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

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6 **Abbreviated title:** Review on avian eggshell biomineralization

7 **Summary**

8 The eggshell is a critical barrier against mechanical stresses and microbial penetration. Its
9 integrity is essential to maintain the hygienic quality of this basic human food and to limit the
10 number of downgraded eggs. The eggshell is made of 95% mineral phase (calcium carbonate
11 on calcite form) and an organic matrix (3.5%) mostly containing proteins. Eggshell formation
12 arises from an extra-cellular biomineralization process. We describe in this review, the latest
13 advances in the formation of the eggshell, which takes place in a fluid that contains eggshell
14 precursors and involves a transient phase of amorphous calcium carbonate (ACC). We also
15 describe recent insight on the identification of transient amorphous calcium carbonate
16 explaining this rapid mineralization process. We also report on the advances on the function of
17 shell matrix proteins to interact with mineral, thus determining the crystal polymorph, the first
18 event of nucleation and the final texture of the shell and consequently its resulting mechanical
19 properties. The role of vesicular transport to provide stabilized ACC in chicken uterine fluid
20 where mineralization takes place was also demonstrated recently. These extra-cellular vesicles
21 play a crucial role in eggshell mineralization, in which annexins transfer calcium into vesicles
22 and carbonic anhydrase 4 catalyzes the formation of HCO_3^- , for accumulation of ACC in
23 vesicles. ACC is stabilized by ovalbumin and/or lysozyme or additional proteins identified in
24 vesicles in this study. Finally, EDIL3 and MFGE8 are proposed to guide EVs to the
25 mineralization site.

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

27 **Introduction**

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

31 shocks, a thermic protection, it allows gas exchanges and it prevents the penetration of the
32 microbes. The shell is the only non-consumable part of an egg, and a large number of socio-
33 economic issues for the consumer egg industry will depend on its integrity and quality. Thus,
34 cracked shells will lead to an economic loss for the producer and to food infection risks for the
35 consumers. Moreover, in the current context of evolution of the societal demand for rearing
36 systems with outdoor runs and an extension of the production period, the maintenance of the
37 integrity of the shell is then preponderant to guarantee a healthy egg and preserving good
38 mechanical properties (Gautron et al., 2021). The shell quality depends of numerous factors as
39 genetics of the birds, the hen's physiology, the environment, the nutrition and management of
40 hens. Then finally the egg quality is depending of the « insult » that occur in the rearing system,
41 the egg transport and egg sorting. Many of these factors impacting shell quality are perfectly
42 controlled. The use of appropriate genetic, optimal nutrition, limit but do not eliminate the
43 breakage, notably for elderly birds for which the egg percentage breakage can increase to 10-
44 12% at the end of laying period.

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

57 **Eggshell structure, formation and composition**

58 The chicken eggshell contains 1.6 % water, 3.3 to 3.5 % organic matrix when eggshell
59 membranes are included and 95 % inorganic minerals. It is mainly made of calcium carbonate
60 (98.4 % of its mineral part), which is pervaded by an organic matrix corresponding to 2.3 % of
61 the shell weight. From inside to outside, six different layers are observed in the eggshell
62 (Gautron et al., 2021). In chicken, the most documented bird, the eggshell is about 0.4 mm
63 thick. The innermost layers are made of two shell membranes composed of interlacing protein

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

79 Shell mineralisation occurs in the uterine part of the oviduct of birds. When the egg enters the
80 uterus five hours after ovulation of the yolk, it is a soft egg on which mineralisation will start
81 in a process that will last about 17 hours in the laying hen. This process takes place in the lumen
82 of the organ, where the physico-chemical conditions necessary for cell-free biomineralisation
83 are present. Shell formation is temporally controlled, and in chickens four main steps can be
84 identified during the 17 h process (from 5 h to 22 h post-ovulation) (Rodriguez-Navarro et al.
85 2015). They corresponded to the initial stages dominated by amorphous calcium carbonate
86 (ACC) deposition on eggshell membranes (5 h p.o.), its progressive transformation to form
87 calcite aggregates on mammillary knobs surrounded by ACC particle and the growth of large
88 calcite units surrounded by ACC. Calcite crystals rapidly grow to form larger crystal units. The
89 interaction with eggshell organic matrix components inhibits calcite crystal faces parallel to the
90 c-axis, thus causing elongated crystal growth in this direction. Calcite crystals growing with
91 their c-axis nearly perpendicular to the surface block the growth of adjacent crystals with less
92 favourable orientations, resulting in the development of columnar calcite units. Finally,
93 mineralization is terminated and a thin proteinaceous layer (cuticle) is deposited on the shell
94 surface.

