Recent advances on the avian eggshell biomineralization and on involvement of extracellular vesicles

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Summary

The eggshell is a critical barrier against mechanical stresses and microbial penetration. Its integrity is essential to maintain the hygienic quality of this basic human food and to limit the number of downgraded eggs. The eggshell is made of 95% mineral phase (calcium carbonate on calcite form) and an organic matrix (3.5%) mostly containing proteins. Eggshell formation arises from an extra-cellular biomineralization process. We describe in this review, the latest advances in the formation of the eggshell, which takes place in a fluid that contains eggshell precursors and involves a transient phase of amorphous calcium carbonate (ACC). We also describe recent insight on the identification of transient amorphous calcium carbonate explaining this rapid mineralization process. We also report on the advances on the function of shell matrix proteins to interact with mineral, thus determining the crystal polymorph, the first event of nucleation and the final texture of the shell and consequently its resulting mechanical properties. The role of vesicular transport to provide stabilized ACC in chicken uterine fluid where mineralization takes place was also demonstrated recently. These extra-cellular vesicles play a crucial role in eggshell mineralization, in which annexins transfer calcium into vesicles and carbonic anhydrase 4 catalyzes the formation of HCO3-, for accumulation of ACC in vesicles. ACC is stabilized by ovalbumin and/or lysozyme or additional proteins identified in vesicles in this study. Finally, EDIL3 and MFGE8 are proposed to guide EVs to the mineralization site.

Keywords: Chicken, eggshell, biomineralization, calcium supply, extracellular vesicles

Introduction

The eggshell constitutes the external envelope of the eggs and fulfils five essential functions to allow the harmonious development of a chicken embryo. It prevents the dehydration of the internal environment of the terrestrial egg, it ensures a role of physical protection against the

shocks, a thermic protection, it allows gas exchanges and it prevents the penetration of the microbes. The shell is the only non-consumable part of an egg, and a large number of socio-economic issues for the consumer egg industry will depend on its integrity and quality. Thus, cracked shells will lead to an economic loss for the producer and to food infection risks for the consumers. Moreover, in the current context of evolution of the societal demand for rearing systems with outdoor runs and an extension of the production period, the maintenance of the integrity of the shell is then preponderant to guarantee a healthy egg and preserving good mechanical properties (Gautron et al., 2021). The shell quality depends of numerous factors as genetics of the birds, the hen's physiology, the environment, the nutrition and management of hens. Then finally the egg quality is depending of the « insult » that occur in the rearing system, the egg transport and egg sorting. Many of these factors impacting shell quality are perfectly controlled. The use of appropriate genetic, optimal nutrition, limit but do not eliminate the breakage, notably for elderly birds for which the egg percentage breakage can increase to 10-12% at the end of laying period.

Further improvement of the mechanical properties of the shell will be achieved by taking into account not only the mass of the shell, but also mechanisms largely dependent on the ultrastructure of the shell, i.e. the arrangement, shape and orientation of the constituent crystals that give the shell its structure and mechanical properties (Gautron et al., 2021, Nys et al., 2022). This manufacturing process is the result of an interaction between minerals and proteins secreted in the formation environment that control this process. The knowledge of these processes is crucial to allow the integration of this component in new genomic selection programs and also to study nutritional factors such as vitamin D whose metabolism could be limiting at different periods of the hen's life and, which could potentially be corrected by nutrition. The objective of this paper is therefore to review the state of knowledge on the mechanisms of eggshell biomineralization, in order to identify avenues for further improvement.

