liq. Cryst., Vol. 547: pp. 164/[1854]–172/[1862], 2011

ISSN: 1542-1406 print/1563-5287 online
DOI: 10.1080/15421406.2011.572042

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Organogenesis has been given increasing attention in the fields of biomedical and bioengineering. However, the mechanism for a succession process as complex as embryogenesis remains largely unknown. Based on our previous discoveries, liquid crystal may play a crucial role in organogenesis. Here, our results demonstrated that LC droplets were distributed on the pleural area, the bronchus and bronchiole in the developing lung. The lung liquid crystal droplets are capable of phase transitions between liquid crystal, crystal, and isotropic phases which are dependent on the rate of temperature change as previously reported in liver, kidney and other major tissues of the embryo.

Keywords Embryogenesis; liquid crystal droplet; lung development; phase transition

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1. Introduction

In 1979 a systematic description summarized research proceedings on liquid crystalline (LC) in biological organisms [1]. Liquid crystalline structures had been observed in atherosclerotic lesions of 95 patient samples. The major components of these LC were cholesterol, cholesterol ester, and phospholipid [2]. This phenomenon was further confirmed by another group [3] and has also been mimicked in *in vitro* system [4]. Later, these lipid depositions were also found to accumulate in smooth muscle and foam cells [5]. The lipid depositions in the vascular wall were found to be mediated by receptors distributed on the cell surface forming a complex with low density lipoprotein-cholesteryl esters [3,4, 6–9]. Liquid crystalline structures were not only observed in atherosclerotic lesions, but also found in drusons of retina in patients suffering from age related macular degeneration (AMRD) [10–13]. In both these cases, liquid crystalline formation in the tissue was the pathogenic symptom.

In 1978 and 1979, He and Wu reported findings in chicken development revealing the in mass existence of liquid crystalline structures in the liver, yolk sac, blood, and other developing tissues and organs during chicken embryogenesis [14,15]. They later reported that liquid crystal (LC) configuration can also be observed in fish development [16,17]. Chao and Li then reported CaCO3 vaterite existence within LC yolk fluid [18,19]. This result demonstrated that spherical calcified structures found in 1979 is one of three isoforms of calcium carbonate [20]. We have recently demonstrated that this crystallization is associated with LC in yolk sac LC. The chimeras of the LC and vaterite crystal provided solid evidences for this LC to crystallization hypothesis. Two major mechanisms facilitating crystallization for calcium carbonate biomineralization were identified, from-inside-to-outside and from-outside-to-inside accumulation of vaterite on LC molecular laminar structures [21,22]. Subsequent study of avian development proved that hepatic liquid crystalline is omnipresent in the liver during other avian development [23–29].

In this study we report the novel finding of LCs in the respiration system as a Maltese's crosses. These LC are mainly distributed in the pleura area, bronchus, and bronchiole of the lung. Our data exhibits that lung LC is able to transit to crystal and both LC and crystal can also transit into isotropic droplets, which can resume LC status. Summarization of the properties of lung LC will be discussed in parallel with the characteristics of the LC in other tissues and organs genesis during avian embryogenesis.

2. Methodology

The strategy normally used to investigate LC in embryos is as shown in Figure 1. First, freshly collected samples with smear preparation are observed under polarization microscope. If birefringence is detected, pressure-recovery experiment is conducted to test fluidity of birefringent particles. If birefringent particles are crystal, their structural shapes would crashed and not recoverable after pressure is applied. If birefringent particles are LC, their shapes would resume after pressure is released. Cryosection is then employed for histological analysis. Polarization microscopy, combined with histological and cell biology analysis, is used to localize the distribution of LC in embryonic tissue or organs.

A thermal stage is used to record the temperature of phase transitions. If LC droplets are massive in the tissue, X-ray diffraction (XRD) and small-angle X-ray