95

96 **Molecular control of the avian eggshell biomineralization**

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

101 *Role of organic matrix proteins during eggshell biomineralization*

102 During its formation, the shell is bathed in a uterine fluid (UF) secreted by uterine cells that
103 contains the organic and mineral precursors necessary for shell calcification (Gautron et al.,
104 1997). The transition of ions to a crystalline state is achieved through amorphous transitional
105 forms allowing crystallisation under physiological conditions. In birds, calcium carbonate is
106 initially deposited as an amorphous calcium carbonate phase (ACC), which progressively
107 transforms into calcite (Rodriguez et al., 2015). Matrix proteins play a crucial role in this
108 process. They stabilize ACC, promote crystal nucleation, select the calcite polymorph, and
109 regulate the evolution of crystal size and morphology (Gautron et al., 2021 ; Dominiguez-Vera
110 et al., 2000 ; Hernandez-Hernandez et al., 2008). These matrix–mineral interactions determine
111 the orientation of calcite crystals, which results in the complex ultrastructure of the eggshell, its
112 texture, and consequently, its mechanical properties. These observations have largely
113 stimulated research to identify organic matrix proteins by proteomic and transcriptomic
114 approaches. The set of sequences identified were grouped into 904, 697, 622, 475, 484 and 149
115 unique proteins constituting the chicken, turkey, quail, zebra finch, duck and Guinea fowl
116 eggshell proteomes (Gautron et al., 2019; Mann and Mann, 2013; 2015; Mann, 2015; Le Roy
117 et al., 2019). The role and function of these proteins in shell calcification has only been studied
118 in chicken and only for a limited number (Gautron et al., 2021; Hincke et al., 2012). Among
119 this large list of shell matrix proteins, are proteins with an established role in the
120 biomineralization, which directly interact with the mineral phase to stabilize ACC and/or to
121 modify the morphology of crystals that determine the eggshell ultrastructure of avian eggshells
122 and their resulting mechanical properties. Another group is composed of proteins involved in
123 the regulation of proteins directing mineralization. This group is made of uterine fluid proteins
124 that interact with proteins directing mineralization. Indeed, mineralization takes place in an
125 acellular medium and the proteins belonging to this group inhibit or activate proteins of the
126 mineralization milieu. Some of these proteins may be involved in proper folding of eggshell
127 matrix proteins to ensure an appropriate template for calcium and mineral interactions. Protease
128 and protease inhibitors are also belonging to this group. They are believed to play specific and

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

131 *Regulation of calcium supply*

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

142 A comprehensive and further refined model for calcium and carbonate transport to the
143 mineralization site during eggshell formation was recently proposed (Nys et al., 2018; Nys et
144 al., 2022; Gautron et al., 2021). Calcium and carbon dioxide originate from the blood. Blood
145 CO_2 passively diffuses into uterine cells (Hodges and Lörcher, 1967), where it is hydrated by
146 Carbonic Anhydrase 2 (CA2). Alternatively, bicarbonate can be actively transferred into uterine
147 cells using the $\text{Na}^+/\text{HCO}_3^-$ co-transporters SLC4A4-A5-A10 (Nys and Le Roy, 2018).
148 Bicarbonates are actively extruded from cells by the $\text{HCO}_3^-/\text{Cl}^-$ exchanger SLC26A9 (Nys and
149 Le Roy, 2018). Additionally, bicarbonate ions can be directly produced in the uterine fluid by
150 hydration of CO_2 by membrane-bound CA4, which has its active site in the extracellular space
151 (Zhu et al., 1990). The transcellular pathway to secrete calcium and bicarbonate ions into the
152 fluid has been previously described (Jonchère et al., 2012; Brionne et al., 2014). Plasma Ca^{2+}
153 is transferred into uterine cells by transient receptor potential cation channels (TRPVs) and/or
154 otopetrin 2 (OTOP2) and/or ATPase secretory pathway Ca_2^+ transporting 2 (ATP2C2) (Sah et
155 al., 2018; Nys and Le Roy, 2018). Intracellular calcium ions are buffered/transferred by
156 calbindin. Other Ca^{2+} pumps associated with the endoplasmic reticulum could also be involved
157 in this transfer (ATP2A2/3 and ITPR1/2/3). Finally, the $\text{Ca}_2^+/\text{Na}_2^+$ exchangers SLC8A1-3 and
158 the Ca_2^+ pumps ATP2B1-B2 are involved in the apical extrusion of calcium into the uterine
159 fluid (Sah et al., 2018; Nys and Le Roy, 2018). Uterine Ca_2^+ secretion is quantitatively
160 associated with calbindin levels and the regulation of uterine calcium transfer in conjunction
161 with its synthesis has been studied in detail (Nys and Le Roy, 2018; Bar, 2009).

