Role of extracellular matrix molecules in shell formation and structure

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Avian eggshells are natural composite bioceramics containing organic and inorganic phases. The occurrence and role of particular collagens and proteoglycans in the eggshell and their pattern of secretion by the oviduct is discussed. While type X collagen, the main constituent of the shell membranes, functions to inhibit their mineralisation, mammillan, a mammillary keratan sulphate proteoglycan, is involved in nucleation of the first calcite crystals of the shell and ovoglycan, a unique dermatan sulphate proteoglycan, is involved in the growth of the crystalline palisade. Eggshell biomineralisation is effected by specialised oviduct cell populations in a defined topographically and temporally regulated process.

Keywords: Biomineralisation; eggshell; dermatan sulphate; keratan sulphate; mammillan; oviduct; ovoglycan; type X collagen

Introduction

The avian eggshell is a microenvironmental compartment which provides physical protection to the embryo and regulates gas, water and ionic exchange. It is a natural composite bioceramic containing organic (3.5%) and inorganic (95%) phases (Heuer *et al.*, 1992; Arias *et al.*, 1993) and is composed of a two-layered membrane and calcified extracellular matrix which are sequentially assembled during the 22 hours the egg moves along the oviduct (Fernandez *et al.*, 1997). Ultrastructurally, eggshells are composed of shell membranes, mammillary knobs, palisade and cuticle (see Arias *et al.*, 1993).

The avian oviduct involved in egg formation is a tubular organ responsible for the transport of the egg and the secretion of the components surrounding the yolk. It is organised into five regions: from proximal to distal, the *infundibulum* which receives the ovum, the *magnum* which secretes albumen, the *isthmus* which

secretes precursors of the shell membranes, the *red isthmus* or *tubular shell gland* where the mammillary knobs are formed and the initial process of calcium deposition is targeted specifically at them and, finally, the *shell gland* or *uterus* which adds calcium to the shell, forms the cuticle and increases the egg weight by the addition of "plumping" fluid to the albumen (Solomon, 1991). The mucosa of the shell forming region of the oviduct comprises a lining epithelium with ciliated and non-ciliated cells and tubular glands (Breen and De Bruyn, 1969; Draper *et al.*, 1972; Wyburn *et al.*, 1973; Solomon, 1983).

Although other reviews have focused on the formation, structure, organisation, and chemical composition of eggshells (Solomon, 1991, 1999; Arias *et al.*, 1993; Roberts *et al.*, 1994; Nys *et al.*, 1997, 1999; Fernandez and Arias, 2000; Lavelin *et al.*, 2000), the present review attempts to provide an integrated summary of what our laboratory, alone or in a collaborative work, has done on the cell biology, morphological organisation, crystallography, chemical composition and process of biomineralisation of avian eggshells.

Structural organisation of the chicken eggshell

The structure of the eggshell is shown diagrammatically in *Figure 1*. The first layer to be formed in the eggshell comprises the shell membranes. These are two nets of fibres composed of a core surrounded by a fuzzy material referred to as a mantle (Masshoff and Stolpmann, 1961; Simons, 1971) containing the inner net of thinner fibres. Sparsely deposited on the outer side of the shell membranes are the mammillary knobs which are the sites where crystal nucleation takes place (Robinson and King, 1963; Stemberger *et al.*, 1977), forming together the so-called mammillary layer. Mamillary knobs consist of the calcium reserve assembly (CRA) and crown region. The CRA consists of a dense, flocculent material partially embedded within the outer shell membrane. It is capped by a centrally located calcium reserve body (CRB) sac containing numerous 100–300 nm electron dense spherical vesicles and a base plate closely associated with the shell membranes (Dieckert *et al.*, 1989; Dennis *et al.*, 1996). The calcified layer proper, or

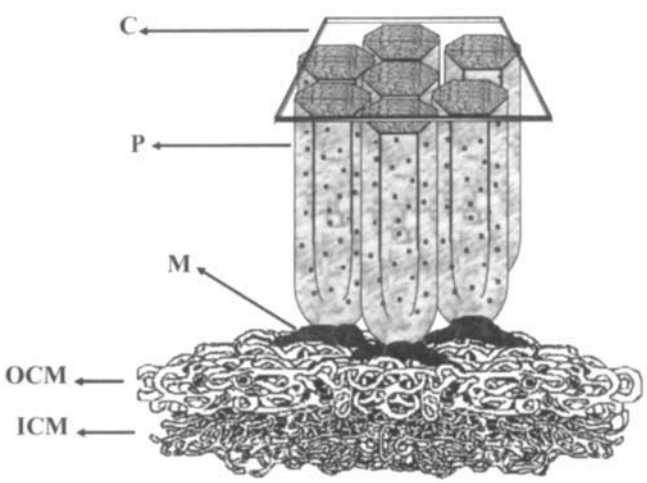

Figure 1 Diagram of a transverse section of chicken eggshell. C, cuticle; P, palisade; M, mammillae; OCM, outer shell membrane; ICM, inner shell membrane.

