Faraday Discussions

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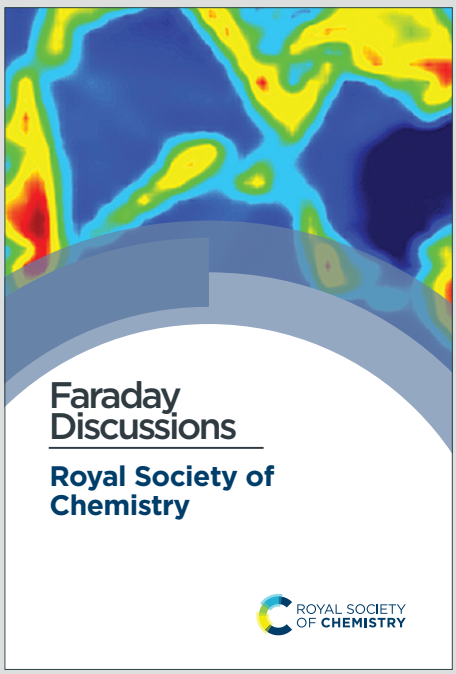

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Advanced imaging techniques in biomineralization research: concluding remarks

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These Faraday Discussions have shown that a wide range of physical techniques allows imaging biominerals whatever their size, mineralogy and complexity. These techniques cover an extensive spectral range from infrared to hard X-rays and enable the micro- and nanostructures of these exceptional biological materials to be described in great detail. All scales are covered, with numerous overlaps, from the millimetre to atomic scales. The next frontier will be to map in 3D the organic matrix components associated to biominerals in order to understand their function in biomineralization.

Introduction

From time immemorial, human beings have felt the need to represent the world as they perceive it, whatever the scale of observation. Biomineralization - a scientific discipline that took shape at the end of the 1960s but whose oldest foundations date back to the 17th century - is no exception to this universal rule. Indeed, the observation of biominerals with the naked eye or by microscopic means has almost always been accompanied by their representation in two or three dimensions. Biominerals are complex objects in terms of shape, structure and composition: they therefore require precise mapping. Indeed, the notion of biomineral is intrinsically linked to the notion of imaging. Yet, surprisingly, few scientific events have formally associated the two concepts, biomineral and imaging. The present Faraday Discussions that took place in Edinburgh, have gone some way towards filling this gap. This scientific event, held from May 14 to 16, 2025, brought together dozens of specialists from a wide variety of backgrounds and with no fewer problems, but whose common denominator was to use sophisticated techniques - most often borrowed from physics - to produce images of biominerals.

As I pointed out a few lines above, biomineralization as a scientific discipline has its origins in the very first microscopic observations of biomineralization, following the invention of the first optical microscopes, probably by Zacharias Janssen somewhere in the first half of the seventeenth century. It is fair to say that the first description of biominerals – which could be considered as pathological - was made by Robert Hooke in his famous work *Micrographia* (1665) in his *Observation XII: Of Gravels in Urine*[1]. This was only the beginning, with other descriptions following, notably those of Clopton Havers, who was the first to report the structure of bone in *Osteologia nova, or some new Observations of the Bones, and the Parts belonging to them, with the Manner of their Accretion and Nutrition* (1691)[2]. The 18th and 19th centuries saw a profusion of descriptions of biomineralization using optical microscopy, which are beyond the scope of this article. However, we should mention the remarkable work of William Benjamin Carpenter, one of the first to rigorously image the microstructure of mollusc shells in his work *Report on the state of science on the microscopic structure of shells* (1845)[3]. I cite this author in particular because he worked during his doctoral years at the University of Edinburgh (from 1835 to 1839), a few miles from the venue of this conference, before moving to London where his monograph was published. The 20th century would be the century of biominerals imaging in electron microscopy, with the successive inventions of the transmission electron microscope (TEM) in 1931 by Ruska and Knoll, followed by the development of the scanning electron microscope (SEM) by Knoll himself a few years later (1935). While the first technique provides ultra-thin cross-sectional images, *i.e.*, two-dimensional representations, the second, by producing surface images of biomineralization, provides access to the third dimension. From the 1960s onwards, among the flood of publications, the short-lived *Biomineralization Research Reports*, published from 1970 to 1979, presented remarkable illustrations of biomineral structures obtained using these two techniques. No less remarkable was the multi-author work edited by Joseph G. Carter (1990), *Skeletal Biomineralization: Patterns, Processes and*

Evolutionary Trends in two volumes[4], a veritable atlas illustrating the skeletal microstructure of representatives of most metazoan mineralizing phyla, with a profusion of SEM images. Finally, I should mention the extraordinary work published by Bevelander & Nakahara between 1969 and 1991, presenting a bivalve's mother-of-pearl ('brick wall') in very high-resolution TEM. These images of the interface between forming nacre tablets surrounded by their organic sheath and the mineralizing epithelium with its microvilli remain, several decades after their publication, an unmatched technological feat[5,6].

