Erschienen in: Biophysical Reviews ; 8 (2016), 4. - S. 309-329
https://doi.org/10.1007/s12551-016-0228-4

Mineralization and non-ideality: on nature's foundry

Ashit Rao[1] · **Helmut Cölfen**[2]

Abstract Understanding how ions, ion-clusters and particles behave in non-ideal environments is a fundamental question concerning planetary to atomic scales. For biomineralization phenomena wherein diverse inorganic and organic ingredients are present in biological media, attributing biomaterial composition and structure to the chemistry of singular additives may not provide a holistic view of the underlying mechanisms. Therefore, in this review, we specifically address the consequences of physico-chemical non-ideality on mineral formation. Influences of different forms of non-ideality such as macromolecular crowding, confinement and liquid-like organic phases on mineral nucleation and crystallization in biological environments are presented. Novel prospects for the additive-controlled nucleation and crystallization are accessible from this biophysical view. In this manner, we show that non-ideal conditions significantly affect the form, structure and composition of biogenic and biomimetic minerals.

Keywords Biomineralization · Crystallization · Liquid phase · Molecular crowding · Non-ideality · Nucleation

Introduction

In the living world, steady, successive changes of simpler cellular forms have led to fascinating morphologies and internal organizations (Oparin 1938). In view of the physico-chemistry of cellular life, 'microspaces' with multicomponent, multiphase and crowded conditions in aqueous electrolytes are presumed crucial for enabling sustained biochemical processes (Spitzer et al. 2015; Spitzer and Poolman 2009). Such crowded and confined environments render macromolecular reaction rates and equilibria complex, especially in view of the heterogeneous composition of biological spaces (Zhou et al. 2008). At the mesoscale, the impact of non-ideality is evident from the liquid-like behavior of membraneless cellular components (Brangwynne et al. 2009, 2011; Hyman and Brangwynne 2011). Associated subcellular phase transitions fundamentally alter the perception of biomolecular interactions and bring to the center-stage the multiphasic aspects of biochemical regulation. Although chemical non-ideality is vital for cellular processes (Minton 2001; Zhou et al. 2008), several in vitro experiments often apply relatively low contents of biomolecular reactants and may neglect a faithful replication of the biophysical scenario. Therefore, the states and dynamics of the physico-chemistry in niche biological environments can offer significant insights into the workings of life.

Biological and geological processes, the origins of life, and the machinery of biomolecules involve complex interactions of organic and inorganic constituents operating at different time scales. Among biological processes, biomineralization is unique because it encompasses a dynamic interface between

✉ Ashit Rao
ashit.rao@frias.uni freiburg.de

✉ Helmut Cölfen
helmut.coelfen@uni konstanz.de

[1] Freiburg Institute for Advanced Studies, Albert Ludwigs University of Freiburg, 79104 Freiburg im Breisgau, Germany

[2] Physical Chemistry, Department of Chemistry, University of Konstanz, D 78457 Konstanz, Germany

organic molecules and different forms of inorganic matter, i.e.. ions, ion-clusters, amorphous phases and crystals. Given the interdisciplinary nature of biomineralization, relevant to fields such as biochemistry, material science, physiology, medicine and geology, an understanding of mineral formation under conditions of non-ideality is essential. Therefore, in this review, we explore the consequences of physico-chemical non-ideality on material nucleation and growth.

Facets of biophysical non-ideality

In ideal solutions, the chemical behavior of a dissolved species correlates with its concentration, i.e. the value of the activity coefficient is unity. Usually, only dilute solutions are considered ideal, the conditions of which significantly deviate from cellular environments. Therefore, the extrapolation of concentration-dependent equilibria and rate constants determined for ideal conditions may not provide an accurate representation of cellular biophysical processes. For instance, in cellular interiors, macromolecules occupy between 20 and 40 % of the cytoplasmic volume (Ellis 2001; Ellis and Minton 2003). Under such conditions, the equilibrium constants for protein self-association can change by tens of orders of magnitude depending on the activity coefficient of associating species, which in turn depends on the concentration, shape and molar mass distribution of macromolecules (Zimmerman and Trach 1991). Deviations in protein equilibria and stability are also identified for high concentrations of small co-solutes such as polyols (Davis-Searles et al. 2001; Patel et al. 2002). From this perspective, it is important to study metabolic and regulatory pathways in living systems in the context to the actual physio-chemical environments.

Several forms of non-ideality are ubiquitous in biological systems. One of these, the excluded volume or molecular crowding effect, originates from non-specific steric repulsions arising from the mutual impenetrability of solute molecules (Ellis 2001). Studies show that, under crowding conditions, activity coefficients and conformational states can be significantly altered, thus affecting reaction equilibria and rates (Chebotareva et al. 2001; Zimmerman and Minton 1993; Zimmerman and Trach 1991). For instance, sedimentation equilibrium centrifugation using labeled macromolecular solutes reveals that fibrinogen activity in a crowded environment is about an order of magnitude larger than that in simple buffer solutions (Rivas et al. 1999). Molecular crowding also modulates homo-/hetero-association and conformational behavior. For example, the apparent binding constants for interactions between oligonucleotides and a certain DNA binding protein (TyrR) is enhanced in the presence of crowding induced by small co-solutes (Poon et al. 1997). The development of distinct aggregation and conformational states is suggested to be a trade-off between crowding-induced aggregation and diffusion-limitation effects that operate in relation to molecular molar mass of the crowding agent (Shearwin and Winzor 1990a; Winzor and Wills 2006; Ellis 2001). Conditions of crowding can also enhance the stability of biomolecules against denaturation and inactivation (Winzor and Wills 1986; Winzor et al. 1992; Hall et al. 1995). The effects of molecular crowding also encompass extracellular environments which are rich in secreted macromolecules such as fibrous proteins and proteoglycans. For example, the nucleation and growth of collagen fibrils as well as the emergent physical properties of mechanics and porosity are dependent on prevailing crowding conditions (Dewavrin et al. 2014). Such environments are suggested to stimulate the formation and self-assembly of collagen into triple helices as demonstrated in vitro (Lareu et al. 2007). As the fundamental roles of molecular crowding in living systems are gradually being deciphered, its applicability in fields such as tissue engineering and pathology is gaining emphasis (Dewavrin et al. 2014; Shtilerman et al. 2002).

Distinct from crowding, molecular confinement is another form of non-ideality applicable to biological design and function. Macromolecular confinement refers to the behavior of a solute with respect to volume exclusion by a fixed boundary (e.g., cytoplasmic meshwork or extracellular matrix), whereas macromolecular crowding refers to effects of volume exclusion with reference to inter-solute interactions (Zhou et al. 2008). Confinement of macromolecules within recesses or pores can result in significant size- and shape-dependent changes of solute chemical potential. Along these lines, equilibrium constants of reactions and associations significantly vary in confinement relative to the bulk phase (Minton 2001). Weak, non-specific interactions between solutes in restricted volumes and confining surfaces are suggested to affect solute self- or hetero-association driven by adsorption-based interactions with the confining surface (Minton 1995). Statistical–thermodynamic models predict that aggregates with extended morphologies (linear or discoid) are favored under confinement in comparison to globular shapes favored under crowded conditions (Minton 1992). Conditions of confinement (such as in chromatography resins, Anfinsen cages of chaperones, and ribosomal interiors) are also suggested to stabilize the protein molecules against reversible unfolding affected by the amino acid chain length and volume of confinement (Zhou and Dill 2001).

Cell-associated liquid-like states and associated phase transitions represent an important paradigm shift in cellular physico-chemistry. Membraneless sub-cellular compartments such as nucleoli, centrosomes and Cajal bodies exist as phase-separated, liquid-like droplets (Hyman and Brangwynne 2011; Hyman et al. 2014). Demixing phase transitions can lead to liquid-like, hydrogel or other colloidal nano-phases depending on electrostatic, dipolar and short-range directional interactions, molecular concentrations and conformations as

well as physiological conditions (Brangwynne et al. 2015; Lin et al. 2015; Xiang et al. 2015). Liquid–liquid phase transitions in macromolecular solutions are also of interest to the field of protein crystallization (Wills and Winzor 2005; Deszczynski et al. 2006). Phase separation-based cellular organization is not limited to aqueous solutions. Phase-transitions of lipid mixtures also provide a basis for the lateral organization of cell membranes involving cholesterol-rich lipid rafts floating in a relatively disordered liquid phase (Lingwood and Simons 2010). The impact of sub-cellular phase behavior on spatial organization and biochemical regulation is gradually being recognized, and raises interesting questions on aspects of soft matter physics operating in vivo at different length scales. In all, molecular crowding, confinement and mesoscopic phase transitions represent important forms of non-ideal physico-chemistry prevalent in biochemical niches.