Eggshell structure, formation and composition

The chicken eggshell contains 1.6 % water, 3.3 to 3.5 % organic matrix when eggshell membranes are included and 95 % inorganic minerals. It is mainly made of calcium carbonate (98.4 % of its mineral part), which is pervaded by an organic matrix corresponding to 2.3 % of the shell weight. From inside to outside, six different layers are observed in the eggshell (Gautron et al., 2021). In chicken, the most documented bird, the eggshell is about 0.4 mm thick. The innermost layers are made of two shell membranes composed of interlacing protein

fibers. There are two of them and they are entirely made up of organic matter. The inner shell membrane is 20 µm thick and is in contact with the egg white. It is from the outer shell membrane (50 µm) that the mineralisation of the shell is initiated to give rise to the mammillary layer. The mamillary layer of about 70 µm is the innermost part of the calcified layer. Its base consists of the mamillary knobs which are organic clusters deposited on the surface of the outer shell membrane and from which mineralisation is initiated. The mineralisation continues outwards, initially forming a cone or mamelon-like structure. The palisade layer begins when the multidirectional growth of the cones of the mamillary layer leads to a fusion of adjacent cones. The palisade layer is therefore a compact layer of minerals associated with an organic framework. This continuity is broken at the level of the pores which cross the shell from one side to the other to allow the gas exchanges necessary for the development of the embryo. A surface layer of small adjacent single calcite crystals is then deposited vertically on the surface of the palisade layer under the cuticle. The cuticle is the outermost layer of the egg and consists of organic material. The cuticle closes the pores and thus prevents the penetration of bacteria into the egg. Gas exchange is made possible by cracks that appear in the dried cuticle.

Shell mineralisation occurs in the uterine part of the oviduct of birds. When the egg enters the uterus five hours after ovulation of the yolk, it is a soft egg on which mineralisation will start in a process that will last about 17 hours in the laying hen. This process takes place in the lumen of the organ, where the physico-chemical conditions necessary for cell-free biomineralisation are present. Shell formation is temporally controlled, and in chickens four main steps can be identified during the 17 h process (from 5 h to 22 h post-ovulation) (Rodriguez-Navarro et al. 2015). They corresponded to the initial stages dominated by amorphous calcium carbonate (ACC) deposition on eggshell membranes (5 h p.o.), its progressive transformation to form calcite aggregates on mammillary knobs surrounded by ACC particle and the growth of large calcite units surrounded by ACC. Calcite crystals rapidly grow to form larger crystal units. The interaction with eggshell organic matrix components inhibits calcite crystal faces parallel to the c-axis, thus causing elongated crystal growth in this direction. Calcite crystals growing with their c-axis nearly perpendicular to the surface block the growth of adjacent crystals with less favourable orientations, resulting in the development of columnar calcite units. Finally, mineralization is terminated and a thin proteinaceous layer (cuticle) is deposited on the shell surface.

Molecular control of the avian eggshell biomineralization

There are two physiological processes that allow the mineralisation of the shell. They are the transfer mechanisms of the large quantity of minerals necessary for the formation of the shell and the biomineralisation process controlled by the organic matrix to give an ordered structure with exceptional mechanical properties.

Role of organic matrix proteins during eggshell biomineralization

During its formation, the shell is bathed in a uterine fluid (UF) secreted by uterine cells that contains the organic and mineral precursors necessary for shell calcification (Gautron et al., 1997). The transition of ions to a crystalline state is achieved through amorphous transitional forms allowing crystallisation under physiological conditions. In birds, calcium carbonate is initially deposited as an amorphous calcium carbonate phase (ACC), which progressively transforms into calcite (Rodriguez et al., 2015). Matrix proteins play a crucial role in this process. They stabilize ACC, promote crystal nucleation, select the calcite polymorph, and regulate the evolution of crystal size and morphology (Gautron et al., 2021 ; Dominiguez-Vera et al., 2000 ; Hernandez-Hernandez et al., 2008). These matrix–mineral interactions determine the orientation of calcite crystals, which results in the complex ultrastructure of the eggshell, its texture, and consequently, its mechanical properties. These observations have largely stimulated research to identify organic matrix proteins by proteomic and transcriptomic approaches. The set of sequences identified were grouped into 904, 697, 622, 475, 484 and 149 unique proteins constituting the chicken, turkey, quail, zebra finch, duck and Guinea fowl eggshell proteomes (Gautron et al., 2019; Mann and Mann, 2013; 2015; Mann, 2015; Le Roy et al., 2019). The role and function of these proteins in shell calcification has only been studied in chicken and only for a limited number (Gautron et al., 2021; Hincke et al., 2012). Among this large list of shell matrix proteins, are proteins with an established role in the biomineralization, which directly interact with the mineral phase to stabilize ACC and/or to modify the morphology of crystals that determine the eggshell ultrastructure of avian eggshells and their resulting mechanical properties. Another group is composed of proteins involved in the regulation of proteins directing mineralization. This group is made of uterine fluid proteins that interact with proteins directing mineralization. Indeed, mineralization takes place in an acellular medium and the proteins belonging to this group inhibit or activate proteins of the mineralization milieu. Some of these proteins may be involved in proper folding of eggshell matrix proteins to ensure an appropriate template for calcium and mineral interactions. Protease and protease inhibitors are also belonging to this group. They are believed to play specific and