Representing biomineral structures through imaging, a series of challenges

Imaging biomineral structures involves tackling the complexity of biological systems. Unlike their purely chemical equivalents, biomineral structures exhibit intrinsic properties that make them much more difficult to study. Analysing them for imaging purposes therefore presents a number of real challenges that can be summarized as follows:

- Challenges due to the multiscale structure of biominerals. Depending on the scale at which they are observed, biominerals do not display the same morphological characteristics; in other words, they offer fascinating hierarchical properties. Everyone is familiar with the example of bone organization with its seven hierarchical levels, from the tropocollagen molecule (itself formed of three monomers wound into a triple helix) to the complete bone with its cortical and trabecular components[7]. What is true for bone is also true for organisms that appear simpler but are also capable of producing hierarchical mineralized structures: this is particularly the case for red coral (*Corallium rubrum*), in which seven hierarchical levels, from the nanometric to the centimetric scale have been described by Vielzeuf and coworkers[8] (Fig. 1). It is therefore clear that the means of observation is of paramount importance in understanding this hierarchy, and it is often through a combination of different means of observation that the complexity of mineral structures can be revealed in detail.

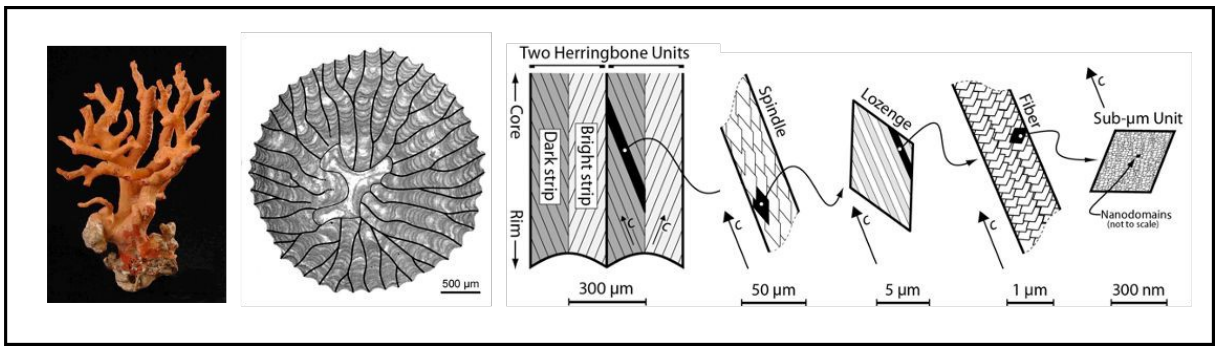

Fig. 1. The seven levels of hierarchy of the skeleton of the red coral *Corallium rubrum.* Redrawn from ref. 8.

- Challenges due to the heterogeneity of biominerals. Biominerals are composite materials made of a mixture of organics and mineral. The mineral phase can be crystalline or amorphous. It is generally hard and brittle. The organic fraction, on the other hand, is soft and ductile. This organic fraction can be of two types: 1) an extracellular matrix in the case of acellular biomineralized skeleton (e.g., mollusc shells). However, this matrix is very complex and heterogeneous, as it is itself composed of a mixture of macromolecules (proteins, polysaccharides, lipids) and smaller molecules (peptides, metabolites, pigments, etc.). 2) or, in addition to a matrix, living, differentiated, spatially localized cells in the case of cellular biomineralization, which is particularly the case for bone, with its osteoblasts, osteoclasts, and osteocytes. Processing such heterogeneous objects requires adequate imaging preparations. In addition, the mineral-organic mixture is responsible for the emerging properties (particularly mechanical properties) that most biomineral materials possess. As a reminder, one talks about emerging properties when the finished product (in this case, the biomineral) has characteristics that are not simply the sum

of those of its constituent parts taken separately (in this case, the mineral alone and the organics alone) but go far beyond them.