Biomineralization

By definition, biomineralization encompasses biochemical mechanisms for the selective extraction and uptake of elements from proximal environment of the organism and their incorporation as functional materials (Lowenstam and Weiner 1989; Mann 2001). In other words, the biomineralization machinery is an evolutionarily optimized system dedicated to the regulation of nucleation, phase transitions and morphogenesis concerning inorganic constituents of (bio)minerals (Fig. 1). While the chemical diversity of biominerals is very limited, with calcium carbonate ($CaCO_3$), calcium phosphate and silica being by far the most abundant (Lowenstam and Weiner 1989), the diversity of biomolecules interacting with the inorganic phase (ions, ion-clusters, amorphous and crystalline particles) is remarkable, as suggested by a plethora of biochemical elements associated with biominerals (Mann et al. 2006, 2010; Marie et al. 2010). For simplicity, the collective of biomolecules (e.g. skeletome or shellome) contributing to the mineralization machinery is broadly categorized based on function:

1. Biomolecules perform ion-sequestration and transport of mineral precursors. For example, in simple planktons, biochemical pathways regulate seawater endocytosis and transportation to mineralization sites (de Nooijer et al. 2009). Important members include ion transporters such as Ca^{2+} ATPase pumps and anion exchangers (Fan et al. 2007; Mackinder et al. 2011) as well as enzymes such as carbonic anhydrase. The activities of these biomolecules condition the pre-nucleation and early mineralization stages, for instance by regulating supersaturation and pH. Such biomolecules can also modulate the stability of mineral precursors as well as regulate nucleation and phase transitions reflecting the multiple roles of (bio)molecules as additives in mineralization processes (Verch et al. 2011; Rao et al. 2014). Here, multiple properties of biomolecular additives such as charge, amino acid/glycan/lipid composition, covalent modifications, conformations, and self-assembly propensity as well as intermolecular synergy play mechanistic roles (Arias and Fernández 2008; Bentov et al. 2010; Chang et al. 2016a, b; Rao et al. 2013, 2015).
2. Closely associated with intermediate inorganic phases, biomolecules tune phase transitions of the inorganic phase. Examples include proteins with acidic residues or phosphorylation, suggested to promote the formation and stabilization of amorphous mineral phases (Addadi et al. 2003; Bentov et al. 2010), and also possibly lipids, in the form of membranes that may encapsulate and enhance the stability of amorphous phases (Tester et al. 2011). Proteinaceous and saccharide additives can also tune the stability of early precursors and favor the formation of a particular phase, i.e. mediate poly(a)morph selection (Cartwright et al. 2012; Chang et al. 2016a; Gebauer et al. 2009; Rao et al. 2013, 2014).

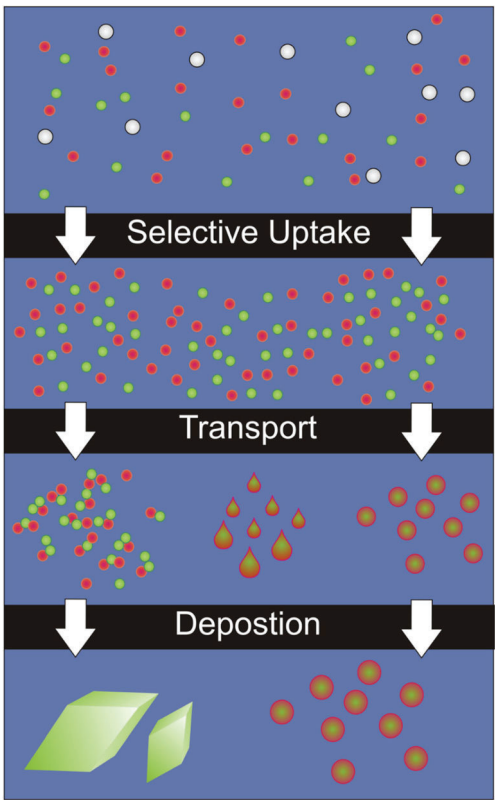

Fig. 1 A simple representation of biomineralization steps involving (*top* to *bottom*) (1) the selective uptake of ions, the fundamental mineral precursors from nutrients and environment, (2) the transport of assimilated ions as well as transient mineral precursors, i.e. ion clusters and (possibly liquid like) amorphous phases, and (3) finally, the growth of amorphous and crystalline matter at the mineralization site

3. Present in several composite biominerals, organic matrices (or scaffolds) are largely constituted by insoluble macromolecules and play critical roles during biomineralization such as (1) providing niche spaces with tuned surface chemistry for mineral deposition, (2) regulating mineral growth and repair at different length scales, as well as (3) elegantly integrating with the inorganic phase to realize extraordinary physical properties. A prime example, from mollusk shells, is an inter-lamellar membrane composed of hydrophobic β-chitin and biomolecules. This matrix provides spatially periodic sites for crystallization and imparts a hierarchical organization to the growing mineral (Addadi et al. 2003; Checa et al. 2016; Nakahara 1991; Pereira-Mouriès et al. 2002).
4. Indirect regulators of biomineralization tune properties such as post-translational modifications and assembly states of mineralization additives. For instance, the glycosylation microheterogeneity of proteins modulates self-association propensity of additives under mineralization conditions and also affects mineral nucleation and growth (Chang et al. 2016a). Secreted forms of kinases tweak the phosphorylation of extracellular proteins and thereby guide biomineralization processes (Tagliabracci et al. 2012). Matrix metalloproteases are important regulators of biomineralization, for instance by suppressing the occlusion of biomolecules in biominerals and regulating the morphology of mineral particles (Prajapati et al. 2016; Qin et al. 2004). Thus, the mineralization 'activity' of biomolecules in terms of their spatial distribution and also interactions with inorganic phases are modulated by regulatory checkpoints.
5. Although a synergetic behavior of macromolecules is crucial for regulating biochemical processes, certain key players are multifunctional and are indispensable for biomineralization. For instance, silicateins from the axial filaments of siliceous skeletons catalyze hydrolysis of silicon alkoxides and self-assemble to form filamentous templates that spatially direct silica deposition (Murr et al. 2009). Certain synthetic macromolecules fulfill multiple roles during mineralization (Verch et al. 2011) so that the multifunctionality of certain mineral-associated biomolecules appears to be no exception.

Considering the functional classes of macromolecules involved in biomineralization and their complex interplay, investigating the underlying mechanisms is a challenge due to limitations in faithfully mimicking the in vivo milieu. For instance, the isolation of biomineral-bound organic matrix is tedious due to a low solubility of biomolecules and insufficient organic content of certain biominerals. Thus methodologies that probe the effects of biomolecular additives on nucleation and crystal growth normally utilize low to moderate additive contents in bulk volumes and in turn may discount mineralization phenomena originating from crowded and confined heterogeneous environments. Also keeping in mind the polymorphism of organic molecules as monomeric and oligomeric species, adopting organized or aggregated forms as solutes, or liquid- or gel-like phases (Fig. 2), the role of organic phase transitions in course of biomineralization can be important. In fact, the biophysical complexity of biomineralization emerges to be spectacular ,considering that organic as well as inorganic phases exhibit distinct forms with characteristic hydration and structure ranging from nanoscopic ion-associates to large solid particles (Fig. 2). Thus, understanding the inter- and intra-phasic spatiotemporal dynamics in biochemical niches is the key to understanding mineralization in nature.

Crowded solutions and confinement: impact on nucleation

Few studies have applied physiologically relevant conditions during mineral nucleation and crystallization. In the case of ovalbumin, a glycoprotein abundant in avian eggs and involved in shell development (Panheleux et al. 1999), physiologically suitable contents (7.5 mg/mL) of this protein extensively stabilize a 'polymer-induced liquid precursor' (PILP) of $CaCO_3$ (Gower 2008; Wolf et al. 2011). An interesting observation here is that lysozyme (with pI = 9.3) destabilizes the

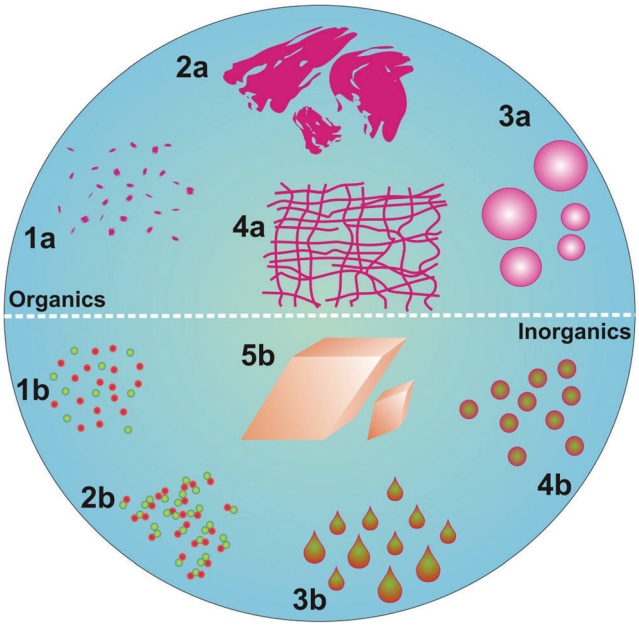

Fig. 2 Diversity of organic and inorganic phases, Organic forms exist as (*1a*) monomeric and oligomeric species, (*2a*) organized and aggregated forms, (*3a*) liquid like phases and (*4a*) hydrogels. The inorganic components are present as (*1b*) ions, (*2b*) ion clusters, and (*3b*) liquid like forms, as well as (*4b*) amorphous and (*5b*) crystalline particles

emulsified state and the acidic protein ovalbumin (pI = 4.7) enhances PILP stability. The role of macromolecular charge in regulating the coalescence of PILP droplets via charge neutralization and depletion-based stabilization is suggested from a colloid chemistry view (Wolf et al. 2011). When lower contents of ovalbumin are applied, the formed calcite crystals are morphologically distinct, with no direct indications of a liquid-like mineral precursor (Wang et al. 2010). Mineralization in the presence of varying ovalbumin contents suggests that, in addition to additive chemistry such as charge and conformation, the physiological content of additive players is an important determinant. The chemistry and shape of crowding agents may also be contributing factors. For instance, lysozyme (monomer: 14.3 kDa) and ovalbumin (monomer: 45 kDa) have distinct molar mass, surface area and shapes (Table S1), as well as exhibit distinct aggregation propensities in dependence of ionic and pH conditions (Ianeselli et al. 2010). For certain proteins, Ca^{2+} ions can modulate self-association states via direct interactions (Shearwin and Winzor 1988; Rao et al. 2013; Seto et al. 2014), suggesting ion-dependent macromolecular conformation bias during mineralization. Further due to distinct density fluctuations and conformations of crowding agents in topologically-frustrated void spaces, crowding induced by different biomacromolecules can have dissimilar consequences (Samiotakis et al. 2009). Therefore, the biophysical aspects of mineralization compartments in terms of macromolecular contents as well as the mineralization-dependent chemistry and conformation of additives require attention. Along these lines, experimental design for investigating biomolecular behavior under non-ideal conditions must also consider interactions with small molecules such as buffer components (Winzor et al. 2007; Scott et al. 2011).