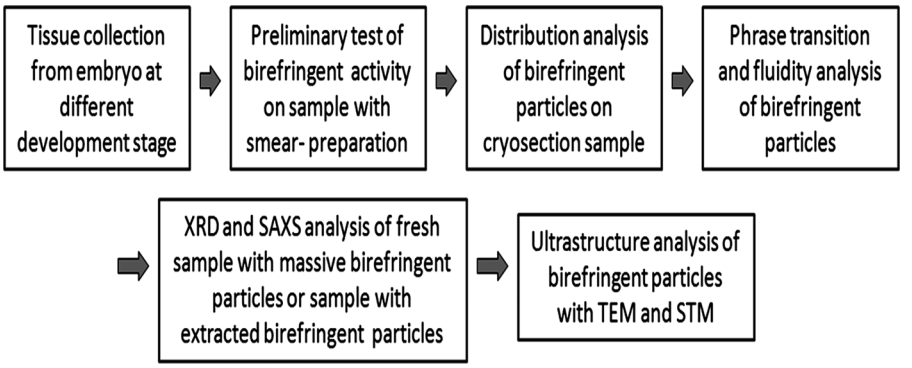

Figure 1. A brief schematic diagram of the strategy for birefringent particle analysis.

scattering (SAXS) will be exercised to examine birefringent activity under various conditions with a broad-angle goniometer of D-max-rA diffractometer in diffraction angle (2θ) of 1–20 degree with $1/6^\circ$ of CuK-alpha, $1/6^\circ$ of graphite monochromator and slits with optional width. To examine ultrastructure, transmission electron microscopy (TEM) and scanning electron microscope (SEM) combined with freeze-etch – preparation methods particularly are used to observe ultrastructure of birefringent particles in of tissue and organ [22–24, 27,30,31]. The XRD analysis in our project is normally carried out with standard parameters within 3 to 40 degree of diffraction angle and data are analyzed with software designed by the Material Institute of Tsinghua University.

All experiments carried out in this study followed the stratagem documented above. Hematoxylin and Eosin (H&E) staining were fulfilled as described [21,32,33] for histology analysis [21]. The sections for H&E staining were cut at a thickness of 5 μm. Cryosection samples at a thickness of 10∼20 μm were for polarization microscopy. In H&E stained slides, red and blue colors are representative of the cytoplasm and nucleus, respectively. Phase transition measurements were applied on thermal stage as previously described [21,22,31].

Animal related experiments were carried out under the guidelines regulated by the University Institutional Animal Care and Use Committee (IACUC). Maintenance and other activities of vertebrate animals for our experiments were carried out mainly in the University Avian Animal Facility located at Luojia Domestic Animal Farm, Wuhan University School of Life Sciences. Only non-survival sampling was involved in this study. Fertilized avian eggs were incubated under standard conditions, at a temperature of 37°C and a humidity of 60%. As usual, E and P are shorthand for the embryonic and postnatal age of the embryo. Number after E or P indicates the incubation day or day after hatching [20,21,30].

3. Distribution of Liquid Crystal in Lung and Other Embryonic Tissues

Birefringence was observed in two major areas of the chicken lung at stage of P18. First is pleura and pleural fluid, where the birefringence is distributed under the outside surface of lung (Fig. 2A to C) compared with H&E staining (Fig. 2D) and pleural area between lobes (Fig. 2E to G) compared with H&E staining (Fig. 2 H). At higher magnification, the surface of the pleural area (Fig. 2I and J) and