- Biomineral structures are dynamic and evolve over time. Over a very short period of time, much less than a second: this is particularly the transition from amorphous to crystalline phases, encountered in many – if not all? - biominerals. Over a short period of time, from a day to a month: this corresponds for example to bone remodelling; it corresponds also to the various transitions between larval and juvenile phases in several calcifying metazoans (sea urchins, molluscs), or to the periodically renewable carapace in crustaceans (due to molting). Finally, over a long period of time (from a few years to a few million years): this corresponds to taphonomic and diagenetic transformation (*i.e.*, fossilization) of biomineral structures that can recrystallize, enrich themselves with exogenous ions, loose a large part of their organics and lithify.
- The last point I just mentioned - the dynamic aspect of biomineralization - prompts me to tackle head-on another challenge that remains at the heart of biomineral research to this day. This challenge involves understanding how biomineralization occurs, namely the transition from the precursor liquid (which may be a gel or a colloid) to the finished product, the solid biomineral. Significant conceptual advances have been made over the past 25 years. As a first approximation, most studies conducted on various models have demonstrated that an amorphous-crystalline transition seems to be the shared rule. But is this a universal rule? Kanmani Chandra Rajan's work on the Hong Kong oyster (this volume[9]) seems to show that it may not. With regard to amorphous precursors, can we talk about "polyamorphism"[10], *i.e.*, that for the same mineral system (calcium carbonate, for example), there are different types of amorphous forms that predetermine crystallization into calcite or aragonite? The PILP (Polymer-Induced Liquid Precursor) process proposed by Gower and Odom[11] remains an attractive hypothesis 25 years after its publication. It assumes that polyanionic polymers are capable of stabilizing transiently a viscoelastic precursor phase (gel, colloid, etc.) that can then be molded and transformed into a crystalline phase. The strength of the PILP process is that it can mimic forms that exist in nature *in vitro*[12]. Recently, this concept has been updated under the acronym CAT (Colloid Assembly and Transformation[13]). Independently, the concept of mesocrystals and oriented attachment has been proposed[14], suggesting that biomineralization involves the assembly of nanograins (amorphous or crystalline) surrounded by soluble organics. These grains bond together to form (meso)crystals with a monocrystalline appearance. This concept has more recently been extended to numerous possible mineralization pathways[15], all grouped under the term non-classical crystallization pathways.

Of course, these challenges determine the preparation of biomineralization for imaging - I will come back to this point later - but above all, they determine the technique to be used depending on the scientific question underlying the research. Today, one can only be amazed by the diversity of physical techniques for analysing biomineralization that have been developed over the last two decades. Table 1 below provides a non-exhaustive list of the main technical approaches used to image biomineralization. This table details their maximum resolution and indicates the type of information extracted from these techniques.

Technique	Maximal Resolution	*Information Provided*
Scanning Electron Microscopy (SEM)	1-10 nm	*Surface morphology, crystal shape*
Cryo-SEM	1-10 nm	*Surface morphology, crystal shape*
Transmission Electron Microscopy (TEM)	0.1-0.2 nm	*Internal structure, crystal lattice, diffraction*
Cryo-TEM	0.1-0.2 nm	*Internal structure, crystal lattice, phase transformation*
Atomic Force Microscopy (AFM)	0.1 nm (vertical), ~1 nm (lateral)	*Surface topography, mechanical properties*
Confocal Laser Scanning Microscopy (CLSM)	~200 nm (lateral), ~500 nm (depth)	*3D structure with fluorescence labeling*
Fourier Transform Infrared Spectroscopy (FTIR)	~10 µm	*Chemical bonds, organic/inorganic composition*
Raman Spectroscopy	~1 µm (can be enhanced to ~300 nm)	*Molecular structure, mineral phase mapping*
X-ray Absorption Spectroscopy (XAS)	Element-specific, ~0.1 nm	*Elemental composition, local structure*
Solid State Nuclear Magnetic Resonance (ss-NMR)	Atomic level, ~0.1 nm	*Molecular environment, organic-inorganic interactions*
Electron Energy Loss Spectroscopy (EELS)	Sub-nm (elemental analysis in TEM)	*Elemental composition at nanoscale*
X-ray Diffraction (XRD)	Limited by crystal size (~nm scale)	*Crystal structure, phase identification*
X-ray Fluorescence (XRF)	<< 1 µm	*Elemental composition, sub ppm*
Electron back-Scattered Diffraction (EBSD)	30-100 nm	*Crystallographic orientation of nanocrystals*
3D Bragg ptychography	10-25 nm in 3D	*3D mapping of internal crystal structure*
Micro-Computed Tomography (Micro-CT)	~1-5 µm	*3D internal mineral distribution*
Nano-SIMS (Secondary Ion Mass Spectrometry)	50-100 nm	*Elemental/isotopic mapping*
Atom Probe Tomography	0.2 - 0.3 nm	*Elemental composition*
Small/Wide-Angle X-ray Scattering (SAXS/WAXS)	1-100 nm (SAXS), 0.1-1 nm (WAXS)	*Nano to microstructure, orientation of crystals*

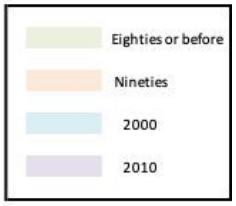

Table 1. Classical imaging techniques used for biominerals. Their resolution and the information they provide are indicated. On the right, the colour code indicates the period from which each technique was commonly used.