controlled roles during the calcification process, either by degrading proteins or regulating processing of proteins into their mature forms.

Regulation of calcium supply

The calcium metabolism linked to egg formation in birds is strongly exaggerated. Indeed, there is no calcium storage in the shell gland (uterus) before shell formation (Nys et al., 2022). Calcium is directly provided by ionic blood calcium, to supply daily the necessary 2 g of shell calcium. Calcium is provided by the hen diet, directly by intestinal absorption, although 40% of this is derived from bone mobilisation because of desynchronization between the period of feed intake (daytime) and shell formation, which mainly takes place during the night (Nys et Le Roy, 2018: Nys et al., 2022). Both components of the shell mineral (Ca_2^+ and CO_3^{2-}) are continuously supplied during eggshell formation via the blood plasma, firstly by trans-epithelial ionic transport through the uterine epithelium and secondly, by vesicular secretion of ACC mineral particles.

A comprehensive and further refined model for calcium and carbonate transport to the mineralization site during eggshell formation was recently proposed (Nys et al., 2018; Nys et al., 2022; Gautron et al., 2021). Calcium and carbon dioxide originate from the blood. Blood CO^2 passively diffuses into uterine cells (Hodges and Lörcher, 1967), where it is hydrated by Carbonic Anhydrase 2 (CA2). Alternatively, bicarbonate can be actively transferred into uterine cells using the Na^+/HCO_3^- co-transporters SLC4A4-A5-A10 (Nys and Le Roy, 2018). Bicarbonates are actively extruded from cells by the HCO_3^-/Cl^- exchanger SLC26A9 (Nys and Le Roy, 2018). Additionally, bicarbonate ions can be directly produced in the uterine fluid by hydration of CO^2 by membrane-bound CA4, which has its active site in the extracellular space (Zhu et al., 1990). The transcellular pathway to secrete calcium and bicarbonate ions into the fluid has been previously described (Jonchère et al., 2012; Brionne et al., 2014). Plasma Ca^{2+} is transferred into uterine cells by transient receptor potential cation channels (TRPVs) and/or otopetrin 2 (OTOP2) and/or ATPase secretory pathway Ca2+ transporting 2 (ATP2C2) (Sah et al., 2018; Nys and Le Roy, 2018). Intracellular calcium ions are buffered/transferred by calbindin. Other Ca^{2+} pumps associated with the endoplasmic reticulum could also be involved in this transfer (ATP2A2/3 and ITPR1/2/3). Finally, the Ca_2^+/Na_2^+ exchangers SLC8A1-3 and the Ca_2^+ pumps ATP2B1-B2 are involved in the apical extrusion of calcium into the uterine fluid (Sah et al., 2018; Nys and Le Roy, 2018). Uterine Ca_2^+ secretion is quantitatively associated with calbindin levels and the regulation of uterine calcium transfer in conjunction with its synthesis has been studied in detail (Nys and Le Roy, 2018; Bar, 2009).