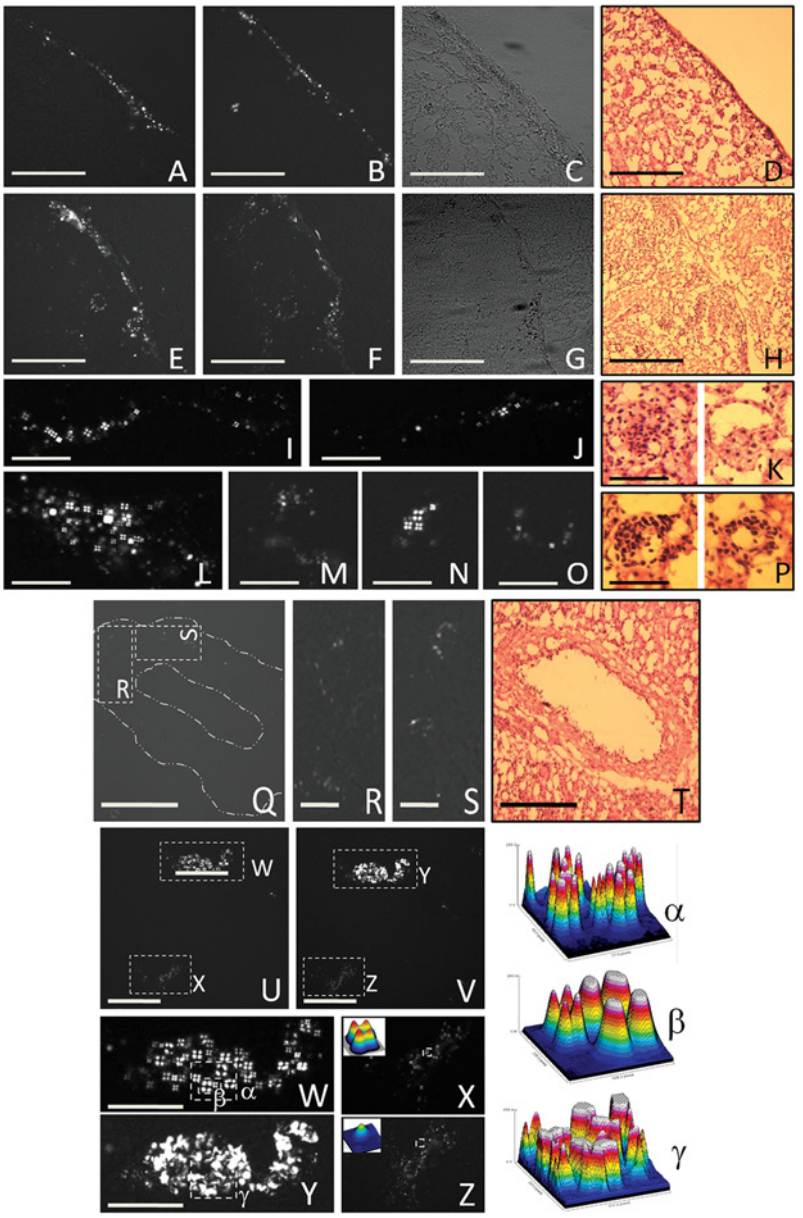

Figure 2. Distribution of birefringent crystal and LC in developing lung of chicken embryo at D18. Under polarized microscope, crystals (B, F, V, X and Z) observed in cryosections are corresponding to LCs (A, E, I, J, L to P, Q to S, U, W and Y) after transitions of crystal to isotropic status, then to LC in various locations. Birefringence is mainly situated in the pleural area (A to D) and pleural area between two PP (E to H). In these areas, higher magnification exhibites various size of Maltese crosses (I and J). Within the lung pleural area, LC droplets are found in branchiols (U to Z) and small branchiols (M to P). In large branchiols, liquid crysatl droplets are located in the wall (Q to T). Birefringent density and patterns of crystal (γ) and LC droplets (α and β) are significantly different. Bars in A to G, Q to T are 100 μm; Bars in R, S, U and V are 20 μm; Bars in I to P, W, X, T, Y and Z = 10 μm. (Figure appears in color online.)

pleural area between lobes (Fig. 2L) contain birefringent LC Maltese's crosses droplets. The second area where the birefringence locates is located inside the lung. Under polarization microscope, some LC droplets cluster and form unclosed ring structures (Fig. 2M to O). H&E staining showed that these ring structures are developing small bronchus and bronchiole (Fig. 2K and P). In some bigger bronchuses the LC droplets form dotted one or two layer of LC droplets outside elastin layer (Fig. 2Q to T). Birefringence clusters at different size can be observed in lung (Fig. 2U to Z).

LC are found in a wide variety of animal embryos including Chinese honeybee (*Apis cerana*) chrysalis [27], fish [17,33], reptile [unpublished data] and avian [21,22,30]. The distribution of LC in both invertebrates and vertebrate are in tissues or organs related to energy metabolism, such as fatty body of insects [27], yolk sac of fish and avian, and liver in of avian [22,29]. In addition to organs involved in energy metabolism, the LC can be also observed in other developing organs and tissues of avian embryos such as meso- and metanephros, and lungs.

In chicken development, more than twenty different organs and tissues exhibit LC droplets at certain developmental stages. The presence of LC normally lasts to early postnatal stages [12,13]. The earliest LC droplets appear on the inner embryonic disc during the second day of development [14]. The LC droplets eventually vanish within three to four weeks into postnatal development of the kidney and liver [23,24,30].