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

178 *Extracellular vesicles to transport and stabilize transient forms of calcium*

179 More recently, Stapane et al (2019-2020), have demonstrated a vesicular mechanism to stabilise
180 the transient forms of calcium carbonate necessary for calcite crystal formation. Evaluation of
181 CaCO_3 vesicular transport in chicken uterus was initiated following the observation of high
182 levels of vesicular protein markers (EDIL3 and MFGE8) in eggshell and in uterine fluid during
183 shell formation (Marie et al., 2015a). Bioinformatics tools, mRNA levels and protein
184 quantification were used to explore the role of EDIL3 and MFGE8 in chicken eggshell
185 biomineralization. In avian uterus, transmission electron microscopy (TEM) observations
186 demonstrated the presence of intracellular vesicles (100 to 500 nm) in the cytoplasm of the
187 epithelial ciliated cells (Stapane et al., 2020). Vesicles accumulate at the apical plasma
188 membrane and bud to secrete extracellular vesicles (EVs), which were revealed in uterine fluid
189 adjacent to the apical region of uterine cells (Stapane et al., 2020). The presence of calcium
190 carbonate as ACC in the vesicles was confirmed by electron energy loss spectroscopy (EELS)
191 and by energy-dispersive X-ray spectroscopic (EDS). Electron diffraction on EVs extracted
192 from uterine fluid indicated that the calcium carbonate inside vesicles was amorphous, similar
193 to the ACC previously identified at the initial stage of eggshell formation (Rodriguez-Navarro
194 et al., 2015). This observation was further explored by studying the presence of major EV

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

197 EDIL3 and MFGE8 bind to EVs budding from uterine cells into the uterine fluid, in order to
198 guide vesicular transport of stabilized ACC for delivery to the mineralizing site and moreover
199 prevent non-specific precipitation. Three annexins (Anxa 1, 2 and 8) are expressed at high levels
200 in the uterus at the onset of shell formation (Stapane et al., 2020), in agreement with previous
201 proteomics studies (Mann, 2006; Jonchere et al., 2012; Marie et al., 2015b), and were revealed
202 in the epithelium (Anxa 1, 8) and tubular glands (Anxa 8) by immunochemistry. Annexins are
203 Ca channels proposed to contribute to uptake of Ca for intra-vesicular ACC formation. EDIL3
204 is overexpressed in the uterus and is specific to the uterine fluid EV fraction (Stapane et al.
205 2019; 2020). This protein possesses an EGF-like calcium- binding domain and is hypothesized
206 to guide EVs to the mineralisation front. Carbonic anhydrase 4 (CA4) is present in the epithelial
207 cells and in EVs and is highly expressed at the early stage of shell formation. CA4 catalyzes
208 the reversible hydration of CO₂ forming HCO₃ and might contribute to accumulation of ACC
209 in vesicles.

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

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

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

227 also involves the uterus, which will have to transfer to the calcification site (uterine fluid), large
228 quantities of calcium necessary for the formation of the shell while maintaining cellular
229 homeostasis. Vitamin D and in particular its active metabolite (1.25(OH)₂D₃), will play a crucial
230 role in the regulation of calcium transfers at the intestinal and bone level. Vitamin D is first
231 hydroxylated to 25-hydroxycholecalciferol (25-OH-D₃) in the liver before being hydroxylated
232 to 1.25(OH)₂D₃ in the kidney (Christakos. et al, 2010). The use of the hydroxylated form (25-
233 OH-D₃) in the feed has a metabolic advantage by avoiding the initial hepatic step and would
234 allow for better availability of the intermediate vitamin D metabolite. This role at the uterine
235 level has been little explored and it is generally accepted that vitamin D would have no effect
236 at the uterine level in the hen. In a recent study (Gautron et al., 2022), hens were fed with
237 vitamin D₃ and hydroxylated form (25-OH-D₃) and the expression level in the uterus was
238 analyzed for 91 genes. Of these, 17 genes encode organic matrix proteins known to play a major
239 role in shell mineralization and 65 encode transporters of calcium, bicarbonate and other ions
240 necessary for mineralization. Additionally, 21 overexpressed genes code for paracellular
241 transport proteins and 44 allow transcellular transfers. It is particularly notable that all of these
242 genes are stimulated by 25-OH-D₃. This study clearly shown that vitamin D plays an important
243 role in the regulation of calcium and mineral transfers in the hen's uterus. This role is not limited
244 to calcium transfers to the gut and bone as previously described. Furthermore, this study shows
245 that the use of the hydroxylated form of vitamin D₃ as 25-OH-D₃ allows an overexpression of
246 many genes involved in the transcellular, vesicular and paracellular calcium transfer pathways,
247 as well as an overexpression of genes encoding organic matrix proteins.

248

249 **Conclusion**

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

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

263

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