Although the physical behavior of single ions is largely unaffected by macromolecular crowding, mineral species existing at larger length scales are significantly influenced based on the geometry of crowding agents as well as their chemical properties (Rao and Cölfen 2016). According to the conventional theory, nucleation in a homogenous supersaturated solution occurs via stochastic microscopic density fluctuations and a balance between the bulk and surface energy of an emerging phase (Frenkel 1939). However, recent studies indicate that ion-associates such as pre-nucleation clusters and liquid-like phases play vital roles in determining nucleation phenomena associated with several minerals (Bewernitz et al. 2012; De Yoreo et al. 2015; Dey et al. 2010; Gebauer and Cölfen 2011; Gebauer et al. 2008, 2014; Jolivet et al. 2006; Wallace et al. 2013). Potentiometric titration-based studies on $CaCO_3$ nucleation have identified distinct crowding niches leading to either enhanced or suppressed ion-association (Rao and Cölfen 2016). For instance, in the presence of BSA (0.5 mM), the nucleation of $CaCO_3$ particles involves an equilibrium shift towards ion-clusters and an additive concentration-dependent inhibition of nucleation. This effect is not easily attributed to a single additive property but likely emerges via multiple factors including protein chemistry-dependent cluster stabilization, inter-particle distance favoring confinement-based stabilization of ion associates and ionic conditions-based conformational dynamism of macromolecules. As suggested for liquid-like mineral precursors formed in the presence of ovalbumin, the dynamics of interfacial protein layers as well as charge neutralization and depletion stabilization may also be critical factors (Wolf et al. 2011). As shown by previous studies, the modulation of the nucleation regime by charged additives including the inhibition of nucleation and transient stabilization of highly soluble mineral intermediates cannot be explained solely by ion-complexation (Gebauer et al. 2009; Rao et al. 2014). This is validated by the experimentally determined effective valence being only one-third of that estimated from the structure of charged polysaccharides such as dextran sulfate and heparin (Winzor et al. 2004). Similar indications arise for proteinaceous additives wherein the direct correlation between amino acid constituents and net charge of the macromolecule is weak on account of parameters such as pH, ionic strength and buffer composition playing crucial roles (Winzor 2004). Therefore, the conformational and co-operative aspects of ion binding sites presented by mineralization additives emerge crucial. Further, macromolecules regulating biomineralization appear to be biochemically tuned in being conformationally responsive to changes in ionic conditions. For instance, distinct ionic conditions lead to β-turn or β-sheet conformations in the low-complexity disordered proteins involved in sea urchin skeletogenesis (Xu and Evans 1999; Rao et al. 2013). Interestingly, sequentially similar proteins derived from gluten extracts also exhibit distinct homo- and hetero-association behavior tuned by solution ionic strength (Winzor 1966).

The role of protein-based macromolecular crowding in the formation and stabilization of metal ion clusters is well evident in literature. For instance, high contents of denatured BSA enable stabilization of Ag ion-associates under crowded conditions, due to favorable interactions with cysteine residues (Guo and Irudayaraj 2011). Also, highly fluorescent Au nanoclusters are stabilized by BSA contents of about 25 mg/mL (Xie et al. 2009). A similar synthesis approach for Cu quantum clusters is also demonstrated, suggesting the role of protein secondary structure in cluster stabilization (Goswami et al. 2011). Concerning nucleation in confined geometries, stabilization effects towards precursor and intermediate mineral forms become significant approaching nanoscopic-length scales. For instance, fetuin A forms a transient stable colloid wherein the protein units surround ion-associates, temporally stabilizing these against crystallization (Heiss et al. 2010). Although moderate protein concentrations are applied, a local confinement effect occurs because the protein units form a densely packed shell encapsulating the mineral precursors

(Heiss et al. 2007). A similar confinement-based stabilization of metal clusters is exhibited by ferritin nanocages that contain functional nanopores (Behera and Theil 2014). A nacre-associated protein (n16) self-associates in the presence of mineral precursors to form a hydrogel. The gel structures provide a confined space for mineral nucleation and growth (Perovic et al. 2014). Other biological examples of ion-cluster stabilization in relation to biomacromolecular super-assemblies include a $MoFe_7S_9$ cofactor cluster with nitrogenase enzyme and a Fe_4S_4 cluster with the heme group in sulfite reductase (Beinert et al. 1997).

Vesicles are another form of confinement-based stabilization relevant to mineral systems. For example, vesicular structures containing mineral precursors are involved in bone development and sea urchin spiculogenesis (Mahamid et al. 2011; Vidavsky et al. 2014). Although the effects of macromolecule- or vesicle-based confinement at different length scales on mineralization are not completely clear, some possible explanations for the distinct nucleation behavior are (1) a confinement size-dependent interplay between homogenous and heterogeneous nucleation (Woo et al. 2007), (2) critical size constraints corresponding to crystal nuclei (or ion-associates) and related polymorph selectivity (Ha et al. 2004), and (3) size constraint-driven thermodynamic crossovers in phase stability (Hamilton et al. 2008; Navrotsky 2004; Raiteri and Gale 2010), as well as distinct solvent dynamics and physicochemical conditions in restricted environments and the bulk solvent (Brubach et al. 2001; Moilanen et al. 2007; Rao and Cölfen 2016). Taken together, biomacromolecular crowding conditions as well as the interfacial aspects and size regime of confinement appear evolutionarily optimized for modulating the nucleation regime including associated phase transition events.

Macromolecular crowding also enables neutral macromolecules to regulate mineral nucleation and growth. For instance, polyethylene glycol (PEG)-induced crowding suppresses ion association and inhibits nucleation. An extreme diffusion limitation and charge screening towards ion-association, preferential hydration of mineral precursors and limiting void space are suggested factors (Rao and Cölfen 2016). To examine this nucleation behavior under conditions of crowding induced by small molecules, we tested the effect of high sucrose contents on the equilibrium between free ions and ion-clusters during mineral nucleation (Fig. 3). In the course of $CaCO_3$ nucleation at high sucrose contents, the development of free Ca^{2+} ions indicates a shift in equilibrium towards free ions as well as a significant inhibition of nucleation of solid $CaCO_3$ particles. These observations are similar to those in the presence of high PEG contents and indicate that factors such as additive-modulated hydration of mineral precursors as well as excluded volume-based anomalous diffusion and charge screening affect the early nucleation regime in terms of the stability of pre-nucleation clusters and nucleation

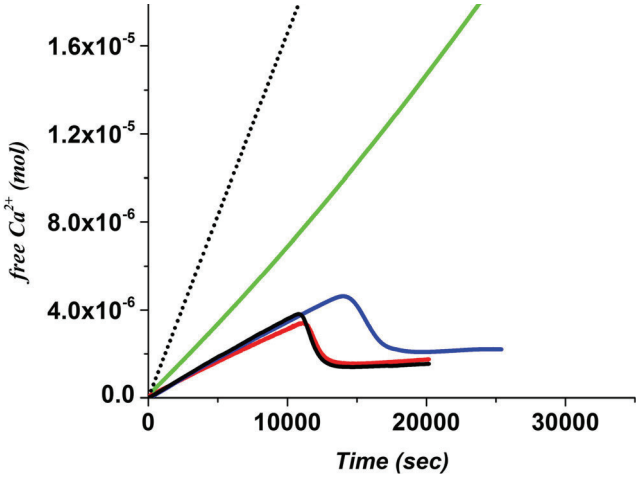

Fig. 3 Development of free Ca^{2+} in carbonate buffer (10 mM, pH 9.0) in the absence (*black*) and presence of sucrose (1 wt% (*red*), 5 % (*blue*) , 10 % (*green*)). Added calcium is represented by the *dashed line*

period (Rao et al. 2014; Rao and Cölfen 2016). Thus, we see two opposite outcomes of molecular crowding on mineral nucleation using high contents of either charged (BSA) or uncharged (PEG or sucrose) molecules as mineralization additives. This indicates that the mineralization modifiers such as soluble biomolecules and matrix constituents likely have homeostatic levels of expression, destabilization of which may perturb interactions with ions or ion-associates, nucleation and particle growth. Biologically tuned contents of nucleation and crystallization additives are also supported by a significant loss in structural mobility for concentrated protein solutions, i.e. under conditions of self-crowding (Erlkamp et al. 2015) and by an in situ concentration-dependence of biological activity (Dill et al. 2011).

Crowded solutions: on crystallization

Methodologies applied for pharmaceutical and protein crystallization strategically tune contents of crystallization additives in order to select crystal shape, size, phase transitions and polymorph properties. For instance, at sufficient concentrations, certain polymers promote protein–protein interactions and crystal nucleation driven by stearic hindrance related to the excluded volume effect (Arakawa and Timasheff 1985; Bolen 2004). In instances wherein the bioavailability of a drug is limited by low solubility of its crystalline forms, amorphous particles are the preferred polymorphs. Given the thermodynamically unstable nature of amorphous phases and susceptibility to crystalize, high contents of polymeric co-solutes are applied in order to retard crystal nucleation and growth from amorphous particles (Konno and Taylor 2006). An impediment towards the transport of crystal precursors (decreased mass transport) by polymer molecules is the suggested mechanism for suppressed crystal nucleation and growth rates.