palisade, is the region formed by calcite columns beginning above each mammilla and ending below the cuticle. Closely associated with the columns is an organic material known as the shell matrix which is composed of vesicular structures interspersed with a fibrillar matrix material. Crystallographic and morphological studies of the palisade reveal three subzones (cone, central, and vertical crystal layers) which differ in their crystal orientation (Arias *et al.*, 1993). This region contains many vesicles between the crystals (Simons, 1971; Wyburn *et al.*, 1973; Arias *et al.*, 1993; Dennis *et al.*, 1996; Fraser *et al.*, 1998). The last formed layer, the cuticle, covers the entire shell and is made out of glycoproteins, a large part of the pigments in coloured eggs, and hydroxyapatite crystals (Nys *et al.*, 1991; Dennis *et al.*, 1996; Fraser *et al.*, 1999).

Chemical composition of eggshells

We have shown that, among other molecules described elsewhere (Pines *et al.*, 1994; Hincke, 1995; Hincke *et al.*, 1995, 2000; Gautron *et al.*, 1996, 1997, 1998; Lavelin *et al.*, 1998; Mann and Siedler, 1999; Nys *et al.*, 1999; Mann, 1999; Panheleux *et al.*, 2000) whose role in the calcification process has not yet been elucidated, the organic phase of the eggshell contains particular collagens and proteoglycans (Arias *et al.*, 1991a, 1992, 1997a; Carrino *et al.*, 1996, 1997; Panheleux *et al.*, 1999a, b) whose occurrence depends on a defined topographical and temporal deposition (Fernandez *et al.*, 1997).

Although early studies suggested that the shell membrane is composed of keratin (see Baker and Balch, 1962), we have found that this suggestion is inaccurate (Arias *et al.*, 1991b) and have shown that it contains mainly type X collagen which, once secreted by the isthmus tubular gland cells (Arias *et al.*, 1991a, c), constitutes the core of the shell membrane fibres (Fernandez *et al.*, 2001). This particular collagen type has previously been described only in association with chicken bone formation (Kielty *et al.*, 1985; Schmid and Linsenmayer, 1987). Our observation agrees with a previous description showing the occurrence of collagenous cross links (desmosine and isodesmosine) in the shell membrane protein (Starcher and King, 1980) and lysyl oxidase, an enzyme resposible for cross linking, in the isthmus (Harris *et al.*, 1980). In fact, inhibition of this enzyme results in deleterious consequences in shell formation (see Chowdhury and Davis, 1995), especially due to alteration in type X collagen cross linking (Arias *et al.*, 1997b). The core of the shell membrane fibres also contains keratan sulphate, a glycosaminoglycan polyanionic molecule that is thought to be part of the glycosylated group of type X collagen (Fernandez *et al.*, 2001). Although it has been suggested that the composition of the mantle differs from that of the core (Simons, 1971; Draper *et al.*, 1972), we do not know if the recent finding of lysyl oxidase coupled with catalase in the shell membrane (Akagawa *et al.*, 1999) corresponds with the chemical composition of the mantle.

Mammillae are discrete aggregations of organic matter that intermix with the fibrillar material of the outer shell membrane fibres. We have shown that mammillae, especially the 100–300 vesicles of the CRB, contain a calcium binding molecule known as *mammillan* which is a keratan oversulphated proteoglycan (Arias *et al.*, 1992; Fernandez *et al.*, 1997; Fernandez and Arias, 1999).

