

CryoTEM as an Advanced Analytical Tool for Materials Chemists

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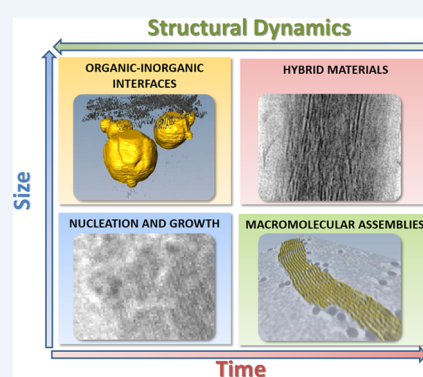
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CONSPECTUS: Morphology plays an essential role in chemistry through the segregation of atoms and/or molecules into different phases, delineated by interfaces. This is a general process in materials synthesis and exploited in many fields including colloid chemistry, heterogeneous catalysis, and functional molecular systems. To rationally design complex materials, we must understand and control morphology evolution. Toward this goal, we utilize cryogenic transmission electron microscopy (cryoTEM), which can track the structural evolution of materials in solution with nanometer spatial resolution and a temporal resolution of <1 s.

In this Account, we review examples of our own research where direct observations by cryoTEM have been essential to understanding morphology evolution in macromolecular self-assembly, inorganic nucleation and growth, and the cooperative evolution of hybrid materials. These three different research areas are at the heart of our approach to materials chemistry where we take inspiration from the myriad examples of complex materials in Nature. Biological materials are formed using a limited number of chemical components and under ambient conditions, and their formation pathways were refined during biological evolution by enormous trial and error approaches to self-organization and biomineralization. By combining the information on what is possible in nature and by focusing on a limited number of chemical components, we aim to provide an essential insight into the role of structure evolution in materials synthesis. Bone, for example, is a hierarchical and hybrid material which is lightweight, yet strong and hard. It is formed by the hierarchical self-assembly of collagen into a macromolecular template with nano- and microscale structure. This template then directs the nucleation and growth of oriented, nanoscale calcium phosphate crystals to form the composite material. Fundamental insight into controlling these structuring processes will eventually allow us to design such complex materials with predetermined and potentially unique properties.



1. INTRODUCTION

Self-assembly provides an elegant strategy to create functional, highly complex, and hybrid materials for a myriad of applications. In particular, living systems, through evolution, have achieved exquisite control over the deposition of both organic and inorganic building blocks to create hierarchical, composite materials with exceptional properties. Nature does this under ambient conditions, utilizing compartmentalization and confinement of chemical environments to control the pathway of formation, realizing structures and shapes that are not readily achievable in synthetic systems. If materials chemists are ever able to have this level of control, it will come from a deep understanding of general mechanisms and pathways that govern the self-assembly of hierarchical and hybrid structures in complex solution environments.¹ We aim to gain this deep understanding by investigating materials formation processes in synthetic and bioinspired systems to avoid the trial-and-error approaches common in materials science. This is a highly interdisciplinary task combining synthetic, analytical and theoretical approaches. From an analytical standpoint, imaging

techniques that enable direct observations of structure and chemistry in solution with atomic or nanoscale resolution provide an essential perspective. To achieve this, cryogenic transmission electron microscopy (cryoTEM),^{1,2} liquid phase EM,^{3,4} liquid phase atomic force microscopy (AFM),^{5,6} super-resolution light microscopy (LM),^{7,8} and X-ray microscopy^{9,10} can be utilized. CryoTEM provides a unique perspective in that it can be used to determine the structure of materials in liquids with (sub)nanometer resolution in two and three dimensions with subsecond time resolution.^{11–14} This is enabled by plunge freezing a thin layer of reaction solution,¹⁵ which vitrifies the liquid and the transient structures within at a single time point. For the materials chemist this means that the formation process in almost any reaction can be followed to understand the evolution of shape, size and crystallinity of materials. As we will show, complemented by chemical, theoretical, and bulk analysis, cryoTEM can provide the link between the underlying