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Models and techniques used

The four half-days devoted to imaging in biomineralization research featured 23 guest speaker presentations (in addition to Laurie Gower's introductory lecture) divided into four themes: crystal nucleation in biominerals, interfaces at the microscale, interfaces at the nanoscale, and finally, connecting length scales. In what follows, I will not repeat this outline, but will highlight two different divisions, one focused on study models in the broad sense, the other on the major scientific questions addressed through the models.

A) Models - Figure 2 highlights four series of study models. The first, “atoms, ions, and molecules,” includes four publications addressing calcium[16], aspartic acid[17], a synthetic peptide P_{11}-4[18], and a bone matrix protein, osteopontin (OPN)[19]. The second cluster covers all calcium phosphate models, from calcium phosphate solutions[20] to animal models – bones and teeth – studied in various vertebrates (sharks, mice, quails, cattle)[19, 21-26]. The third group, numerically the largest and most eclectic, is that of calcium carbonate, with models of coccolithophores[27], corals[28, 29], molluscs[9,21, 29-33], echinoderms[34] and eggshells[35]. Finally, two other models make up the fourth group (non-Ca-P, non-$CaCO_3$), with sorghum silica[36] and organic guanine crystals[37] from the ocular system of zebrafish. Of course, despite the remarkable eclecticism of the models discussed, not all biological systems were represented in these Faraday Discussions: the bacterial world (Fe- / Mn-oxidizing bacteria, cyanobacteria precipitating calcium carbonate), other protists (diatoms, foraminifera), or even some metazoans forming a calcareous skeleton, such as sponges, brachiopods, bryozoans, annelids and crustaceans.