The palisade corresponds to the thickest layer of the eggshell and is composed of integrated inorganic and organic components (shell matrix). It contains hyaluronic acid (Nakano *et al.*, 2001) and *ovoglycan*, a unique 200 kDa dermatan sulphate proteoglycan (Arias *et al.*, 1992; Carrino *et al.*, 1997; Fernandez *et al.*, 1997;

Fernández and Arias, 1999) with a core protein of 120 kDa (Carrino *et al.*, 1996, 1997) known as ovocleidin-116 and has recently been cloned and ultrastructurally localised in the same shell matrix vesicles as those shown to be reactive to dermatan sulphate (Hincke *et al.*, 1999; Fernandez *et al.*, 2001), and glycosaminoglycan chains containing dermatan sulphate with a mean molecular weight of 22 kDa (Carrino *et al.*, 1996, 1997; Dennis *et al.*, 2000). It appears that *ovoglycan* is a copolymeric proteoglycan containing non-sulphated chondroitin and dermatan sulphate glycosaminoglycans (Carrino *et al.*, 1997; Nakano *et al.*, 2001). Constituents of the cuticle have been studied elsewhere (see above).

Eggshell formation

Eggshells are formed by combining particular components of extracellular matrix molecules with a crystalline calcite filler while the egg is moving along the oviduct to produce a multilayered mineral-organic composite. Eggshell formation is shown diagrammatically in *Figure 2*.

Shell membranes are formed in the isthmus region of the oviduct 3–5 hours after oviposition – that is, the time elapsed after the last egg was laid (Fernandez *et al.*, 1997). Formation of the inner shell membranes occurs 3–4 hours after oviposition and, coincidently, the electron dense vesicles of the isthmus tubular gland cells show a positive reaction to type X collagen (Fernandez *et al.*, 2001). The same vesicles contain keratan sulphate at 4–5 hours when the outer shell

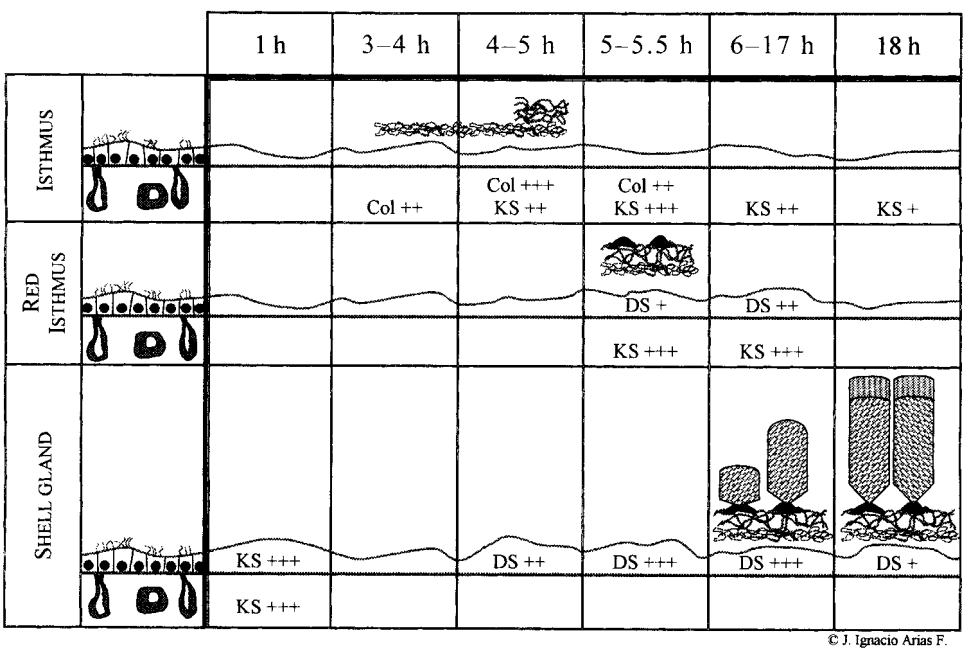

Figure 2 Diagrammatic representation of eggshell sequential formation, secretion pattern and localisation of particular macromolecules in different oviduct regions. The times at the top of the diagram indicate the number of hours after oviposition, and on the left the oviduct regions with lining epithelium and tubular glands are shown. KS, keratan sulphate; Col, type X collagen; DS, dermatan sulphate.

membrane fibres are formed. The isthmus secretion of both molecules decreases later. Type X collagen not only forms the shell membrane fibres but also inhibits calcium deposition on the shell membranes (Arias *et al.*, 1997a).

Mammillary formation occurs 5.25–5.5 hours after oviposition when the egg is in the red isthmus. Mammillan is detected in the vesicles of the CRB sac after being produced by the tubular gland cells of the red isthmus. Small amounts of ovoglycan are produced by the epithelial cells lining the red isthmus. At the end of this period calcium crystals are formed in close association with the occurrence of mammillan in the CRB sac, together with the start of secretion of ovoglycan by the epithelial cells lining the red isthmus (Fernandez *et al.*, 1997, 2001). These initial crystals are more randomly orientated than those of the palisade layer that will form later.