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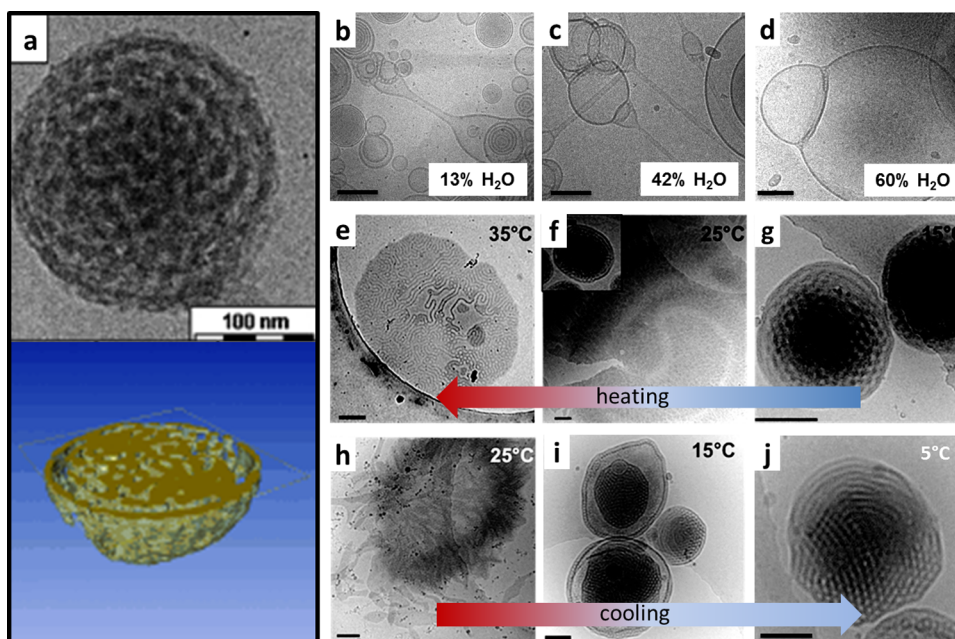


Figure 1. CryoTEM image and 3D rendering of a PNOEG-PNGLF bicontinuous nanoparticle. (a) CryoTEM image of PEO-*b*-PODMA dispersions at different water concentrations. (b–d) PEO-*b*-PODMA dispersions at constant water content; (e–g) increasing temperature; (h–j) decreasing temperature. Scale bars in (b–j) are 200 nm. Panel (a) is adopted from ref 22. Copyright Wiley-VCH. Panels (b–d) and (e–j) reproduced, respectively, from the Supporting Information and main text of ref 23 with permission from the Royal Society of Chemistry.

solution chemistry of synthetic precursors, transient assemblies, and complex materials, offering unique insights and directions for future research and innovation.

2. COMPLEX MACROMOLECULAR ASSEMBLIES IN SOLUTION

Understanding the self-assembly of macromolecules in solution is important in many fields, including organic photovoltaics, biomedical and food technologies, and interface and colloid science.^{16–18} Self-assembly processes are typically studied using conventional, dry state TEM, and scattering experiments. However, these offer only limited insight due to the poor stability of soft structures upon drying and the complexity of the scattering models needed to interpret their assembly processes,^{17–21} in particular for particles with complex internal morphologies. Indeed, cryoTEM pioneer Jacques Dubochet compared studying a liquid system with dry TEM to looking at an aquarium after removing all the water from it. Here we will discuss how the use of cryoTEM and cryo-electron tomography (cryoET, 3D cryoTEM) can provide the essential details regarding the structure and formation of synthetic macromolecule systems. While cryoTEM is becoming a more and more widespread technique for the characterization of a wide variety of biological structures, cryoET is still relatively unexplored for synthetic organic materials, as is exemplified by our study of bicontinuous polymer nanoparticles discussed below.^{20,22–24} We also use this example to illustrate the possibility of using cryoTEM to monitor self-assembly in the presence of organic solvents, and eventually show how cryoET can even be used in a fully organic solvent to reveal the 3D structure of P3HT nanowires as well as to monitor the development of crystallinity in the system.²⁵