Under such conditions, coacervates or droplets rich in polymer and small molecules are possibly stabilized (Konno and Taylor 2006). These studies hint at a fundamental role of macromolecular crowding in the growth and structure of minerals.

Few mineralization investigations have employed a wide range of additive concentrations to study particle structure and form. Considering the suppression of phase transitions in presence of high polymer contents (Konno and Taylor 2006) and Ostwald ripening-based growth of minerals, one might expect an emergence of phase behavior favoring metastable forms (i.e. polymorph selection) under conditions of macromolecular crowding. However, mineral growth in the presence of low to moderate lysozyme contents indicate a lower stability of the metastable phase (vaterite) at higher additive contents (Wang et al. 2009). A similar relationship is presented for the selective formation of $CaCO_3$ polymorphs (calcite, vaterite or aragonite) in the presence of an anionic sulfonated copolymer (PSS-co-PNIPAM) in an additive concentration dependent manner (Xu et al. 2008). Note that the thermoresponsiveness of PNIPAM is altered by mineral precursor species, potentially inducing local crowding and confinement (Xia et al. 2012). In contrast to these studies, with high contents of PEG, a relatively inert additive, vaterite is stabilized which indicates that non-equilibrium mineral forms can also emerge on account of macromolecular crowding (Xie et al. 2006). It appears that polymorph behavior as well as structural properties of minerals are significantly affected by local or bulk macromolecular crowding and confinement conditions, in addition to additive chemistry. Also, as shown for nucleation behavior (Rao and Cölfen 2016), crowding situations induced by charged and neutral additives may have distinct consequences for mineral form and structure.

To better elucidate these phenomena, we observe products of double-jet-based mineralization of $CaCO_3$ performed in the presence of varying concentrations of non-interacting PEG additives. In the presence of high PEG contents (Fig. 4), a distinct tendency towards mineral particles with small sizes and lower specific surface area is observed. The calcite rhombohedra formed at lower and moderate PEG concentrations exhibit surface porosities (Fig. 4, arrows), which may be on account of the dissolution of intermediate (possibly amorphous) mineral phases, suggesting involvement of dissolution-reprecipitation processes. We validate that PEG-induced crowding leads to polymorph selection towards metastable phases (Fig. S1). PEG induces vaterite formation under conditions of crowding, whereas calcite is the predominant mineral product at low additive contents. Also, the effect of molar mass is not very pronounced for the range tested. A lower molar mass of PEG appears to support vaterite precipitation in a mixture with calcite while the higher molar mass only leads to calcite (Fig. S1). Experiments performed in the presence of sucrose indicate similar phenomena, although less pronounced (Fig. S2). Although calcite formation is suppressed at high additive contents, the corresponding effect on mineral size and shape is not as obvious compared to PEG-induced crowding. These observations show that even neutral macromolecules can regulate the shape and phase of mineralization products selectively, at optimal conditions of concentration and molar mass.

For comparison, the effects of charged macromolecules on the $CaCO_3$ formation are also evaluated. Using BSA as a crowding agent, an inclination towards protein and mineral aggregation is observed at higher additive contents, possibly due to surface charge neutralization by the ions and protein

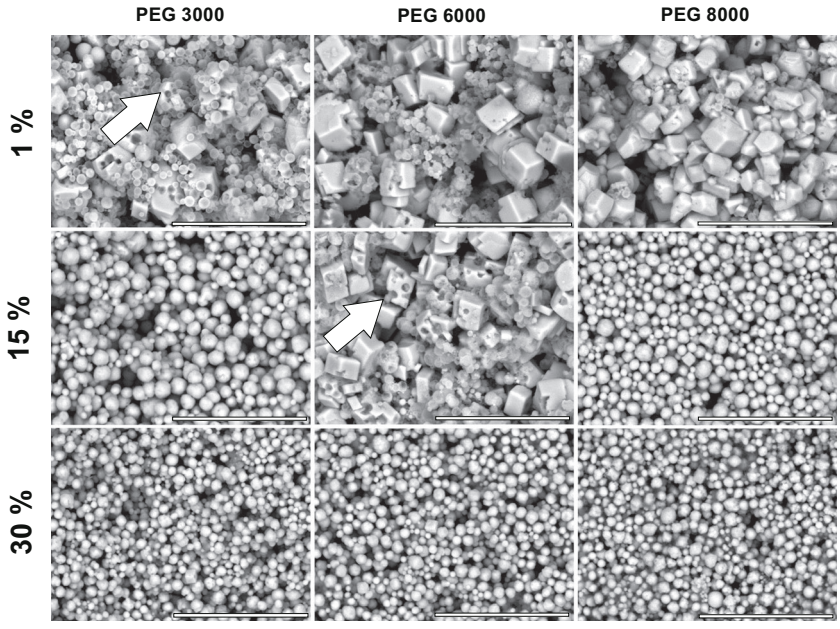

Fig. 4 Representative SEM images of crystals grown in the presence of PEG of distinct molar masses at different concentrations (wt%). *Scale bars* 30 μm

molecules (Fig. 5a). Smaller particle sizes are observed from low (0.1 %) to moderate (1 %) protein contents; however, at high additive levels (3 %), large aggregates of proteins and mineral particles are evident (Fig. 5a, arrow). In crowded conditions, particles reminiscent of a liquid-like phase (e.g., droplet-shaped and viscous fingering-related structures) suggest the transient stabilization of an intermediate Ca^{2+}-rich liquid-like phase (Fig. S3). Another distinctive feature is the formation of calcite with BSA-induced crowding, which contrasts mineralization in the presence of PEG-induced crowding producing vaterite. With poly(4-styrenesulfonate) (PSS), as a relatively simple charged additive, calcite formation and a decrease in the size of spherical particles occurs at high polymer contents (Fig. 5b). Together, these observations indicate that non-classical crystallization pathways involving particle attachment and liquid-like transient phases operate under the applied crowding conditions especially for charged additives (Cölfen and Antonietti 2008; De Yoreo et al. 2015; Niederberger and Cölfen 2006). Although a confident conclusion cannot be drawn without screening several additives, we also note an interesting relationship between the formation of metastable mineral forms and the relative stability of pre-nucleation clusters (Rao and Cölfen 2016). In the case of PEG, crowding leads to an equilibrium shift towards free ions in the pre-nucleation stage and also leads to metastable crystallization products such as vaterite (Rao and Cölfen 2016; Xie et al. 2006). On the other hand, BSA-induced crowding leads to a relative stabilization of pre-nucleation clusters and possibly intermediate precursors, and does not form vaterite as the predominant product after crystallization. This indicates that distinct crystallization pathways can emerge by conditioning the pre-nucleation regime, i.e. the stability of mineral precursors under the appropriate physico-chemical conditions.

In comparison to these experiments, crowding induced by a heterogeneous solute mixture is a complex but realistic portrayal of biochemical spaces. The physico-chemical properties of environments in which the pathways of nucleation and crystal growth operate appear to be tuned via synergistic additive–additive and mineral species–additive interactions. For instance, interactions with ions can induce the self-assembly of proteins or polysaccharides into matrices that subsequently regulate crystal growth (Perovic et al. 2014; Rao et al. 2016). With crowding, such effects can be accentuated for instance via a preference towards more compact molecular conformations and aggregates or oligomeric species via non-specific or specific interactions (Spencer et al. 2005). To simulate this behavior for a biomineralization additive, we derived the molar mass distributions of SUMO-CTL by fitting the analytical ultracentrifugation (AUC) data to the Lamm equation model, assuming a partial specific volume of 0.718 ml/g (Schuck 2003). AUC is a powerful method for the quantitative analysis of molecular size, shape and density, and is useful to understand biochemical processes in complex solutions such as crowded but also interacting media (Cölfen et al. 1996, 1997; Cölfen and Winzor 1997; Wilson et al. 1997). The recombinant fusion protein is based on the N-terminal C-type lectin domain of a key biomineralization protein (SM50) involved in sea urchin skeletogenesis (Rao et al. 2013). Sedimentation velocity profiles are measured in the absence and presence of PEG-induced crowding (Fig. 6a, b). In the simultaneous absence of Ca^{2+} ions and crowding conditions, a single peak at 26 ± 2 kDa (2.8 S) corresponding to monomeric species is observed (Rao et al. 2013). An increase in Ca^{2+} content (2 mM) leads to protein species with molar masses of 32 ± 5 and 70 ± 39 kDa, respectively (Fig. 6a). The species with molar mass intermediate between monomeric and dimeric aggregation states indicate reversible interactions comprising the time average of the different oligomers. With 10 mM Ca^{2+} ions, an observed species with a molar mass of 70 ± 39 kDa corresponds to the protein trimer. Hence, Ca^{2+}