Two organs, which exhibit massive birefringent particles at certain stages during chicken development, are liver and yolk-sac. The yolk-sac birefringence is caused by LC droplets and calcium carbonate vaterite [20,16], which are surround by stromal cells in yolk-sac fluid. The LC droplets were proved to be the precursor of the vaterite calcium carbonate through the discovery of chimerical structures containing a hybrid of each form [20,21]. In liver, the hepatic birefringent particles are LC droplets mainly composed of cholesteryl oleate, cholesterol, lecithin and an unidentified component [22–24]. These LC droplets are found in hepatocytes of the hepatic cord region. In chicken kidney development, LC droplets can exist in the cytoplasm of epithelial cells and the lumen of proximal tubules in the mesonephros and metanephros during kidney organogenesis [30].

4. Phase Transitions of Liquid Crystals and Crystals in Embryo are Common

In the lung, LC droplets in smear sample can be observed under polarization microscope as Maltese crosses. However, when cryosection was applied on chicken lung at age of P18, LC could not be observed under the scope. These LC droplets transited to crystal in frozen, and were found in pleural area, small bronchus and bronchiole (Fig. 2B, F, V, X and Z). Thermal stage combined with polarization microscopy revealed the ability fo these lung LC droplets to phase transit from crystal to isotropic droplet, from isotropic droplet to LC, and from isotropic droplet to crystal (Table 1). When the transitions were complete, the LC droplets were observable in the pleural area, small bronchus and bronchiole (Fig. 2A, E, V, X and Z) corresponding to the same locations where the crystals were observed. Birefringence density patterns of LC droplets (Fig. 2 α and β, and inset in γ) are significantly different than from those of the crystals (Fig. 2 γ, and inset in Z).

Table 1. Comparison of phase transitions between liquid crystal, crystal and isotropic droplets in liver mesonephros, lung and yolk sac of chicken embryo

Phase transition (Temperature °C)	LC to crystal	Crystal to isotopic	Isotopic to crystal (temperature slow decreasing)	Isotopic to LC	LC to isotopic
Liver	Overnight Frozen	39.8∼42.1	35.8∼36.1	Fast Cool	37.3∼41.4
Kidney (Metanephros)	Overnight Frozen	38.0∼40.6	35.2∼35.8	Fast Cool	36.8∼40.4
Lung	Overnight Frozen	38.6∼41.7	35.0∼35.4	Fast Cool	36.9∼41.0
York sac	Frozen	44.0∼46.0	46.0∼46.8	NON	38.9∼42.1

Since these phase transitions were discovered in chicken hepatic LC droplets [23,24], they have been confirm in different avian hepatic LC droplets [22] as well as generally observed in meso- and meta-anaphors during chicken kidney organogenesis [30]. Interestingly, decreased speed of temperature change is critical factor, which determines the direction of phase transitions to from isotropic to LC or crystal. When the rate of temperature change is rapid, isotropic droplets will resume to LC droplets, while slow rates of temperature change will change isotropic droplets back to crystals.

Although these transitions are common is most tissues and organs, this phenomenon could not be detected in yolk-sac LC. In this case, the LC droplets are mediating precursors for calcium carbonate vaterite crystallization [20,21].

5. Size Variation of Liquid Crystals in Embryonic Tissues and Organs

As discussed above, more than twenty tissues and organs display LC droplets, appear as birefringent Maltese crosses, at certain stages in embryonic development and earlier stages of postnatal development [15]. Despite the similarities in appearance, the sizes of Maltese crosses in liver and yolk-sac are much bigger than those found in other tissues.

In this study, we documented the sizes of Maltese's crosses in yolk sac, kidney (meta-nephros), liver and lung using chicken embryo at D18. Comparison of the LC droplets is shown in Figure 3. Excluding yolk sac LC droplets, the majority of the LC droplets in the different tissues ranged from roughly 4 μm to 800 μm in size. The smallest LC droplets, with diameters less than 80 μm, are found in the kidney, pleural area and internal area of lung. There are two sizes of LC droplets populating in the inside the lung, one ranging from 30∼40 μm and another around 300 μm.