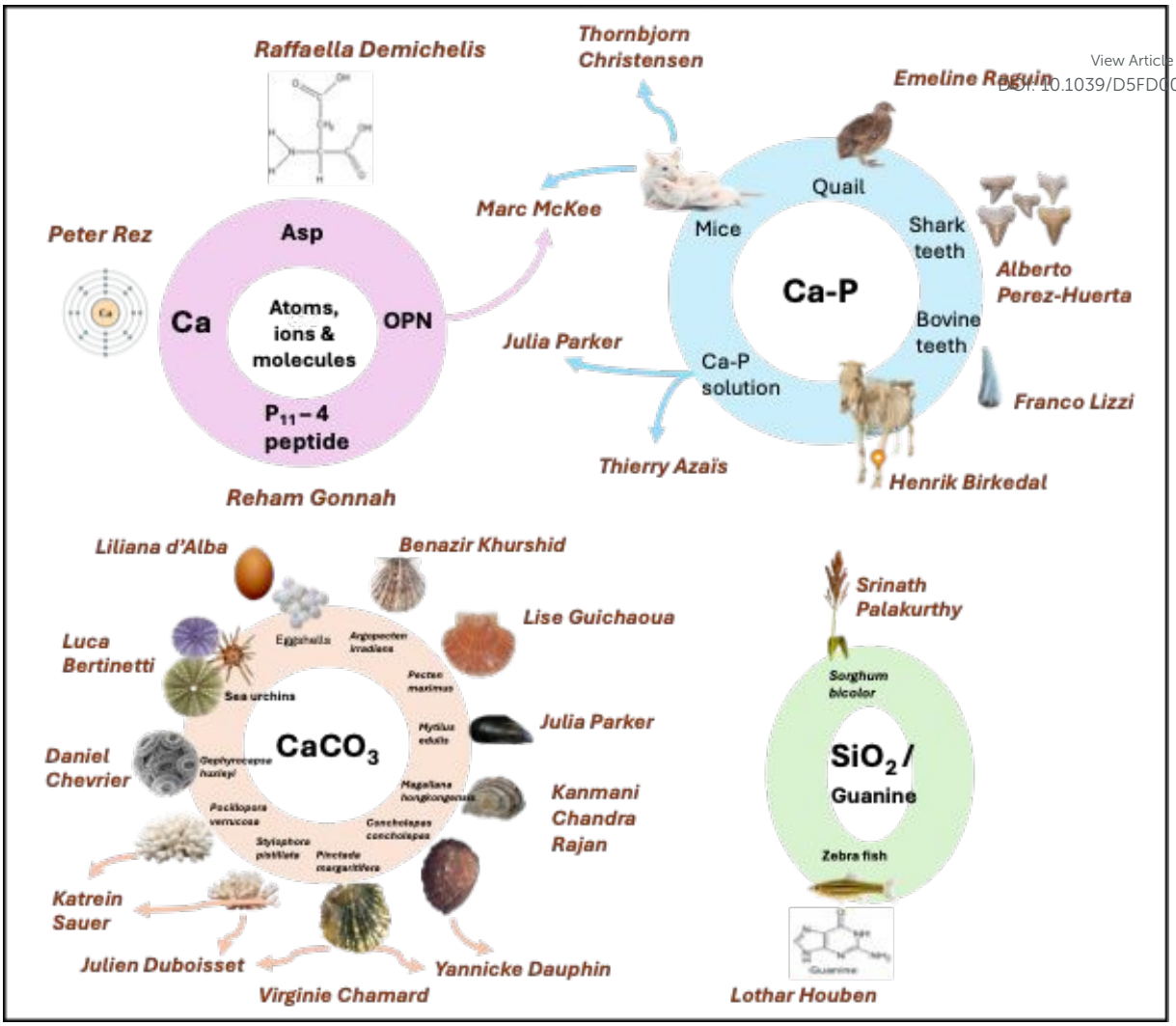

Fig. 2. The different study models discussed during the Faraday Discussions. Note the preponderance of calcium carbonate models taken classically from the metazoan non vertebrate world. On the opposite, sorghum with its silica deposits and zebra fish with its eye system containing guanine crystals are original models that have been little studied until recently in biomineralization field. The names indicated in brown are those of the speakers.

B) Techniques – Table II below lists all the techniques used by participants of the Faraday Discussions. It is interesting to note that, with a few exceptions, most of the studies presented are based on multi-technique approaches.

Techniques / Speakers	SESSION 1						SESSION 2							SESSION 3			SESSION 4						
	PR	RD	TA	HB	RG	APH	VC	LDA	JP	DC	LH	JD	KS	LB	SP	KCR	MMK	ER	TC	YD	LG	BK	FL
References	16	17	20	25	18	26	30	35	21	27	37	29	28	34	36	9	19	23	22	31	32	33	24
Computer simulation																							
Light microscopy UV-fluorescence																							
Cryo-CLEM																							
s-SNOM																							
TEM / FIB TEM																							
SEM / FIB-SEM / correlative SEM																							
Cryo-4D STEM																							
X-ray tomogr. / scanning transm Elect																							
EDX																							
EELS																							
ss-NMR, cryo																							
XRD / nano-XRD																							
µ / nano XRF																							
X-ray phase contrast																							
X-ray photoabsorption																							
X-ray photoelectron spectro (XPS)																							
EBSD																							
µ / nano FTIR - srFTIR																							
O-PTIR																							
Raman spectroscopy																							
Atom Probe Tomography																							
3D-X-ray Bragg Ptychography																							
3D Stimulated Raman Scattering (SRS)																							
AFM																							

Table 2. Diversity of the technical approaches used by the speakers of the Faraday Discussions for imaging biominerals. The initials of the speakers' names are indicated on top.

Scientific issues addressed during these Faraday Discussions

I have chosen to group the presentations into four themes: the first concerns technological advances, the second the biomineralization process itself, the third, health, ecology and environmental issues, and the last one, long-time scale, involving evolutionary and fossilization issues. I briefly summarize in few lines the central tenets of the 23 presentations and name the scientist who presented the work.

A) Technological advances -

- The fundamental issue of the calcium detection threshold was addressed by Peter Rez[16], notably through the use of X-ray photoabsorption, EELS, and TEM-EDX techniques. In TEM, EDX can detect lower Ca concentrations (~0.05 mM) than EELS (~1 mM) due to its lower background. Soft X-ray absorption spectroscopy offers lower spatial resolution (~20 nm) but superior sensitivity, detecting Ca down to 35 nM in aqueous environments.
- Aspartic acid is a key amino acid in calcium carbonate biomineralization. It has three functional groups (two carboxylate groups and one amine group) that can interact with the mineral surfaces of carbonate biomineral. Its complex behaviour on calcium carbonate mineral surfaces (vaterite) has been studied in detail and refined through atomistic simulations carried out by Raffaella Demichelis and her team[17].
- Solid-state NMR (ss-NMR) is a powerful technique for describing the state and dynamics of a liquid or solid at the atomic and molecular scale. Presented by Thierry Azaïs[20], a new analytical module (stop flow + freeze-quench) makes it possible to study unstable intermediate species by freezing them (after 20 ms of reaction) and preserving their native environment (hydration, pH, ionic strength). This is a major technical advance in understanding the very transient stages leading to biomineralization.
- Another major advance concerns 4D transmission electron microscopy, currently applied to guanine biocrystals contained in the iridosome of zebrafish. This powerful technique, presented by Lothar Houben[37], provides a unique way of imaging nanometric crystals embedded in a thick organic matrix.
- Stimulated Raman scattering, presented by Julien Duboisset[29], is a technique that is sensitive to molecular vibration of carbonate polymorphs and organics and is compatible *in vivo*. It allows the mineralogical evolution of a system to be seen spatially, at the micron scale, for example the following transition: ACC - $CaCO_3$ hemihydrate - aragonite in the scleractinian coral *Stylophora pistillata*.

