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Leading Opinion

Subtleties of biomineralisation revealed by manipulation of the eggshell membrane☆

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ABSTRACT

Biocalcification of collagen matrices with calcium phosphate and biosilicification of diatom frustules with amorphous silica are two discrete processes that have intrigued biologists and materials scientists for decades. Recent advancements in the understanding of the mechanisms involved in these two biomineralisation processes have resulted in the use of biomimetic strategies to replicate these processes separately using polyanionic, polycationic or zwitterionic analogues of extracellular matrix proteins to stabilise amorphous mineral precursor phases. To date, there is a lack of a universal model that enables the subtleties of these two apparently dissimilar biomineralisation processes to be studied together. Here, we utilise the eggshell membrane as a universal model for differential biomimetic calcification and silicification. By manipulating the eggshell membrane to render it permeable to stabilised mineral precursors, it is possible to introduce nanostructured calcium phosphate or silica into eggshell membrane fibre cores or mantles. We provide a model for infiltrating the two compartmental niches of a biopolymer membrane with different intrafibre minerals to obtain materials with potentially improved structure-property relationships.

1. Introduction

Calcification of the eggshell is among the most rapid biomineralisation processes known, with precise spatiotemporal control of its sequence of events [1]. As the egg yolk traverses the oviduct, it acquires egg white in the magnum followed by deposition of a fibrous eggshell membrane (ESM) in the isthmus. In the distal part of the isthmus, proteoglycan-rich mammillary knobs are secreted over the ESM to serve as sites for deposition of columnar calcite crystals that form the palisade layer of the eggshell [2]. The ESM is divided into an inner interlacing network of thinner fibres and an outer network of thicker fibres. Each fibre is traditionally conceived to be made up of a collagen-rich core and a glycoprotein-rich mantle [2,3]. Fibre cores from the outer ESM contain predominantly type I collagen while those from the inner ESM contain types I and V collagen [1]. Type X collagen has also been identified from both membrane layers and is postulated to function as a mineralization inhibitor to prevent the underlying egg white and yolk from being mineralized [4]. Despite immunohistochemical identification of these collagen variants, fibre cores from the ESM appear homogeneously stained at the electron microscopical level and lack substructural fibrillar characteristics or the 67-nm cross striations seen in fibrillar collagen [5]. This may be due to masking of these avian collagens with a cysteine-rich eggshell membrane protein (CREMP) that contains multiple disulphide bonds [6].

Scientists find biomineralisation intriguing because amorphous and crystalline structures created through interactions between proteins and minerals are considerably more advanced than what may be achieved by contemporary materials engineering [7,8]. As

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the ESM does not mineralise *in-situ*, it has been utilised as a biomineralisation model [1,9] or as a biological template for surface modification of crystal growth [10–12]. However, biomimetic mineralisation within the ESM matrix has not yet been achieved even when pepsin is employed to remove its purported mineralisation inhibition components [4]. Nevertheless, mineralisation of pepsin pre-treated ESMs in the presence of a biomimetic analogue of matrix phosphoproteins resulted only in the deposition of extrafibre apatite crystals on the ESM surface [8].

The recent discovery of the involvement of calcium phosphate prenucleation clusters has considerably advanced our understanding of the biomineralisation of collagen [13]. Using polycarboxylic acid analogues of extracellular matrix proteins to stabilise pre-nucleation clusters-derived amorphous calcium phosphate as plastic, liquid-like precursor phases [14], it is possible to take advantage of the templating properties of type I collagen to introduce intrafibrillar apatite crystallites into collagen fibrils [15]. Likewise, biosilicification of diatom frustules is under the precise control of highly-phosphorylated biomolecules and long-chain polyamines that produce plastic protein-stabilised silica phases [16,17]. In this work, we utilised the eggshell membrane as a universal biomineralisation model to test the hypothesis that it is possible to differentially introduce biominerals into the different compartmental niches of a biopolymer membrane (i.e. calcium phosphate in ESM fibre cores and silica in ESM fibre mantles) by using biomimetic analogues to create stabilised amorphous phases of the corresponding mineral.

2. Materials and methods

2.1. Retrieval of eggshell membranes

Eggshell membranes were obtained from commercial breeding lines of Gallus gallus. The outer membranes were carefully removed using forceps and washed with Milli-Q water (18.2 mΩ-cm). The membranes were stored in water to avoid dehydration and used within 24 h after harvesting. The ESMs were cut while immersed in water into 1 cm × 1 cm specimens for the biomineralisation experiments.

2.2. Biocalcification

Polyacrylic acid-stabilised amorphous calcium phosphate precursors were prepared using a concentrated calcium phosphate mineralising medium containing 10.5 mM $CaCl_2 \cdot 2H_2O$ and 6.3 mM K_2HPO_4 in HEPES buffer (pH 7.4). They were prevented from spontaneous precipitation by incorporating 500 μg/mL polyacrylic acid (Mw 1800, Sigma-Aldrich, St. Louis, MO, USA) as an apatite nucleation-inhibiting agent. The concentration of polyacrylic acid used was based on the minimal amount required for the solution to remain stable and visibly clear for at least 1 month. This was monitored with optical density measurements taken at different time intervals with a 96-well plate reader at 650 nm.

Prior to biocalcification, ESM specimens were treated with 1.25 N 3-mercaptopriprionic acid (MPA, Sigma-Aldrich) dissolved in 10% acetic acid for 3 h. After rinsing with Milli-Q water, they were incubated in a 5 wt% sodium tripolyphosphate solution (Mw 367.9, Sigma-Aldrich) at room temperature for 1 h and further rinsed with Milli-Q water. The phosphorylated ESMs were then calcified by immersing each ESM square in 1 mL of stabilised amorphous calcium phosphate precursors at 37 °C for 14 or 28 days, with daily change of the calcifying medium.