Palisade forms 6–18 hours after oviposition when the egg is in the shell gland and, coincidently, a large amount of ovoglycan is secreted by the epithelial cells lining this region. Ovoglycan is detected in 250–300 nm vesicles of the palisade shell matrix (Hincke *et al.*, 1999; Fernandez *et al.*, 2001). The secretion of ovoglycan has a crucial effect on crystal orientation, morphology and growth. In fact, crude or semipurified extracts of eggshell matrix alter calcite crystal morphology deposited *in vitro* to approximate that seen naturally (Arias *et al.*, 1991d, 1992; Wu *et al.*, 1992, 1994; Gautron *et al.*, 1996). The dermatan sulphate chains of ovoglycan are polyanionic and acidic, having a high calcium affinity. After experimentally inhibiting sulphation in the laying hen, the palisade of laid eggs shows severe structural alterations (Fernandez *et al.*, 2001). These alterations are reversed only after 43 days of inhibitor withdrawal when ovoglycan recovers its normal degree of sulphation.

A keratan sulphate molecule not identical to mammillan has been immunohistochemically detected in the cuticle (Arias and Fernandez, 1995) and in the shell gland cells 1 hour after oviposition which corresponds to residual secretion of the cuticle (Fernandez *et al.*, 2001). Although the role of keratan sulphate in the cuticle has not been fully established, involvement of this kind of molecule in calcium phosphate (hydroxyapatite) deposition and removal has been suggested. In fact, avian medullary bone, which acts as a reservoir for resorbable calcium in the laying hen, also contains keratan sulphate as its major glycosaminoglycan (Fisher and Schraer, 1980, 1982). In addition, phosphorus, either as part of macromolecules, probably osteopontin (Nys *et al.*, 1991; Pines *et al.*, 1994) or as hydroxyapatite (Dennis *et al.*, 1996), has been found in the cuticle, which suggests it has a role in the process of arresting shell formation.

Conclusions

Eggshell biomineralisation is effected by specialised oviduct cell populations in an assembly line sequence as the egg passes along the oviduct (Heuer *et al.*, 1992; Arias *et al.*, 1993; Nys *et al.*, 1999). In fact, it has been observed that many macromolecules of the eggshell are deposited in a defined temporospatial order (Arias *et al.*, 1991c, 1992; Pines *et al.*, 1994; Hincke *et al.*, 1995, 1999; Fernandez *et al.*, 1997; Gautron *et al.*, 1996, 1997; Lavelin *et al.*, 1998), with shell membrane type X collagen being responsible for the inhibition of mineralisation of this fibrillar structure (Arias *et al.*, 1997a) and mammillan being mainly involved in nucleation of the first randomly orientated and easily removable crystals of the mammillary layer (Arias *et al.*, 1992) while ovoglycan, a unique dermatan sulphate proteoglycan, is involved with the growth of the crystalline palisade (Wu *et al.*,

1992, 1994; Fernandez *et al.*, 1997, 2001). It has been shown that the calcium reserve body is more susceptible to *in vitro* decalcification than the palisade (Agarwal *et al.*, 1993).

However, distinctive oviduct cells not only produce particular macromolecules involved in eggshell shaping, but their secretion is also transiently regulated in such a way that their production in a particular region of the oviduct is not necessarily dependent on the location of the egg in the corresponding region, although their maximal occurrence coincides with the presence of the egg in that region. Moreover, our studies indicate that the sulphation status of eggshell proteoglycans plays a key role in the crystallisation of the eggshell (Fernandez *et al.*, 2001). Although mechanical strain regulates the expression of eggshell osteopontin (Lavelin *et al.*, 1998), the precise mechanism involved in the regulation of the expression of type X collagen and proteoglycans in the oviduct cells during eggshell formation must be determined.

Further work is needed to establish how, when and where these sulphated macromolecules interact with other molecules such as ovocleidin (Hincke *et al.*, 1995), osteopontin (Pines *et al.*, 1994) and others (Gautron *et al.*, 1997) which have been found in the eggshell and/or the uterine fluid during eggshell calcification in order to understand more fully the molecular control of eggshell biomineralisation and to provide a basis for understanding various anomalies that are seen in several types of eggshell abnormalities.

Acknowledgments

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