The strength of cryoET for studying synthetic macromolecules in solution was first demonstrated for tripeptide-containing amphiphilic double-comb diblock copolymers,

which were shown to form spherical aggregates with internal bicontinuous structure (Figure 1a).²² Later on, the formation of similar bicontinuous polymer nanoparticles (BNPs) was shown for poly(ethylene oxide)-*b*-poly(octadecyl methacrylate) (PEO-*b*-PODMA), for which the entire assembly pathway was visualized using cryoTEM (Figure 1b–j). The different morphological stages were determined as a function of the solvent composition (water/THF ratios) and temperature during the polymer assembly processes.^{20,23,24} This allowed us to observe that, contrary to expectations, the bicontinuous nature of the particles is not formed upon changing solvent composition, but results (after complete removal of the organic solvent) from a reversible temperature transition in pure water.^{23,24} Complementary analysis by small-angle X-ray scattering (SAXS) showed that this ordering is associated with an interdigitation of the PODMA side chains upon cooling and that full crystallization of the PODMA chains is inhibited, most likely imposed by the geometrical constraints on the polymer inside the nanostructure. This means that the formation of the pore structure is essentially a bulk phase separation process more akin to an order–disorder transition in bulk block copolymer assembly,^{24,26} rather than a more complex solution assembly process. Accordingly this allowed the internal morphology to be tuned via minor alterations in molecular structure.²⁰ Moreover, the insight that the bicontinuous morphology here is controlled by an order–disorder transition allows us to further investigate BNP formation based on bulk phase separation principles such as “tapered” block copolymers and homopolymer blending.²⁷ Although numerous morphologies, such as multilamellar vesicles, worms, and spheres, were also observed during the early stages of water addition, at 63 vol % water, large micrometer sized nonstructured aggregates were found, from which discrete 100–300 nm nonstructured aggregates bud off during complete THF removal via dialysis. The clear separation of these two processes—discrete particle formation by budding

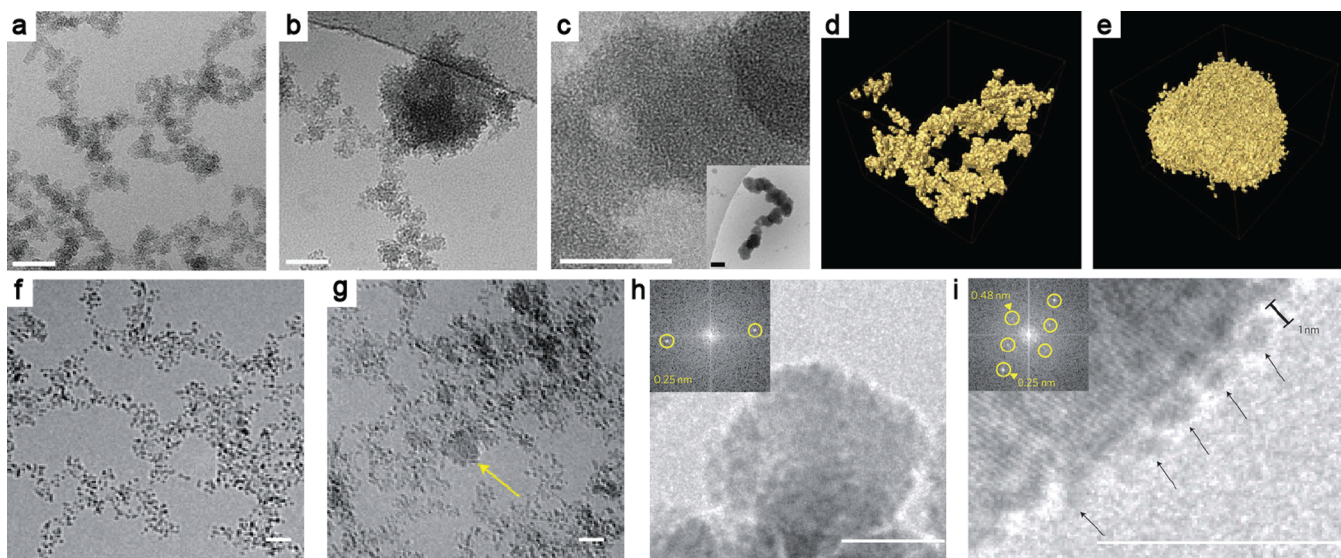


Figure 2. CryoTEM observation of nucleation and growth processes. (a–e) Morphological transformations of calcium phosphate. (a, b) Branched polymeric and densified nodulelike assemblies of prenucleation species, respectively. (c) Amorphous calcium phosphate (ACP) spheres. (d, e) 3D isosurface rendering of cryoET data of the polymeric assemblies and ACP sphere, respectively. (f–i) Nucleation and growth process of magnetite. (f, g) Primary-particle and magnetite-nanoparticle aggregates imaged after 2 and 6 min, respectively. Yellow arrows in (g) indicate early formed crystalline magnetite nanoparticles. (h) Images of a magnetite nanoparticle. (i) Image of primary particles (arrows) attaching to the surface of a magnetite nanoparticle. Insets in (h, i): Fast Fourier transform indicating the crystallinity of the particles. Scale bars: (a–c) 50 nm, inset of (c) 100 nm, (f–i) 10 nm. Panels (a–e) are from ref 35 with permission from Nature Publishing Group. Panels (f–i) are from ref 39 with permission from Nature Publishing Group.