The biggest LC droplets in chicken embryos were recorded in the yolk sac. Here diameters varied from 50 μm to 2500 μm, also containing two main populations, one with a diameter around 200 μm, the other around 2000 μm. The yolk sac LC droplets are different from the others because it involves the crystallization of calcium carbonate vaterite in two different patterns (inside-out and outside-in). Analysis of

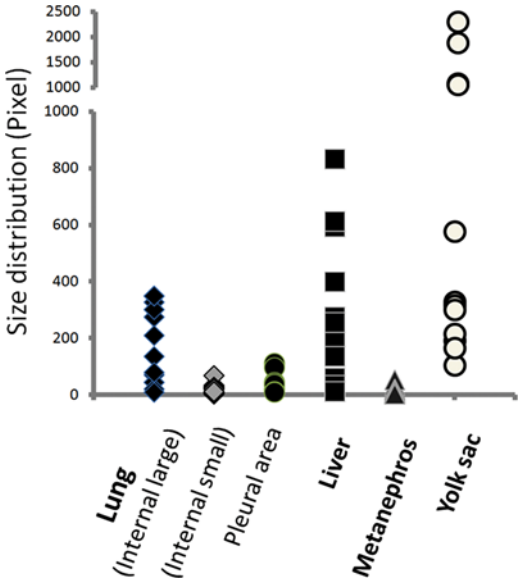

Figure 3. Size distribution of liquid crystal Maltese's crosses in different tissues in D18 embryo. (Figure appears in color online.)

Table 2. XRD comparison of hepatic crystal from different avian embryos

Newborn chicken		Newboi pheasant		Newborn taihe		Newborn quail		Newborn duck	
I/I°	d (Å)	I/I°	d (Å)	I/I°	d (Å)	I/I°	d (Å)	I/I°	d (Å)
100	19.49	100	19.24	100	19.32	100	19.36	100	19.32
3	17.11	2	16.98	12	17.05	5	17.11	8	17.05
4	3.73	4	9.67	4	9.65	4	9.71	3	9.73
2	5.94	2	5.92	10	5.91	4	5.91	6	5.91
1	5.13	1	5.12	2	5.11	1	5.13	2	5.12
11	4.91	13	4.9	35	4.9	21	4.91	23	4.9
2	4.G	2	4.59	10	4.6	4	4.59	6	4.59
3	4.09	2	4.06	**8**	4.09	5	4.09	8	4.03

Newborn pigeor				Newborn oriental dove			
Match		Non-match		Match		Non-match	
I/I°	d (Å)	I/I°	d (Å)	I/I°	d (Å)	I/I°	d (Å)
32	19.48	30	7.21	60	19.48	32	7.22
		33	5.01			32	5.09
		56	4.32			54	4.33
17	5.39	44	4.39	33	5.89	46	4.46
100	5.13	22	4.48	100	5.12	18	4.01

two chimeric structures with polarization microscopy revealed that this crystallization process is a unique mechanism associated with LC facilitated biomineral crystallization [21,22].

6. XRD Analysis of Embryonic Hepatic Liquid Crystal in Different Species

In chicken embryo, the LC droplets in liver are massive and appear as large numbers of birefringent Maltese's cross particles. When these hepatic LC particles transit to crystals after freezing overnight at –70°C, the crystals remains in crystalline form at room temperature [21,24,25]. Utilizing X-ray diffraction, multiple diffraction peaks of these crystals can be obtained at room temperature. Samples collected from embryos of different species of avian, displayed the same X-ray pattern as chicken. In table 2 we listed the 8 strongest diffraction peaks of the samples obtained from 6 species. The XRD patterns from the hepatic crystal of chicken, pheasant, Taihe, quail and duck are same (Table 2, top) showing the same Bragg distance [d(Å)] although the strengths of the diffractions (I/I0) are different. In pigeon and oriental dove (*S. orientalis*), among XRD diffractions, the embryonic hepatic crystal exhibits three diffractions corresponding to the above, and other five diffractions does not match the rest of the peaks (Table 2, bellow). This data indicates that the components of the hepatic LC droplets of pigeon are different from that of the other avian.

7. Conclusion and Perspectives

The LC droplets in found in embryonic chicken lung, observed during development, were distributed on the pleural area of the lung, including the pleura and pleural fluid as well as on the bronchus and bronchiole, where the LC droplets formed open rings corresponding to the developing bronchus and bronchiole. These LC droplets become crystals after samples are preserved through freezing. These LC droplets and crystals will transition to isotropic droplets regardless of the rate of temperature change. After these isotropic droplets have been transitions to birefringent droplets, the speed of temperature decrease is a critical factor in determining the subsequent formation of LC or crystal. In summation, although the sizes of the LC Maltese's crosses vary depending on tissue type, these phase transitions are common characteristics for embryonic LC droplets in other developing tissues and organs. LC droplets in the yolk-sac with ten to hundred fold larger sizes than those found in other tissues can be categorized separately, as they serve a purpose in calcium carbonate biomineralization through vaterite crystallization.

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