A paracellular Ca2+ uptake pathway is present in intestine [95] and acts to replenish calcium from dietary sources during eggshell biomineralization when soluble calcium in the intestinal lumen creates a favorable gradient for passive absorption This intestinal paracellular pathway involves claudins (CLDN), occludins (OCN), junctional adhesion molecules (JAM) and tight junction proteins (TJP) (Gloux et al., 2019). RNA-Seq analysis reveals the expression of several genes of this paracellular pathway (Tjp1, Cldn1, Cldn10, Ocln, Jam2) (Gautron et al., 2020). Moreover, expression of Cldn10 has also been detected in chicken uterus (Sah et al., 2018; Yin et al., 2019). This paracellular pathway is probably contributing to the secretion of water and ions for osmotic regulation (K, Na) during the process of eggshell formation. The ionic calcium concentration in uterine fluid ranges from 6 to 10 mM depending of the stage of calcification (Nys et al., 1991), which is 6 times higher than blood calcium levels (1-2 mM); consequently, the concentration gradient is not in favor of calcium movement towards the uterine fluid through the paracellular pathway (Nys and Le Roy, 2018). However, Bar (2009) suggested that the electrical potential difference could invert this gradient, allowing some paracellular transfer of calcium into the uterine fluid. Consequently, the paracellular pathway could participate to maintain ionic and osmotic homeostasis.

Extracellular vesicles to transport and stabilize transient forms of calcium

More recently, Stapane et al (2019-2020), have demonstrated a vesicular mechanism to stabilise the transient forms of calcium carbonate necessary for calcite crystal formation. Evaluation of CaCO3 vesicular transport in chicken uterus was initiated following the observation of high levels of vesicular protein markers (EDIL3 and MFGE8) in eggshell and in uterine fluid during shell formation (Marie et al., 2015a). Bioinformatics tools, mRNA levels and protein quantification were used to explore the role of EDIL3 and MFGE8 in chicken eggshell biomineralization. In avian uterus, transmission electron microscopy (TEM) observations demonstrated the presence of intracellular vesicles (100 to 500 nm) in the cytoplasm of the epithelial ciliated cells (Stapane et al., 2020). Vesicles accumulate at the apical plasma membrane and bud to secrete extracellular vesicles (EVs), which were revealed in uterine fluid adjacent to the apical region of uterine cells (Stapane et al., 2020). The presence of calcium carbonate as ACC in the vesicles was confirmed by electron energy loss spectroscopy (EELS) and by energy-dispersive X-ray spectroscopic (EDS). Electron diffraction on EVs extracted from uterine fluid indicated that the calcium carbonate inside vesicles was amorphous, similar to the ACC previously identified at the initial stage of eggshell formation (Rodriguez-Navarro et al., 2015). This observation was further explored by studying the presence of major EV

proteins using transcriptomics, proteomics and immunochemistry to decipher the origin and mechanisms of vesicle formation and function.

EDIL3 and MFGE8 bind to EVs budding from uterine cells into the uterine fluid, in order to guide vesicular transport of stabilized ACC for delivery to the mineralizing site and moreover prevent non-specific precipitation. Three annexins (Anxa 1, 2 and 8) are expressed at high levels in the uterus at the onset of shell formation (Stapane et al., 2020), in agreement with previous proteomics studies (Mann, 2006; Jonchere et al., 2012; Marie et al., 2015b), and were revealed in the epithelium (Anxa 1, 8) and tubular glands (Anxa 8) by immunochemistry. Annexins are Ca channels proposed to contribute to uptake of Ca for intra-vesicular ACC formation. EDIL3 is overexpressed in the uterus and is specific to the uterine fluid EV fraction (Stapane et al. 2019; 2020). This protein possesses an EGF-like calcium- binding domain and is hypothesized to guide EVs to the mineralisation front. Carbonic anhydrase 4 (CA4) is present in the epithelial cells and in EVs and is highly expressed at the early stage of shell formation. CA4 catalyzes the reversible hydration of CO2 forming HCO3 and might contribute to accumulation of ACC in vesicles.