during dialysis and phase separation during cooling—are key to defining future research directions in the synthesis of these and even more complex morphologies.

As indicated above, cryoTEM can also be used to observe macromolecular structures directly in pure organic solvents.^{28,29} This is particularly useful in fields such as photovoltaics where, due to their high conjugation, polymeric materials are inherently water insoluble. Our investigation of poly(3-hexylthiophene) (P3HT) self-assembly in organic solvents by cryoTEM, and cryoET showed ordered nanowires (20–30 nm diameter, >100 nm in length) are present in both toluene and *ortho*-chlorobenzene (oDCB) at 1 wt %.²⁵ These assemblies form upon cooling due to π – π stacking interactions, which create ordering that can be monitored by low dose electron diffraction (LDED). By a combination of these techniques, we were able to show that compared to oDCB, using toluene as the solvent leads to (1) a higher fraction of nanowires and (2) a higher degree of crystallinity on account of the lower solubility of P3HT in this solvent.

The ability to observe macromolecular and potentially transient assemblies in their native solution state is essential to understanding structure formation, e.g., during processing steps such as heating, cooling, drying, printing, spin coating, and so forth. Although these observations are powerful, to gain control over these processes, we must understand the kinetics and thermodynamic driving forces that decide the pathway. This will require the utilization of cryoTEM in combination with other theoretical and experimental techniques that can probe dynamics, such as SAXS. In this regard, much progress has been made in the analysis of mineral formation, discussed in the next section, as well as some recent developments for in situ, liquid phase electron microscopy.¹ In summary, as shown above, cryoTEM can be used to follow macromolecular assembly processes in solution and is particularly suited to study pathways in which different structures coexist.

3. NUCLEATION AND GROWTH OF INORGANIC MATERIALS

Crystallization or other phase transformation processes involved in materials synthesis can, in most cases, be divided into two distinct stages: nucleation and growth. Although a thermodynamic description of nucleation and growth was already developed by Gibbs in the 19th century,³⁰ understanding the details of these processes is still an enormous challenge, mostly due to our inability to obtain information with sufficient spatial and time resolutions.³¹ In recent years, it has become clear that a wide variety of kinetic pathways are possible for the nucleation and growth of crystals, which often proceed via attachment of precursor particles instead of “monomers” (i.e., single ions, atoms or molecules).^{32,33} Such precursor particles are often disordered, transient, and highly hydrated, which makes their structural characterization and chemical analysis via conventional methods extremely difficult; and their evolution is not easily described with classical theories. Additionally, the precursor particles are small (often < 5 nm), which puts them at the detection limit of almost all techniques. These challenges and limitations further highlight that direct visualization by cryoTEM offers a unique window in the study of nucleation and growth processes and provides new insights into material formation mechanisms. Undoubtedly, cryoTEM has played a key role in visualizing such nonclassical pathways to crystallization, due to the possibility of observing transient (amorphous) precursor phases as shown by our studies of several inorganic systems including calcium carbonate,³⁴ calcium phosphate,^{35–38} and iron oxide.³⁹

Our investigation into calcium phosphate nucleation by cryoTEM showed nanometer sized prenucleation species which appear in the first few minutes after the addition of a phosphate solution to a buffered solution of calcium ions.³⁵ These prenucleation species first aggregated into polymeric and then