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- Using a combination of approaches (nano-XRF, DPC imaging, nano-XANES, 3D ptychography X-ray tomography), Julia Parker[21] was able to image the organization of a set of biominerals across different structural levels. The emphasis was put on an important aspect in imaging, sample preparation.
- Non-contact specular reflectance spectroscopy (srFTIR) allowed Franco Lizzi[24] to map large surfaces of highly polished and dehydrated human or bovine teeth. This simple, fast, non-destructive imaging technique uses a broad range of wavelengths with high spectral resolution.
- Finally, μXRF & μXRD mapping (MAX IV Synchrotron) employed by Thorbjorn Christensen[22] allowed imaging of large biomineralized objects such as mouse femora and showed the spatial differences in their crystalline structure, orientation, and composition, in particular between cortical and trabecular bone.

B) Dynamics of biomineralization processes -
- As shown by Reham Gonnah[18] in an *in vitro* model, the synthetic peptide P_{11}-4 adsorbed on Si-nitride surfaces interacts with the formation of calcium phosphate and mimics the role of enamel matrix proteins by catalysing the nucleation of ACP-like particles.
- Marc McKee and his team[19], by using 3D FIB-SEM tomography and EELS, highlighted the role of OPN in controlling the microstructure of bone mineralization, by showing that on two types of genetically engineered mice, an excess of OPN expression induced either an incomplete bone mineral tesselation (space filling), corresponding to an inhibition of mineral growth or the stopping or the delaying of the amorphous-to-crystalline transition.
- Emeline Raguin[23] demonstrated the role played by vesicles in the mineralization of femurs of quail embryos, by using cryo–CLEM and FIB-SEM (and *in vivo* immuno-labelling). These vesicles located within the capillary lumen transport the mineral precursors in blood vessels.
- Using synchrotron XRD and XRF, Henrik Birkedal[25] studied the remodelling of bovine metacarpe bone and evidenced the correlation between the presence of zinc in the growth front and that of MMP-13, a bone-degrading enzyme.
- 3D X-ray Bragg ptychography microscopy allows to observe the spatial modification of a system during its mineralization. Applied by Virginie Chamard[30] to the calcitic prisms of the pearl oyster, it revealed minor mineralogical reorganizations like lattice tilting and emphasized the key-role played by magnesium (expelled during crystallization process) along the growth axis of each individual prism.
- Yannicke Dauphin[31] employed a series of novel techniques (DRIFT, Synchrotron IR mapping, s-SNOM, O-PTIR) to image the intricate spatial relationships between organics and minerals, in the pearl oyster (shell + pearl) and the Chilean abalone.
- Finally, Kanmani Chandra Rajan[9] revealed the mineralogical transformation of Asian edible oyster at different larval stages, showing puzzlingly the absence of ACC and the coarsening of mineral grains with maturation.

C) Health, environment and ecology concerns -
- The unicellular coccolithophore algae are key-regulators of the carbonate cycle at global scale and represent a major sink for carbon. Dan Chevrier[27], by using nanobeam-scanning XRF microscopy, evidenced for the first time intracellular Ca- and P-rich bodies containing also Mn, Fe, Zn, which may be involved in coccolith formation or may be used as detoxifying granules.
- Katrein Sauer[28] imaged the soft and skeletal tissues of two scleractinian corals submitted to lead (Pb) contamination. She evidenced a partition of this element, with a high concentration in the soft tissues but its absence in the skeleton.
- Lise Guichaoua[32] studied the myostracal layer of two batches of scallops from non-contaminated and heavy metal contaminated areas. EBSD mapping of this layer could detect microstructural differences between the batches, with smaller grains of polluted samples, which may influence the muscle attachment.
- In the context of future warmer seawaters, Benazir Khurshid[33] analysed the shell parameters of two populations of Atlantic bay scallops grown at two different temperatures (2°C difference). Via EBSD, she observed a higher grain misorientation of specimens grown at higher temperature.

- Sorghum, native to Africa, is a plant of major importance for human and livestock nutrition and can be an alternative to other cereals at world scale. Its lignin tissues contain bound silica biominerals, which contribute to the plant's resistance to drought. By studying different mutants, Srinath Palakurthy[36] evidenced that lignins of high-Si genotypes exhibit a higher catalytic activity of silicic acid polymerization, an interesting property to select in the context of global warming.

D) Long time scales: evolution and fossilisation –

As A. Knoll wrote 22 years ago, "*Biology is chemistry with a history* "[38]. This maxim could also apply to biomineralisation: 'biomineralisation is mineralisation with a history'. The last three papers clearly have implications for the long-time scale, evolution and fossilisation.