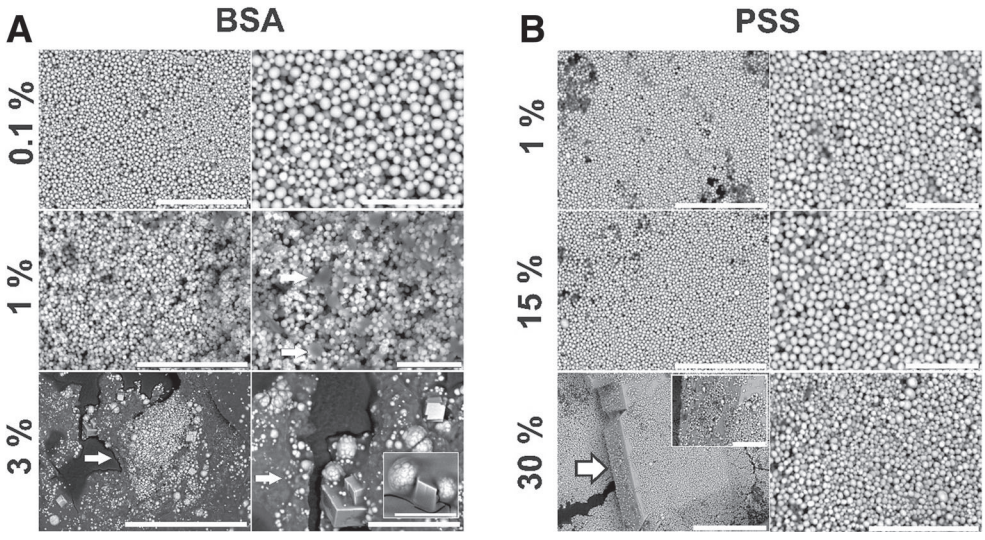

Fig. 5 Representative SEM images of $CaCO_3$ crystals formed in presence of different **a** BSA and **b** PSS contents (wt%). *Insets* show mineral growth by particle attachment and *arrows* show **a** protein aggregation and **b** calcite crystals. *Scale bars* (**a**) 200 μm (*left panel*), 50 μm (*right panel*) and 30 μm (*inset, lower right*); (**b**) 30 μm (*top and middle, left panel*), 100 μm (*bottom, left panel*), 10 μm (*right panel*) and 30 μm (*inset, lower left*)

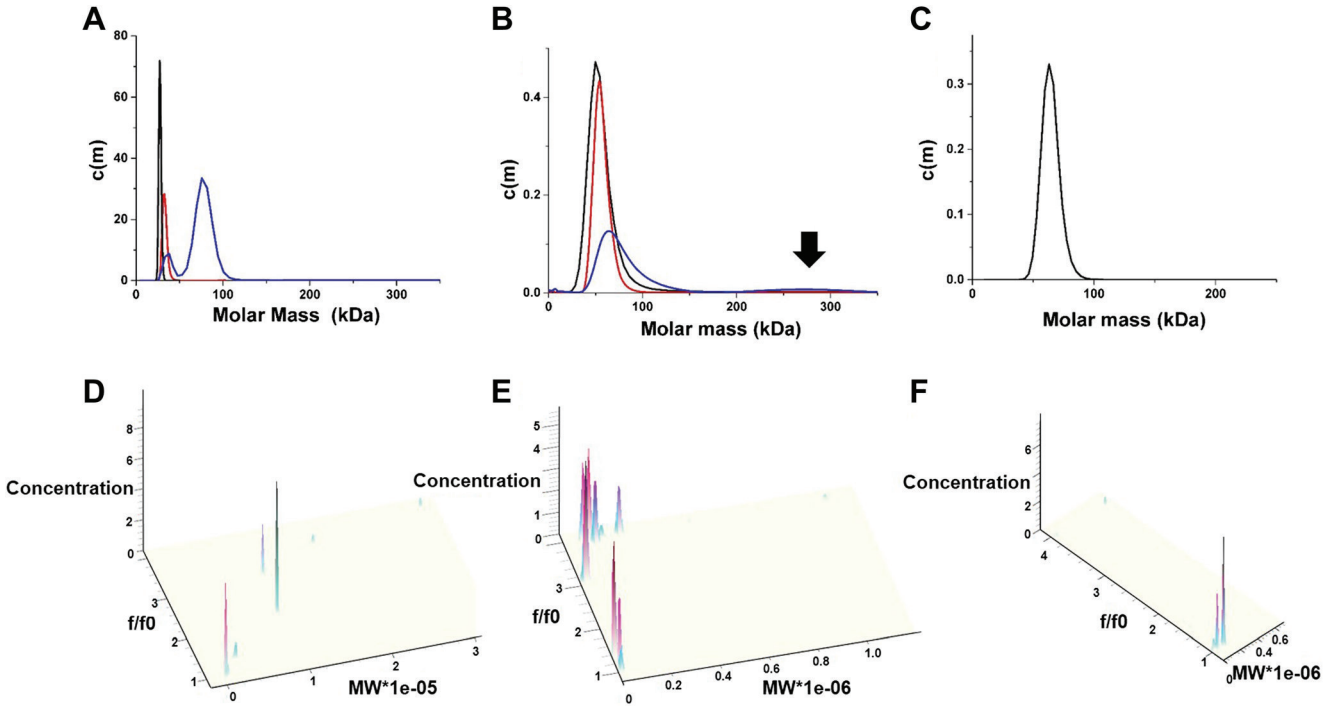

Fig. 6 Molar mass distributions for the SUMO CTL protein in HEPES buffer (10 mM, pH 7.4) and $CaCl_2$ contents of 0 (*black*), 2 (*red*) and 10 (*blue*) mM in **a** the absence and **b** the presence of PEG (20 wt%). *Arrow* indicates broad distribution of aggregates in the presence of PEG. **c** Molar mass distribution for aqueous bovine fetuin. Ultrascan 2 DSA derived plots for molar mass and frictional coefficient distributions for **a**, **b** SUMO CTL and PEG solutions containing 2 and 10 mM $CaCl_2$, respec tively, and **c** fetuin in milliQ water

ions promote the self-association of the C-type lectin domain to a certain extent. In comparison to the protein in a simple buffer, protein self-association is enhanced under conditions of crowding. In the presence of PEG and without Ca^{2+} ions, a single peak corresponding to the protein dimer (51 ± 10 kDa) is observed. This suggests that molecular crowding enhances protein self-association even in the absence of mineralization (Zhou et al. 2008). An intermediate Ca^{2+} ion content (2 mM) does not have a significant effect; however, higher ion contents (10 mM) result in an asymmetric (suggesting some reversible protein self-association) and a broad peak corresponding to 64 ± 23 (trimer) and 274 ± 40 kDa (decamer), respectively. Also, lower than expected absorbance intensities for sedimentation profiles suggest the presence of protein associates with larger sedimentation coefficients. The sedimentation behavior of this biomineral-associated protein shows that mineral precursors and crowding conditions synergistically enhance biomolecular self-association. For SUMO-CTL, conditions of crowding appear to switch the ion-induced protein self-association interactions from reversible to irreversible. The frictional ratio (f/f_0 defined as the frictional coefficient of the protein species compared to that of an ideal sphere with identical volume) of SUMO-CTL species that form under simultaneous crowding and mineralization conditions suggest that macromolecular self-association producing asymmetric shapes ($f/f_0 = 4$) such as protein sheets involves intermediate species with prolate and rod-like ($f/f_0 \sim 2$) morphologies (Fig. 6d, e). Under conditions of crowding, an enhanced self-association tendency and stabilization of intermediate forms occur at higher Ca^{2+} ion contents (Fig. 6e). These results show that crowding and mineralization conditions can induce self-association of macromolecules in a manner that tunes the mass, shape, and size distributions of the self-assembly products. Some biomineralization proteins may exhibit self-association behavior even in the absence of crowding conditions and mineral precursors. For instance, bovine fetuin dissolved in water exists predominantly in a monomeric form (67 ± 9 kDa; 3.6 ± 0.6 S), with a globular structure ($f/f_0 = 1$) (Fig. 6c, f). However, 2-DSA analysis identifies oligomeric forms of fetuin that exhibit compact morphologies (Fig. 6f). Therefore, mineralization-related compartment or template formation can occur prior to or during the nucleation and crystallization process in relation to the biomineralization system and prevalent conditions of crowding. In summary, this section shows that macromolecular crowding as well as the properties of crowding agents such as charge and self-association propensity have distinct effects on ion-association, nucleation and mineral properties such as particle size, structure and polymorph. In non-ideal solutions, the

equilibria and stability associated with macromolecules are significantly altered during the course of mineralization. Thus crowded, viscous and entangled physical features of nature's masonry appear biochemically tuned to serve crucial purposes in material formation and require further investigations in this view.

Osmotic conditions in niche spaces

In general, macromolecular crowding tends to favor a reduction of surface to volume ratio (Davis-Searles et al. 2001; Minton 2006). Under such conditions, the osmotic pressure may be significant enough to compress macromolecules. For example, the osmotic compressibility of soft colloidal particles depends on excluded volume-related de-swelling forces and osmotic pressure exerted on the particles (Tan et al. 2005). Along similar lines, considering protein molecules, a solution of 40 % PEG (mol wt 400 g/mol) leads to a 9 % reduction in the volume of superoxide dismutase (Rajapaksha et al. 2015). Enhanced self-association, susceptibility to compact folding states and stabilization against thermal denaturation of certain proteins, are also noted in relation to co-solute polyol size and concentration (Ogston and Winzor 1975; Shearwin and Winzor 1988; Wills et al. 1993; Davis-Searles et al. 2001). To the best of our knowledge, direct consequences of crowding-induced osmotic forces on biomineral nucleation and growth have not been investigated; however, certain insights can be drawn from the existing literature. For mineral morphology based on fibrous extensions from PILP globules, osmotic pressure is a suggested factor. Here, a pressure-induced collapse of the outer (more dense) crust of the globules is suggested for the release of PILP precursors and crystallization as elongated structures (Olszta et al. 2009). In an interesting perspective on rotational behavior of pearls, a high osmotic pressure at the mineral growth front, emerging from the exothermic mineralization processes and asymmetric mass transport, is suggested in the translation of microscopic forces into macroscopic dynamism (Cartwright et al. 2013). In nacre, an aragonitic hierarchically-structured biomineral, the osmotic pressure across inter-lamellar membranes is sufficient to enable the rupture of mineral bridges between the mineral tablets (Checa et al. 2011). Assuming the strength of bulk aragonite (30 MPa), the local osmotic conditions may be significantly larger than physiologically assumed. Given the pressure-induced amorphous–amorphous phase transition concerning proto-aragonite at extreme pressures (Fernandez-Martinez et al. 2013), the contribution of physiological osmotic pressure-related mineral structure and polymorph behavior is intriguing. In bone and tendon tissues, contractile forces in distinct osmotic conditions suggest that mineralization and protein self-assembly-associated dehydration can generate significant tensile forces during biomineral formation (Bertinetti et al. 2015). Overall, these studies indicate that local osmotic conditions may affect mineral growth in complex environments and also lead to variations in mineral properties, including shape, polymorph and structural mechanics. At cellular levels, the osmotic environment serves functions such as tuning mineralization activity and viscoelastic properties of cellular constituents (Guilak et al. 2002; Sottnik et al. 2015).