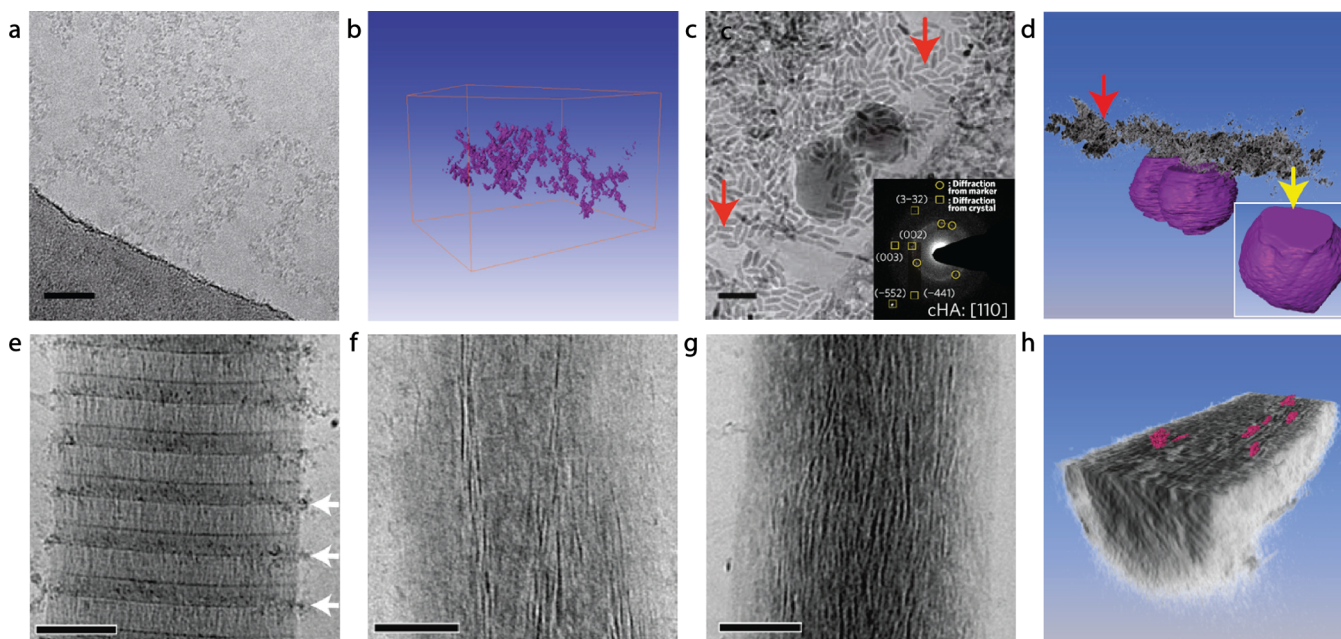


Figure 3. CryoTEM images of mineralization at an organic interface or within an organic matrix. (a–d) CaP mineralized in simulated body fluid under an arachidic acid Langmuir layer. (a, b) Assemblies of pre-nucleation complexes and the 3D surface rendering. (c, d) Final hydroxyapatite (HAp) nanocrystals and the 3D surface rendering. Inset of (c): select area electron diffraction (SAED) pattern of the crystal. The markers for tomography are highlighted by red arrows in (b, d), while the (110) plane of the HAp crystal is highlighted by yellow arrow in (d). (e–g) Collagen fibril during CaP mineralization in the presence of pAsp. (e–g) Images of collagen mineralized for 24, 48, and 72 h, respectively. ACP particles outside the fibril associating with the charged amino bands are highlighted by white arrows in (e), which develop into elongated HAp crystal platelets in (f) and (g). (h) 3D rendering of mineralized collagen fibril (gray) with HAp crystals (pink). Scale bars are (a, b) 50 nm and (e–g) 100 nm. Panels (a–d) are from ref 36 with permission from Nature Publishing Group. Panels (e–h) are from ref 37 with permission from Nature Publishing Group.

nodule-like assemblies (Figure 2a and b) and subsequently densified into solid amorphous calcium phosphate (ACP) spheres (Figure 2c). Ca-Ion selective electrode measurements and liquid phase AFM, in combination with box counting analysis to determine the fractal dimension of the structures from the cryoET reconstructions before and after ACP nucleation (Figure 2d and e), provided insights into the nucleation mechanism. From the combined information it was concluded that the pre-nucleation species shown in Figure 2a and b are not the ACP clusters for which the formula $[\text{Ca}_9(\text{PO}_4)_6]^{4-}$ had been proposed (“Posners clusters”),⁴⁰ but are in fact ion-association complexes of composition $[\text{Ca}(\text{HPO}_4)_3]^{4-}$, which has recently been supported by computer modeling.⁴¹ During nucleation above complexes take up calcium ions to form post-nucleation clusters $[\text{Ca}_2(\text{HPO}_4)_3]^{2-}$ that precipitate into ACP spheres, and subsequently transform via an octacalcium phosphate (OCP) intermediate into crystalline nanoapatite. It became clear that each of these stages represents a separate phase with its own characteristic calcium solubility and the overall pathway toward the final crystal is controlled by kinetic barriers and structural rearrangements.