- Four phyla of metazoans independently produce calcified eggs (chordates, arthropods, molluscs, annelids). Using EBSD imaging, tomography and AFM, Liliana d'Alba[35] investigated the origin and cause of this evolutionary convergence, the lowest common denominator of which is the ubiquitous presence of acidic mucopolysaccharides.
- Sea urchins have unique mesodermal skeleton with a porous microstructure, the stereome, which Luca Bertinetti[34] sought to classify using a microtomography approach (on four clades), followed by advanced mathematical processing. His work showed that stereomes have a constant mean curvature and that the stereome symmetry is independent of the underlying crystallographic orientation of calcite. This robust approach could be extended to all major sea urchin clades (extant, fossil) in an evolutionary perspective.
- Finally, Alberto Perez-Huerta[26] compared the enameloid structures of modern and fossil shark teeth using atom probe tomography and Raman spectroscopy. His analyses suggest that the chemistry of the biomineralisation process between fossil and modern forms may have changed over time. They do not rule out the possibility that the differences recorded are caused by diagenesis, something that will require further testing.

Conclusion and perspectives

As these Faraday Discussions have shown, there is now a very wide range of physical techniques for imaging biominerals, covering an extensive spectral range from infrared to hard X-rays. These techniques enable the micro- and nanostructures of these exceptional biological materials to be described in great detail. All scales are covered, with numerous overlaps, from the millimetre to sub-nanometre scales. The 3rd and 4th dimensions are covered with certain techniques, such as 3D X-ray Bragg ptychography, offering the possibility of visualising the spatial and temporal evolution of mineralising systems. Several imaging techniques also allow the visualisation of samples that have undergone little preparation, in their native state or almost. Finally, imaging does not only focus on biominerals but also on simplified *in vitro* systems and computer simulations.

However, there are few limitations to imaging, related to the samples themselves or to different stages of the analytical process, from sample preparation to analysis and subsequent processing of the data obtained. Each of these limitations can nevertheless be addressed with a view to improvement.

- It cannot be repeated often enough: biominerals are the product of the finely regulated functioning of living cells or tissues, i.e. the result of the coordinated activity of an extremely complex genetic machinery organised into a network, with numerous feedback loops and multiple signalling pathways. Several approaches can be used to reduce this complexity somewhat: a) the development of genetically modified living models in which the expression of key biomineralisation genes is increased or, conversely, suppressed. The mouse[19] and sorghum[36] models presented at these Faraday Discussions thus provide a better understanding of the regulatory pathways of biomineralisation. Comparative imaging of wild-type controls and genetically modified specimens provides a very concrete visualisation of a gene's function (see the role of OPN in 3D bone tessellation). b) Secondly, biomimetic or bio-inspired *in vitro* approaches are a means of reducing the complexity of a biomineralising system by

decreasing the number of reagents and parameters to be controlled. Studies conducted with the P_{11}-4 peptide demonstrate the possibilities of such an approach[18]. c) Finally, sophisticated computer simulations are also a means of overcoming the constraints of biological systems: the presentation on the behaviour of aspartic acid on a mineral surface has demonstrated the full value of this approach[17]. To conclude on this aspect, let us bear in mind that while seeking to reduce the complexity of biomineralising systems is a noble task, it should not lead to a reductionist approach, which is always dangerous in science.

- Sample preparation. Four bottlenecks should be considered: a) procedures for preparing biomineral samples are far from standardised. Very often, publications provide only imperfect descriptions of sample preparation protocols. b) Sample preparation induces artefacts. c) The number of samples to be processed is not commensurate with the time required to prepare them. d) Unstable transitional mineral species (e.g. ACC) are often lost during preparation. For points a and b, based on a suggestion by Tilmann Grünewald, a database on sample preparation for imaging could be created and fed collectively. This database would list the protocols, their adaptations (the "tricks") and list the possible preparation artefacts. For point c), it is obviously necessary to minimise the time taken to prepare samples, or at least to optimise it. Regarding point d), there are now ways of instantly freezing samples to best preserve the most unstable transient mineral species[20].
- Artifacts caused by the analysis itself. High-voltage electron beams and "hard" X-rays can degrade samples, particularly organic parts. These aspects require experimental testing, the results of which could feed into the database mentioned above.
- Finally, data processing is often a tedious operation, especially when there is a large amount of data. Machine learning and artificial intelligence (AI) tools can help to sort images and highlight significant differences between batches of images obtained under different conditions (see the examples of shells from polluted and unpolluted areas[32, 33]).

These Faraday Discussions have presented a wide variety of technical approaches to imaging biomineralisation, to which the following could be added: imaging of nanomechanical tests, Deep-UV luminescence (DUV) analyses and SHG (Second Harmonic Generation) microscopy imaging, which are three complementary approaches to what has been shown in this volume. In particular, the latter two approaches (DUV, SHG) allow the organic constituents of biomineralisation to be visualised as shown by Fig. 3 below.