Mineralization in matrices and gels

Confinement and crowding are characteristic features of material formation in nature and originate from the hierarchical organization of living systems. During biomineralization, organic super-assemblies provide sites for crystal nucleation and growth. Conditions of crowding can confer uniform long-range order to organic matrices. This is suggested by the crowding-induced formation of closely packed and highly-aligned collagen fibrils (Saeidi et al. 2012). Mineralization of a matrix thus derived leads to well-aligned hydroxyapatite nanocrystals with overall structural hierarchy similar to lamellar bone (Wingender et al. 2016). This highlights the importance of macromolecular crowding in establishing a defect-free matrix organization and subsequently a biologically equivalent biomimetic mineral.

In view of the space-filling nature of certain biominerals at the nanoscale (Yang et al. 2011), the physical constraints on account of the matrix organization can drastically affect mineral substructure. For instance, cuticle from the exoskeleton of *Homarus americanus* (lobster) comprises of six hierarchical levels of structural organization ranging from the molecular level to the twisted plywood layer (Bouligand) assembly of fibers at the mesoscale (Raabe et al. 2006; Romano et al. 2007). Here, the superstructural hierarchy and volume fractions associated with the organic and inorganic phases are suggested to modulate the fluid dynamics during mineral growth and material mechanics (Nikolov et al. 2010). Consider collagen, i.e. the bone matrix and the associated role of excluded volume in tuning the mobility of inorganic precursors and crystallization additives. Based on excluded volume in this material and an estimated partial specific volume (0.73 cc/g), globular proteins smaller than a radial size of about 1.2 nm can freely diffuse though the water channels of the matrix (Erickson 2009; Toroian et al. 2007). This suggests that soluble additives regulating crystal nucleation and growth are mobile within the matrix structure, prior to mineralization. Considering the approximate partial specific volume of early mineral species (0.67 cc/g) (Gebauer et al. 2008) and assuming compact morphologies, amorphous phases and pre-nucleation clusters smaller than 1.17 nm can permeate through the excluded volume of the collagen matrix. Infiltration of a collagen fibril by such small calcium

carbonate clusters was indeed experimentally observed by Nudelman et al. (2010). This indicates that the organic matrix of developing biomineral not only provide sites for crystal nucleation but can also regulate the diffusivity of organic molecules and mineral precursors, in a manner that physically regulates mineralization at distinct length scales. Also important to consider are the fluid distribution and pressure in bone material which depend on a hierarchical system of distinct porosities. Studies on collagen mineralization have established that liquid-like, amorphous precursors help realize biomimetic materials structurally similar to the biomineral. Here, the infiltration of a collagenous matrix with mineral is enhanced in the presence of a polymer-induced liquid-precursor and is attributed to capillary effects that draw the inorganic precursors into the grooves and pores of the organic matrix (Thula et al. 2011). In this context, it is likely that factors of permeability and wettability of the organic matrix well complement the properties of liquid precursor phases such as viscosity, density and surface tension, and lead to optimal interfacial interactions. Along these lines, during mineralization of an organic matrix by a liquid precursor of $CaCO_3$, the wettability of the substrate by PILP droplets is tuned by Mg^{2+} ions (Berg et al. 2013). Indeed, the fluid dynamics and porosity in the biomineral matrix appears evolutionarily optimized for fascinating structure–function relationships, not only from the skeletal system down to the cellular level but also to the molecular scale (Knothe 2003). Thus, the properties of niche spaces associated with mineralization provide optimal conditions of crowding and confinement in order to enable effective integration of the organic matrix and mineral counterpart as functional materials.

Studies on crystal growth in gels provide important insights into the role of confined spaces tuned in biomineral matrices for crystallization processes. Properties of the gel matrix such as density, porosity, mechanical stress and chemical nature have a significant bearing on crystal growth. For instance, calcite crystals grown in agarose hydrogels incorporate gel structures depending on the kinetics of crystal growth and the gel strength of the media (Li and Estroff 2009). A weak gel of agarose is pushed away by the growth fronts of the crystal, whereas a strong gel resists deformation, leading to calcite crystals that grow around and incorporate the fibers. However, using a more heterogeneous hydrogel as a matrix for mineral growth, agar (a mixture of agarose and agaropectin), leads to distinctly shaped calcite crystals also in dependence of the gel network. The crystals thus formed can exhibit a hierarchical structure with sub-micrometric and nanocrystalline units; however, exhibiting an overall rhombohedral shape (Oaki et al. 2007). The spatial regulation of mineralization is also determined by gel porosity and chemical functionalization. For example, $CaCO_3$ crystals are either included or excluded from Sepharose beads depending on the degree of cross-linking and the charged nature (Rao and Cölfen 2016). Thus, the physico-chemical properties of niche gel networks can profoundly affect the structure of mineral particles with key factors being (1) crystal growth kinetics, (2) the incorporation or exclusion of the gel matrix by growing crystals, and (3) the chemical nature of the gel network and also the degree of crosslinking (porosity). Depending on these aspects, the structures derived from mineralization can range from polycrystalline aggregates to mesocrystals to single-crystals. Microgels have also been shown to modulate mineral structures. For instance, hollow crystalline shells are formed in the presence of pectin (Butler et al. 2006; Kosanović et al. 2010). This may be attributed to the symmetric Ostwald ripening of composite particles of pectin and inorganic amorphous precursors as well as distinct additive interactions leading to inhibited nucleation and transient stabilization of mineral precursors (Liu and Zeng 2005; Rao et al. 2014). The effects of gels on mineral forms also encompass protein assemblies that exhibit liquid- or gel-like properties. Products of self-assembly of an intra-crystalline protein, AP7, from nacre can lead to calcite crystals with subsurface multiscale porosities and interconnected channels (Chang et al. 2015). Also, a recombinant protein (inspired by C-type lectin domains from sea urchin skeletal elements) forms porous protein hydrogels that tune nucleation as well as crystal growth (Jain et al. 2016). In this manner, several emerging studies indicate that the molecular state of self-association and related physical properties play fundamental roles during biomineralization.

Additives operating in gel environments have radical effects on mineralization on account of simultaneous diffusion as well as crowding and confinement effects. This is indicated by the different morphologies of $CaCO_3$ particles obtained by crystal growth in gels exposed to varying BSA contents (Feng et al. 2016). The formation of transient liquid-like mineral precursors at additive concentrations above 3 g/L is hinted at by droplet-shaped particles and also hollow structures, which might be due to surface-localized phase transformations. Here, the applied protein concentration for generating a transient liquid-like precursors is much lower than for that in the absence of the gel matrix (Figure S3). Also, studies on the mineralization of collagen gels using poly(aspartic acid) report spherical particles with concentric shells and may suggest the role of mineral PILPs and interfacial Oswald ripening (Falini et al. 2000). Thus, confined spaces exhibited by the gel-like composition of biological media can operate in synergy with nucleation and crystallization additives, amplifying or suppressing additive effects, and thus represent a cornerstone for regulating biomineral growth and form.

This discussion suggests the significance of confinement operating at different length scales during biomineralization which is evident from the distinct stabilization of (1) ion-clusters by protein superstructures such as nanocages and (2) amorphous intermediates by vesicles. In addition (3), the void volume presented by gels and matrices lead to unique

crystallization products. Overall, the parameters of confinement such as size regime, hydration and surface chemistry correspond well to the physical nature of mineral precursors (Fig. 7). It emerges that the forms of confinement presented by macromolecular surfaces, super-assemblies and matrices complement the energetics (related to nucleation and transitions) of mineral forms at different length scales in order to yield sophisticated material formation routes.

Mineralization and crowding in quasi-2 dimensions

Crowded environments limited to 2 dimensions are also applied to control crystallization. In an example from pharmaceutical crystallization, exposure of immobilized polymer monolayers to supersaturated solutions can lead to the selective, heterogeneous nucleation of stable polymorphs (Morissette et al. 2004). This enables exploration of the polymorph space in a high throughput manner. Under such conditions, crystallization depends on the surface density of macromolecules, in addition to their chemical functionality and conformation. For instance, in the case of self-assembled monolayers (SAMs), the surface density of attached molecules determines the conformational ensemble being random, partially-disordered or ordered and close-packed (Schwartz 2001). This in turn can affect heterogeneous nucleation and crystal growth. Molecular dynamics simulations also indicate that the packing density (or lateral crowding) of alkyl chains on SAMs can control site-selective deposition of small organic molecules (Xu et al. 2011). Mineralization performed in proximity of monolayers composed of amphiphilic molecules provide similar indications. The growth of mineral particles in simulated body fluid under a Langmuir monolayer of arachidic acid reveals distinct stages of mineral growth involving pre-nucleation clusters and amorphous particles (Cölfen 2010; Dey et al. 2010). Since the mineral precursors associate with the monolayer surface, the interfacial distribution of monolayer constituents possibly influences nucleation and crystallization behavior. Supporting this notion, in a study utilizing amphiphilic peptides, the packing arrangement in the monolayer and the related structural dynamism of individual peptides affected the orientation and morphology of mineral particles (Gong et al. 2014). Similar evidence comes from growth of $CaCO_3$ under monolayers of a particular macrocyclic octacarboxylic acid (Volkmer et al. 2004). Interestingly, at a low surface pressure, the dominant phase is comprised of calcite and vaterite, whereas aragonite crystals are formed at a higher surface pressure. Hence, monolayer conformation and packing which exhibit a high charge density towards mineralizing solutions are suggested to stabilize metastable crystal phases at the interface rather than monolayer–crystal epitaxy which has previously been discussed as the primary reason. During mineralization, the relationship between surface pressure and interfacial packing of amphiphilic molecules is not absolute because ions can induce close-packed monolayer domains even at low surface compression (DiMasi et al. 2003). A factor suggested for the distinct polymorph formation includes the stoichiometry between free ions and those bound to counter-ions and amphiphiles (DiMasi et al. 2003). These observations suggest that lateral crowding is crucial during membrane-associated biomineralization.