CryoTEM has also been used to visualize the early stage of magnetite nucleation and growth, which was shown to occur via the formation of an unstable precursor phase that rapidly converts to low dispersity primary particles of around 2 nm (Figure 2f).³⁹ These primary particles then dehydrate to form secondary particles of 1.0–1.5 nm in size,⁴² which simultaneously aggregate to form 5–15 nm crystals (Figure 2g), displaying a lattice spacing conform with magnetite (Figure 2h, inset), and grow further via attachment and crystallization of secondary particles (Figure 2i). Although the structure of the

primary particles remains unknown, considering that they exhibited no crystallinity before attachment and showed uniformity in their size, as well as in their volume decrease, the individual attachment and crystallization of secondary particles appears to be quite a molecularly uniform process. (Similar observations of a primary particle based growth mechanism were also made for silica, which facilitated size-controlled synthesis combined with high colloidal stability.^{43,44}) More importantly, after a broad theoretical investigation of the relevant parameters to move from atoms/molecule to final crystal structures and the thermodynamic contribution to each pathway, the direct observation of these attachment mechanisms can be explained by a consideration of the relative energy barriers to formation, and hence is still describable within the framework of classical nucleation theory.³⁹

In summary, cryoTEM has been an essential tool for us to resolve the nucleation and growth stages of inorganic materials in liquids which forms the basis to the rational structuring of inorganics with predetermined structures and properties.

4. HYBRID MATERIALS AND INTERFACES

In the previous two sections, we have illustrated how the formation of organic and inorganic materials can be a complex process with many intermediate structures and phases contributing to the final product. In Nature, structure formation of inorganic components is almost always guided by soluble additives and compartmentalization of nonsoluble organic components.⁴⁵ The presence of these tailored surfaces or macromolecular species will of course shift the energy barriers to nucleation discussed above. Through evolution, natural systems have come to steer mineral formation pathways through various energy-economical routes, and generate

biominerals with different polymorphs, microstructures, and properties, depending on their specific biological functions. Understanding how to direct mineral formation processes with the use of surfaces or macromolecular species in solution is one of the great challenges in bioinspired chemistry. To date, only a few studies exist in which detailed organic–inorganic interactions are investigated in living systems.^{46,47} Instead, many studies use biomimetic in vitro systems of reduced complexity with synthetic organics,^{31,34,37,48} but similar difficulties remain even with such simplified model systems. From a characterization standpoint, it requires creating what is known (or hypothesized) to be present at the biological interfaces on a TEM grid and interpreting the detailed structure of hybrid materials whose components display a wide variety of contrast mechanisms.

We explored the potential of cryoTEM and cryoET to study pathological mineralization of calcium phosphate at organic/inorganic interfaces with a biomimetic mineralization model using simulated body fluid (SBF).³⁶ A Langmuir monolayer of arachidic acid was used to simulate the biological calcifiable interface. Assemblies of ~1 nm sized prenucleation complexes were observed in the beginning of the experiment (Figure 3a and b), which later evolve into ACP nanoparticles. These eventually formed hydroxyapatite (HAp) crystals which preferentially nucleated on the (110) plane due to the organic/crystal interaction (Figure 3c and d). The results displayed the vital role of the organic matrix in organizing the prenucleation complexes to form ACP, and subsequently oriented apatite crystals.