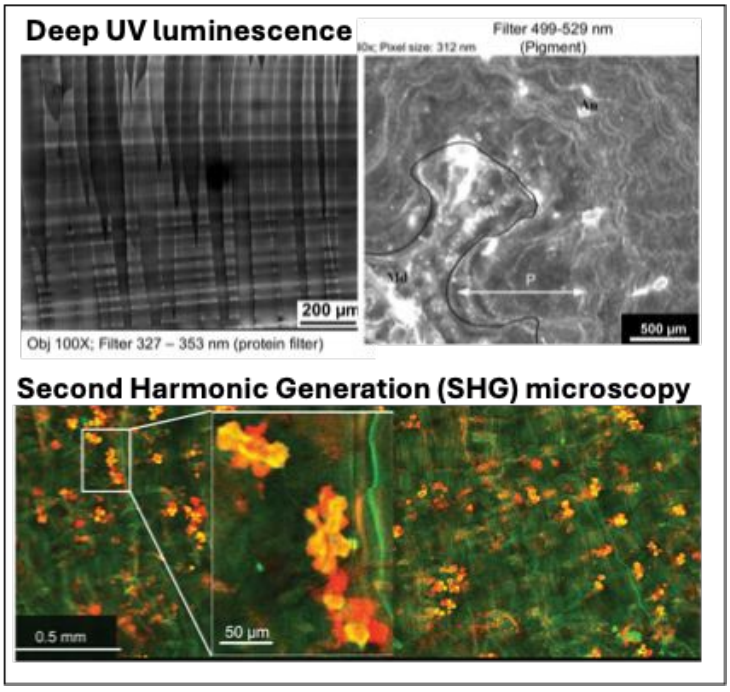

Fig. 3. Examples of Deep UV luminescence imaging (DUV, top) and Second Harmonic Generation microscopy (SHG, bottom). Top left corresponds to the calcitic prisms of *Pinna nobilis* shell, observed longitudinally; top right is a section of the red coral *Corallium rubrum*. Different filters allow revealing either the protein moieties or the organic pigment. Bottom corresponds to SHG imaging of the red coral. Pictures taken from the PhD thesis of B. Khurshid[39].

It is precisely this point that I would like to address in conclusion: imaging the organic constituents of biomineralisation is an aspect that has been relatively overlooked in these Faraday Discussions. We know that the organic matrix is thought to have many functions in biomineralisation[40], but these roles are still under discussion: how does the matrix create a microenvironment conducive to the precipitation of a mineral phase? What is the contribution of the matrix to the selection of one polymorph over another? How does it control the size and shape of biomineral particles, as well as their arrangement into specific microstructures? These questions remain essential when discussing biomineralisation.

According to certain analytical approaches, the organic matrix represents a single entity in relation to the mineral phase. By considering the organic matrix as a whole, there is a risk of failing to take into account its extreme heterogeneity[40] and the differential locations of some of its constituents: for example, in many cases, there is a clear spatial distinction between the intercrystalline matrix (which forms sheaths around crystals) and the intracrystalline matrix (trapped inside “mesocrystals” according to the terminology of Cölfen and Antonietti[14]), these locations reflecting the affinity of these two matrices for the mineral phase. We believe that, on the contrary, the imaging of mineralising matrices needs to be refined, even if this means attempting to differentially locate its molecular constituents taken separately. This difficult task requires the development of molecular tools that specifically target matrix macromolecules, namely antibodies. While many antibodies are already commercially available for mouse or human bone models, the same is not true for invertebrate models. At present, two approaches seem promising to me: immunogold and AFM microscopy with antibody-functionalised tips. We have successfully developed both of these approaches on the calcitic shell of *Pinna nobilis*, a bivalve model [39,41, 42]. The main constraint they impose is that imaging is performed only in two dimensions: on a polished flat surface in the case of AFM, or on a polished surface or fresh fracture in the case of immunogold. We believe that the next technological frontier is the development of spatial (*i.e.*, 3D) proteomics adapted to biomineralisation, with laser ablation systems that will enable the removal of tiny fragments of organo-mineral material, the partial hydrolysis of organic constituents (and their subsequent release from the mineral phase), their ionisation for direct analysis by tandem spectrometry, and finally, the reconstitution of a 3D map localizing each protein. Such a device does not yet exist in biomineralization field but could revolutionise our understanding of the role of organic matrix macromolecules in controlling biomineral deposition.

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Data Availability Statement (DAS)

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No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

The two Tables (Table 1, Table 2) have been specifically prepared for my conclusion paper.

As stipulated in its legend, Figure 1 was redrawn from a paper of Daniel Vielzeuf and coworkers published in 2010 in American Mineralogist.

Figure 2 has been specifically prepared for this paper.

Figure 3 has been prepared from 3 photos taken from the PhD thesis manuscript of my former PhD student, Benazir Khurshid (see legend of the figure).