Several interesting phenomena emerge from biological instances of 2-dimensional crowding. In lipid membranes, the spatiotemporal heterogeneity and distribution of cholesterol, phospho- and sphingolipids depend on lateral crowding, with underlying factors being size, aggregation propensity and hydrophobicity of co-confined biomolecules (Edidin 2003; Lingwood and Simons 2010; Silvius 2003). These factors may converge to have distinct effects such as lateral immiscibility, formation of domains with distinct spatiotemporal patterns and displacement of biomolecules from the membrane interface (Kory et al. 2015; Lingwood and Simons 2010). The effect of lateral crowding on mineralization requires attention in particular for lipid membrane-limited processes. For

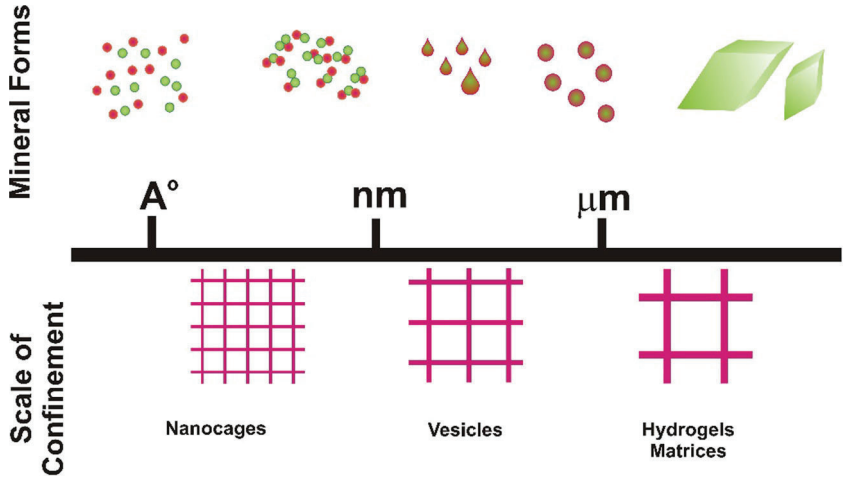

Fig. 7 The complementary nature of size regimes corresponding to mineral forms (*top*) such as ion cluster, liquid like phases and amorphous or crystalline particles and organic interfaces or forms (*bottom*) such as protein nanocages, vesicles and gels

instance, considering the mechanistic role of lateral crowding associated with the magnetosome membrane proteins is relevant. Also, for vesicle mediated confinement and stabilization of amorphous intermediates, the deformation of a smooth membrane during vesicle budding is possibly affected by lateral crowding of membrane proteins (Derganc and Čopič 2016; Rao and Cölfen 2016). Mineralization studies conducted in the vicinity of interfaces provided by SAMs and Langmuir monolayers have undoubtedly provided key insights. However, with emerging knowledge on the complex physico-chemistry of living membranes, it appears that we may have barely scratched the surface of the biophysical regulation involved.

Mineralization and liquid-like phases

Physiologically relevant liquid-like forms of organic as well as inorganic species have recently gained much research attention (Bewernitz et al. 2012; Brangwynne et al. 2015; Demichelis et al. 2011; Gower and Odom 2000; Hyman et al. 2014). As the roles of liquid-like precursors and coacervate phases of inorganic as well as organic systems are gradually being deciphered, their biological consequences emerge as significant in the field of mineralization. Stabilization of a transient inorganic liquid and amorphous phase by polymeric additives establishes a phase which exhibits liquid-like properties such as shape-adaptability (pseudomorphic transformation) and surface tension (Berg et al. 2013; Bewernitz et al. 2012; Gower and Odom 2000; Meldrum and Cölfen 2008; Olszta et al. 2009). Such phases are suggested to play a fundamental role as a precursor phase in the morphogenesis of calcium-based biominerals such as enamel and bone (Olszta et al. 2009). Compositionally distinct, liquid-like phases with organic constituents (organic PILPs or coacervates) are also involved in biomineral growth. In the siliceous diatom cell wall, closely packed polyamine-rich droplets are suggested to spatially modulate silica deposition (Brunner et al. 2004; Sumper 2002, 2004). Here, a phase separation-based model is proposed for the formation of distinct, species-dependent morphologies. Nano-droplets (or micelles) composed of long chain polyamines and polycationic polypeptides are implicated in this template-driven silification. During this process, the coalescence or splitting of the droplets can lead to a dynamic templating effect, which, after silica deposition, leads to an intricate material with distinct pores sizes and a meshwork-like pattern.

In the organic matrix of mollusk shells, chitin substrates with bound silk-fibroin-like proteins have an important role in regulating the morphology and lattice structure of the mineral phase (Sudo et al. 1997; Sun and Bhushan 2012; Weiner and Traub 1980). Several of the attached proteins adopt antiparallel β-sheet conformations and consist of repetitive low-complexity domains (RLCDs) rich in glycine (Evans 2008; Jackson et al. 2010). With similarities to silk fibroin, crowding might have similar effects on these two functional classes originating from distinct sources. In the case of silk fibroin, liquid crystal phases can develop by microphase separation under conditions of self-crowding, which subsequently form filaments with defined structural periodicity (Hamley 2010; Knight and Vollrath 2002). From this perspective, biomineral-bound proteins with RLCDs are not functionally well characterized due to an aggregation-prone nature; however, a cholesteric liquid-crystalline phase of chitin is implicated in spiral geometries and patterns present in developing nacre (Cartwright et al. 2009). This might suggest a comparable liquid–liquid phase separation involving biomolecules that regulate nucleation and crystallization. For instance, the formation of fibrils and other biomacromolecular assembles involving LCDs can occur via a liquid–liquid phase separation step (Molliex et al. 2015). This provides an efficacious route to organic super-assemblies or scaffolds alternate to monomer-by-monomer growth. Although the mechanistic role of organic PILPs in biomineralization needs exploration, the idea of structural hierarchy emerging from a biochemically tuned demixing of a multicomponent phase into co-oriented mineral particles and aligned organic sheets or fibrils is fascinating. From this perspective, the phase behavior of mineralization additives existing as aqueous solutes, insoluble assemblies, coacervates or organic PILPs may be advantageous in spatiotemporally modulating phase transition of the mineral phase and directing mineral deposition.

Given the recent attention towards cell-associated liquid-like organic phases and deficient literature on their mineralization behavior, the effects of organic PILPs or ‘water in ethanol’ emulsions composed of oppositely charged small molecules and polyelectrolytes on $CaCO_3$ formation are discussed (Fig. 8). Three distinct phases (citrate/PEI, caffeine/PAA and arginine/PAA) are produced following a standard protocol (Wohlrab et al. 2005) and represent organic liquid phases formed by liquid–liquid demixing (Fig. S4). As mineralization additives, the organic droplets lead to remarkably distinct mineral morphologies (Figs. 8; S5). The citrate/PEI microdroplets are stable during mineralization and generate core-shell structures (Fig. 8a). The interface of this microphase appears to favor heterogeneous nucleation of the mineral phase leading to shell formation. Similar indications originated from products of gas diffusion-based mineralization wherein the close-packing of sediment droplets serve as a short-lived template for the growth of a micro-porous mineral network (Fig. S6A). Note that the mineral structure is also affected by kinetic aspects of the experiment. For example, fast precipitation of the mineral in presence of the citrate/PEI microphase does not provide optimal interactions between mineral precursors and organic droplets for the ‘soft-templating’ effect (Fig. S6B). Mineralization using caffeine/PAA