A global representation of vesicular transport and molecular actors during eggshell mineralization was proposed (Stapane et al., 2020; Gautron et al., 2021; Nys et al., 2022). Annexins would promote calcium entry into EVs, whereas CA4 would catalyze the hydration of CO2 into bicarbonate ions. ACC accumulates inside EVs and is stabilized by specific proteins. EDIL3 and potentially MFGE8 would serve as guidance molecules to deliver vesicular ACC to the mineralization site. The quantitative contribution of the vesicular secretion of CaCO3, relative to the secretion of each ion by the transcellular pathway, remains to be explored.

Vitamin D and Regulation of the molecular actors involved in the shell calcification

If the calcium contained in the eggshell comes entirely from the food, there is a desynchronization between the need for calcium for the formation of the eggshell during the night and the dietary intake of this calcium during the day. To do this, the hen has a particular bone structure, the medullary bone, which is mobilized during the night to provide part of the calcium necessary for the calcification of the shell. During the day, when the hen has access to its food, the medullary part of the bone will be mineralized again (Nys et al., 2022). The regulation of calcium metabolism during shell formation in the hen involves many organs. First, the gut, which will allow the transfer of calcium to the bone and uterus, via the bloodstream. It

also involves the uterus, which will have to transfer to the calcification site (uterine fluid), large quantities of calcium necessary for the formation of the shell while maintaining cellular homeostasis. Vitamin D and in particular its active metabolite ($1.25(OH)_2D_3$), will play a crucial role in the regulation of calcium transfers at the intestinal and bone level. Vitamin D is first hydroxylated to 25-hydroxycholecalciferol (25-OH-D_3) in the liver before being hydroxylated to $1.25(OH)_2D_3$ in the kidney (Christakos. et al, 2010). The use of the hydroxylated form (25-OH-D_3) in the feed has a metabolic advantage by avoiding the initial hepatic step and would allow for better availability of the intermediate vitamin D metabolite. This role at the uterine level has been little explored and it is generally accepted that vitamin D would have no effect at the uterine level in the hen. In a recent study (Gautron et al., 2022), hens were fed with vitamin D_3 and hydroxylated form (25-OH-D_3) and the expression level in the uterus was analyzed for 91 genes. Of these, 17 genes encode organic matrix proteins known to play a major role in shell mineralization and 65 encode transporters of calcium, bicarbonate and other ions necessary for mineralization. Additionally, 21 overexpressed genes code for paracellular transport proteins and 44 allow transcellular transfers. It is particularly notable that all of these genes are stimulated by 25-OH-D_3. This study clearly shown that vitamin D plays an important role in the regulation of calcium and mineral transfers in the hen's uterus. This role is not limited to calcium transfers to the gut and bone as previously described. Furthermore, this study shows that the use of the hydroxylated form of vitamin D3 as 25-OH-D_3 allows an overexpression of many genes involved in the transcellular, vesicular and paracellular calcium transfer pathways, as well as an overexpression of genes encoding organic matrix proteins.

Conclusion

The shell of chicken eggs is a complex structure and although it is not eaten, it is crucial to allow the marketing of eggs. It is therefore the object of particular attention from the point of view of breeding to improve its mechanical properties, as well as for scientists to understand its calcification. During the last 20 years, considerable progress has been made in terms of understanding the mechanisms of regulation, mineral supply and molecular actors of its biomineralization, which are at the origin of the mechanical properties of this natural biomaterial. This knowledge is already being used by breeders to integrate this component into the precision of genomic selection and to allow new gains other than those integrating shell mass alone. Recently, it has also been shown that shell formation is dependent on vitamin D and its form of intake. All this knowledge opens an important field of perspectives for a genetic-

nutrition interaction in order to improve shell quality in a sustainable way during production cycles maintained at advanced ages and in a strong context of evolution of the production systems of eggs for consumption towards alternative breeding.

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