This study was then extended to the formation of bone, which is a composite material formed through the collagen templated mineralization of CaP.⁴⁹ By reconstituting isolated collagen fibrils onto TEM grids followed by incubation with CaCl₂, K₂HPO₄ and pAsp (nucleation inhibitor), the biomimetic mineralization process was followed by cryoTEM and cryoET (Figure 3e–i).^{12,37} Selective staining procedures were used to precisely localize infiltration and mineralization with respect to the charge density distribution inside the collagen fibril. In this process charged macromolecules temporarily stabilize the ACP in solution, facilitating its infiltration of the collagen. Nucleation of nanocrystals was then shown to only occur at the location of charged amino acids bands within the collagen. Mineralization then proceeds through the fibers forming a composite structure of collagen and ~2 nm thick CaP nanoplates, similar to the one found in bone (Figure 3e–g). Since the ACP/pAsp precursor is negatively charged, the localization of infiltration was attributed to electrostatic interactions with the positively charged C-terminal end of collagen molecules. However, it has also been reported that intrafibrillar mineralization of collagen can be induced by positively charged polymers (e.g., poly(allylamine hydrochloride), PAH),⁵⁰ larger inhibitory proteins (fetuin)³⁷ or without the presence of organics.⁵¹ This implies that electrostatic considerations alone are not enough to explain infiltration, and recently the importance of balancing electro neutrality and osmotic forces has been highlighted.⁵⁰ However, it is clear that a full understanding of the mechanisms behind macromolecular and mineral transformations under confinement will require the combination of high resolution structural and real-space dynamic information. Toward this end, we recently reported the combination of liquid phase TEM and AFM to reveal the nucleation and growth mechanisms behind the formation of calcium carbonate in a nanoscale polystyrene

sulfate matrix.⁴⁸ The in situ imaging techniques allowed for the direct investigation of the role of ion binding in relation to the free energy barrier to nucleation on a single particle level. However, the resolution and 3D information of these techniques is inherently limited compared to cryoTEM and cryoET, making the combination of all these techniques an exciting prospect for understanding materials formation, which has yet to be realized.

5. CONCLUSION

Understanding material formation processes in liquids through segregation of atoms and/or molecules into different phases is a complicated but essential task for materials chemistry. We have shown that cryoTEM can be used to resolve the structural evolution of organic, inorganic, and hybrid materials under a variety of synthetic conditions such as solvent mixing and heating/cooling cycles. The combination of cryoTEM with complementary techniques such as infrared spectroscopy, pH and ion-selective electrode measurements, SAXS/WAXS measurements, liquid phase AFM, and computer simulations can be particularly powerful, to provide a deeper understanding of the molecular configurations present in the structures observed by cryoTEM. While there are many techniques which can be used to gain insight into why and how structurally complex materials form, with LP-EM being a promising recent addition to look at dynamics,⁴⁸ we believe that cryoTEM serves as a necessary baseline in these studies. By providing unbiased snapshots throughout a synthesis, an overview of the (3D) structural evolution, from starting materials to final products, can be readily obtained. This gives a robust platform from which we can choose to deepen our understanding, through the use of theory or further analysis, to a level which either allows us to achieve our synthetic goals or satisfies our scientific curiosity.

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Notes

The authors declare no competing financial interest.

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Yifei Xu obtained his Ph.D. in condensed matter physics from Nanjing University, PRC in 2016 working under the supervision of Prof. Mu Wang. He then joined the group of Prof. Nico Sommerdijk as a postdoctoral researcher. His current research focuses on cryoTEM study of biomineralization processes, and bioinspired hybrid functional materials.

Mohammad-Amin Moradi is postdoctoral researcher at the Laboratory of Materials and Interface Chemistry since 2016. He is interested in investigating vesicle-based nanostructures and multi-materials with controlled assembly via cryoTEM.

Nico Sommerdijk is full professor at Eindhoven University of Technology and chair of the Laboratory of Materials and Interface Chemistry. In 1995 he obtained his Ph.D. (Cum Laude) from the University of Nijmegen for his work on chiral amphiphiles. He did postdoctoral work on sol-gel silicates (1995–1997, University of Kent, UK), on bioinspired crystallization (1997, Keel University, UK), and on macromolecular self-assembly (1997–1998, Nijmegen, -NL). In 1999 he moved to Eindhoven to work on bioinspired hybrid materials through biomimetic mineralization and self-organization. His research uses biological processes such as the formation of bones teeth and sea shells as a source of inspiration for the synthesis of advanced materials. He studies these processes combining (macro)molecular self-assembly and advanced electron microscopy, in particular cryoTEM. His work has been supported by VIDI and VICI Awards from The Netherlands Science Foundation NWO, and he is winner of the RSC Soft Mater and Biophysics Award 2015. He is director of Centre of Multiscale Electron Microscopy, core member of the Institute for Complex Molecular Systems, member of the Eindhoven Polymer Laboratories and the Eindhoven Multiscale Institute.

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