2.3. Biosilicification

Choline-stabilised silicic acid precursors were prepared using a 3% silicic acid stock solution. The latter was prepared by mixing Silbond® 40 (40% hydrolysed tetraethyl orthosilicate; Silbond Corp., Weston, MI, USA), absolute ethanol, water and 37% HCl in the molar ratios of 1.875 : 396.79 : 12.03 : 0.0218 (mass ratio 15 : 182.8 : 2.167 : 0.008) for 1 h at room temperature to complete the hydrolysis of tetraethyl orthosilicate into orthosilicic acid and its oligomers. The 3% silicic acid solution was then mixed with 0.07 M choline chloride (Mw 139.6 m, Sigma-Aldrch) in a 1:1 volume ratio (final pH = 5) under vibration for 1 min. After centrifuging the mixture at 3000 RPM, the supernatant containing choline-stabilised silicic acid was collected for the biosilicification experiments. The MPA-treated ESMs were immersed in a 5 wt% sodium tripolyphosphate solution for 1 h and rinsed thoroughly with Milli-Q water. Each ESM square was silicified in 1 mL of choline-stabilised silicic acid at 37 °C for 4 days with daily change of the silicifying solution.

2.4. Scanning electron microscopy (SEM)

To examine the surface morphology of ESMs before and after MPA pre-treatment, the specimens were desiccated in anhydrous calcium sulphate, sputter-coated with gold/palladium and examined using a field emission-scanning electron microscope (XL-30 FEG; Philips, Eindhoven, The Netherlands) at 10 kV.

2.5. Transmission electron microscopy (TEM)

Eggshell membranes before and after biomineralisation were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ascending ethanol series (50–100%), immersed in propylene oxide and embedded in epoxy resin. Ninety nanometre thick sections were prepared and examined using a JSM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV. Intact or MPA pre-treated specimens without biomineralisation were examined after staining with 2% uranyl acetate and Reynold's lead citrate. Mineralised specimens were examined unstained. Selected area electron diffraction (SAED) was performed on the mineralised.

2.6. Attenuated total reflection – Fourier transform infrared spectroscopy (ATR-FTIR)

Each ESM specimen was desiccated with anhydrous calcium sulphate for 24 h prior to spectrum acquisition. A Nicolet 6700 FT-IR spectrophotometer (Thermo-Scientific, Waltham, MA, USA) with an ATR setup was used to collect infrared spectra between 4000 and 400 cm^{-1} at 4 cm^{-1} resolution using 32 scans.

2.7. Amino acid analyses

Amino acid analyses were performed on untreated ESMs as well as ESMs that were subjected to 3 h and 5 h of MPA pre-treatment to determine if there were common trends in the changes of amino acid profiles. A L8900 Analyser (Hitachi, Schaumburg, IL, USA) equipped with a Hitachi AAA Special Analysis Column (855-4516) was employed for the analyses. The 16 common amino acids together with hydroxyproline, hydroxylysine and cyst(e)ine were analysed. The analyser was calibrated using standard amino acid stock solution (AAS18, Sigma-Aldrich) and additional amino acid standards: trans-4-hydroxy-L-proline, hydroxylysine, L-cysteic acid and pyridylethyl-L-cysteine. L-norleucine was used as the internal standard (Sigma-Aldrich).

The ESMs were hydrolysed with 6 N HCL/2% phenol at 110 °C for 22 h under vacuum into individual amino acid residues. A defined amount of the norleucine internal standard was added to each sample prior to hydrolysis. Cyst(e)ine was oxidised with H_2O_2 and formic acid (1:10 v/v) to cysteic acid prior to analysis. The individual amino acids were separated by ion-exchange chromatography with measurement of the ninhydrin chromophore. Data analysis was performed using the EZChrom Elite software (Version 3.1E; Scientific Software International Inc., Lincolnwood, IL, USA). The data were normalised to the known concentration of the internal standard.

2.8. Electron tomography, serial sectioning and 3-D reconstruction

Electron tomography was performed with 200 nm thick unstained epoxy resin-embedded sections using a Tecnai G2 STEM (FEI, Hillsboro, OR, USA) at 200 kV. Tomographic images were taken from +30° to −30° at 1° increment. Tilt series were created using the Gatan Digital Micrograph software. Image alignment was performed using Reconstruct Version 1.1.0.0. (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm). Three-dimensional reconstruction, segmentation and visualisation of the 3D volume were performed using Amira 5.3.3. (Visage Imaging Inc., Andover MA, USA). For serial sectioning, 120-130 sixty nm thick sections were prepared for TEM imaging, aligned using the Reconstruct software and reconstructed for visualisation using the Amira software programme.

2.9. Scanning transmission electron microscopy-energy dispersive X-ray analysis (STEM-EDX)

Elemental analysis of the mineralised ESMs was performed on the thin sections prepared previously for TEM using the FEI Tecnai G2 STEM at 200 kV. Spectrum acquisition and elemental mapping were conducted using an Oxford Instruments INCA x-sight detector. Elemental mappings were acquired with the FEI TIA software using a spot dwell time of 300 ms with drift correction performed after every 30 images.

2.10. Nanoindentation

Control (non-mineralised) and silicified ESMs were prepared by placing small portions (3 × 3 mm) of the membrane on a glass cover slip. The hydrated specimens were covered within droplets of Milli-Q water to minimise moisture loss. Mechanical properties of the specimens were evaluated by quasi-static indentation using an instrumented nanoindenter (Hysitron Tribinderter 900, Minneapolis, MN, USA) with a 100 μm radius cono-spherical diamond tip indenter. A standard trapezoidal profile was used including a maximum load of 100 μN, indentation hold time of 5 s, and