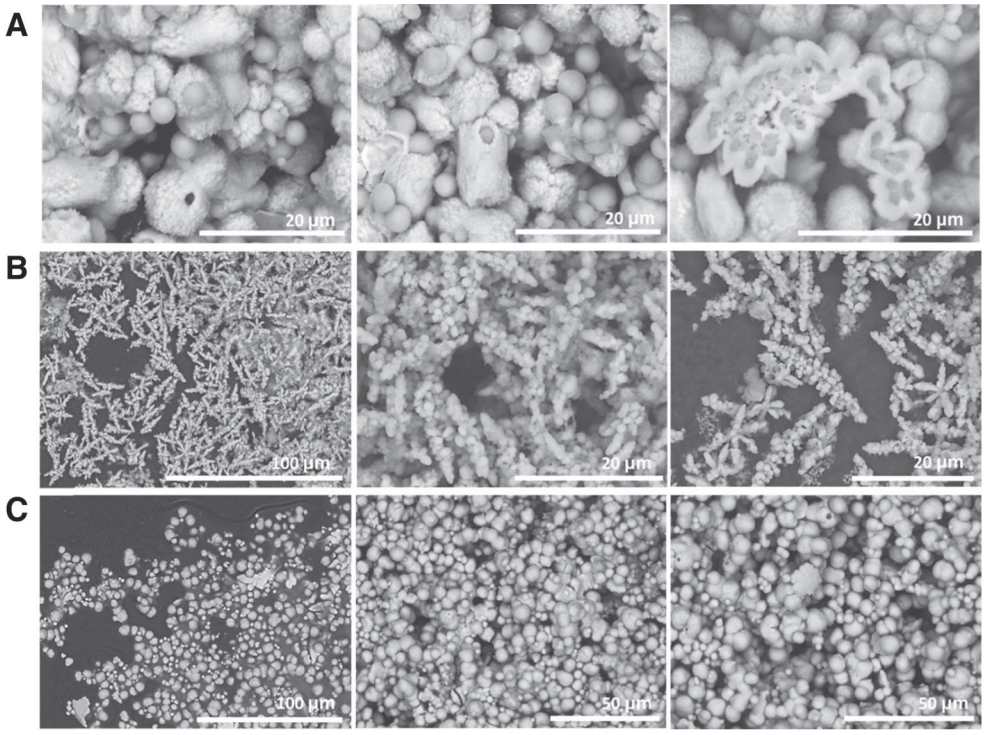

Fig. 8 Representative SEM images of mineral structures grown in presence of liquid like organic phases of **a** citrate/PEI, **b** caffeine/PAA and **c** arginine/PAA following the double jet protocol for $CaCO_3$ formation

microphase produces elongated superstructures composed of smaller particles (Fig. 8b). This morphology is reminiscent of the acicular shape of caffeine crystals (Fig. S4). This suggests that the organic PILP is destabilized during mineral nucleation and that the caffeine crystals thus formed directly template $CaCO_3$ growth or spatially organize the attached mineral. Of the organic liquid phases tested here, the caffeine/PAA systems appears the least stable and crystallizes relatively effectively to caffeine crystals (Fig. S4). A similar destabilization effect of the mineralization process towards organic PILPs is observed with the arginine/PAA system. However, the mineral particles formed are roughly spherical and agglomerated (Fig. 8c). Using conditions of fast precipitation with the arginine/PAA microdroplets leads to mineral structures composed of closely packed mineral units (Fig. S6C). The mineral products grown by gas diffusion in the presence of organic PILPs also present distinct crystallographic properties (Fig. S5). Organic PILP phases of citrate/PEI and caffeine/PAA or arginine/PAA lead to polycrystalline and monocrystalline products, respectively, as deduced using quantitative birefringence imaging. This hints towards a primary role of the charged polymers (PEI and PAA) that constitute the PILP phase in determining the crystallographic properties of mineral structures. Taken together, mineralization in the presence of organic PILPs produces diverse structures based on the (1) stability of the organic microphase during the mineralization reaction, (2) the fate of the organic constituents during PILP destabilization such as the self-association and mineral occlusion or exclusion tendency as aqueous solutes, (3) the role of the liquid–liquid interface in conditioning nucleation and crystal growth, and (4) the kinetics of mineral growth. Hence, the role of organic liquid-like phases in formation of diverse micro- and nano-structured biomineral forms emerges to be crucial.

The stability of organic PILPs in relation to mineralization regimes may also offer an explanation for the scarcity of organic molecules in certain mature biominerals. An ingrained advantage of liquid-like phases is the ease of mobility without the need for extreme concentration gradients of aqueous solutes. For instance, a mineralization-associated destabilization of the organic PILP may form soluble constituents suitable for other biochemical purposes. Along these lines, since the cohesive nature of organic PILPs arises from a balance between Van der Waals and electrostatic interactions as well as depletion forces (Hyman et al. 2014), conditions that disrupt these molecular interactions can induce phase destabilization. To shed some insight into this phenomenon, organic PILPs of citrate/PEI are produced under different conditions of pH (Fig. S7). After isolation of the PILP phase by centrifugation (Wohlrab et al. 2005), a distinct viscosity is seen in relation to pH. We speculate that low pH values affect the speciation of charged constituents within the PILP phase and hamper electrostatic interactions responsible for the cohesiveness of a liquid (Fig. S8). This leads to hydration loss associated with the liquid-like phase and release of constituent molecules as aqueous species. To better envisage the consequences of this phase behavior in terms of mineralization, the PILPs of arginine/PAA are qualitatively monitored using polarization light

microscopy under conditions for mineralization growth. In the presence of sodium carbonate (10 mM, pH 10) alone, the organic PILP is locally destabilized suggested by growth of arginine crystals at the liquid–liquid interface and apparent change in viscosity of the liquid precursor (Fig. 9a, b; Supplementary videos). Phase destabilization driven by direct interactions of PILP constituents with carbonate species cannot be ignored. In contrast to the aging-related destabilization, coalescence and crystallization of organic PILPs (Fig. S4), the carbonate solution induces crystallization of arginine/PAA droplets specifically at the liquid–liquid interface leading to ring-like or spherical crystalline structures (Fig. 9a, b). This also indicates distinct diffusion behavior of ion species in bulk aqueous and organic PILP phases, which might affect the form and structure of mineralization products (Fig. 8). A change in the cohesive properties of the organic PILP also suggests that pH conditions can tune the permeability or capillarity of liquid-like phases in biological matrices, and highlights the functional nature of the transient interface (Fig. 9b). During mineralization (simulated by direct precipitation of $CaCO_3$), a comparable change in viscous behavior is observed which is attributed to the pH decrease during mineral formation (Fig. 8c). This viscosity change occurs in three stages corresponding to an apparent pre-nucleation period, the formation of amorphous mineral particles and the phase transformation to crystals. The decrease in viscosity is more significant during the amorphous to crystalline transformation of the mineral phase (Fig. 9c). This is possibly due to a dynamic equilibrium between bicarbonate and carbonate species associated with mineral intermediates that involves the deprotonation of bicarbonate ions (i.e. decreasing the pH) on onset of crystallization. Another interesting outcome is the spatial inhibition of mineralization in the zone occupied by the organic PILP (Fig. 9c), which hints that the phase separation of ion and ion-associate species towards the arginine/PAA organic PILP is disfavored with respect to the bulk solution. In summary, the organic–inorganic interactions in organic PILP–mineral systems have 'bi-directional' (and multiple) consequences exemplified by (1) mineral species affecting the physical properties of the organic phase including stability and cohesiveness, and (2) the organic PILP spatially regulating mineral growth and structure (Fig. 10). Note that the experimental illustrations provided are of qualitative nature, unless stated

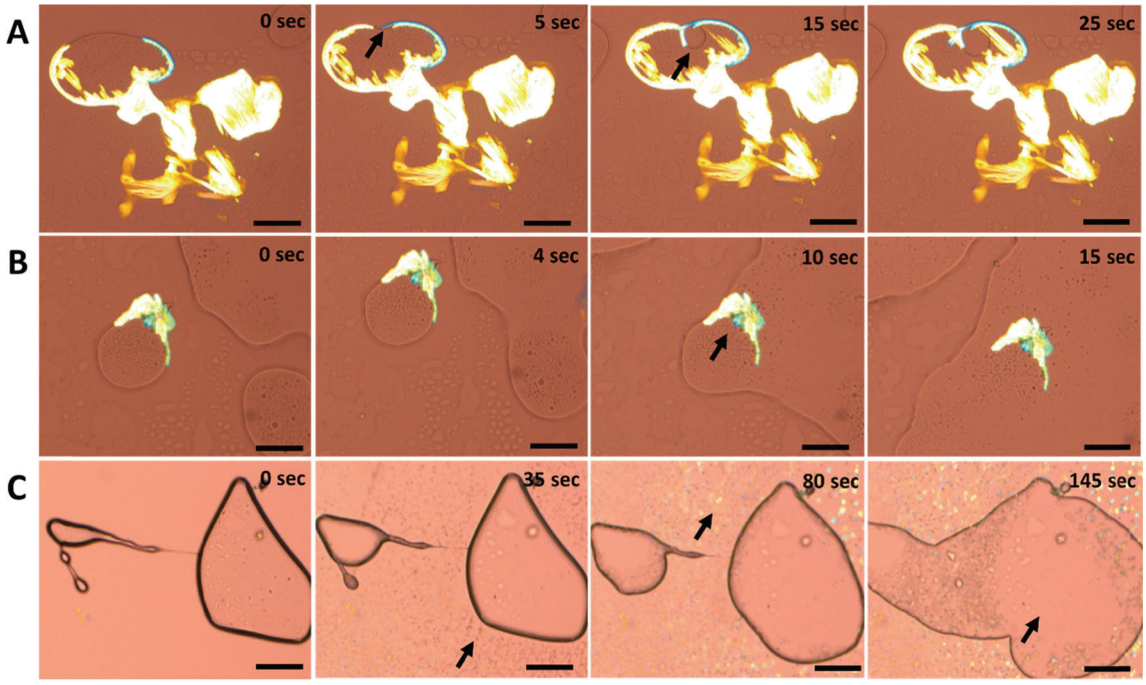

Fig. 9 Time lapse polarization light microscopy images of the behavior of arginine/PAA organic PILPs in presence of **a**, **b** sodium carbonate (10 mM, pH 10) and **c** mineralization induced by mixing sodium bicarbonate (10 mM, pH 8.2) and calcium chloride (10 mM). *Arrows* in (**a**) indicate the formation of arginine crystals directed by the liquid liquid interface and the release of PILP associated hydration, in (**b**) decreased viscosity and coalescence of the organic PILP phase, and in (**c**) stages in viscosity change corresponding to the initial formation of amorphous particles as evidenced by the lack of birefringence, the onset of crystallization and the spatial inhibition of mineral growth by the organic PILP. *Scale bars* 100 μm. The images correspond to the respective supporting videos

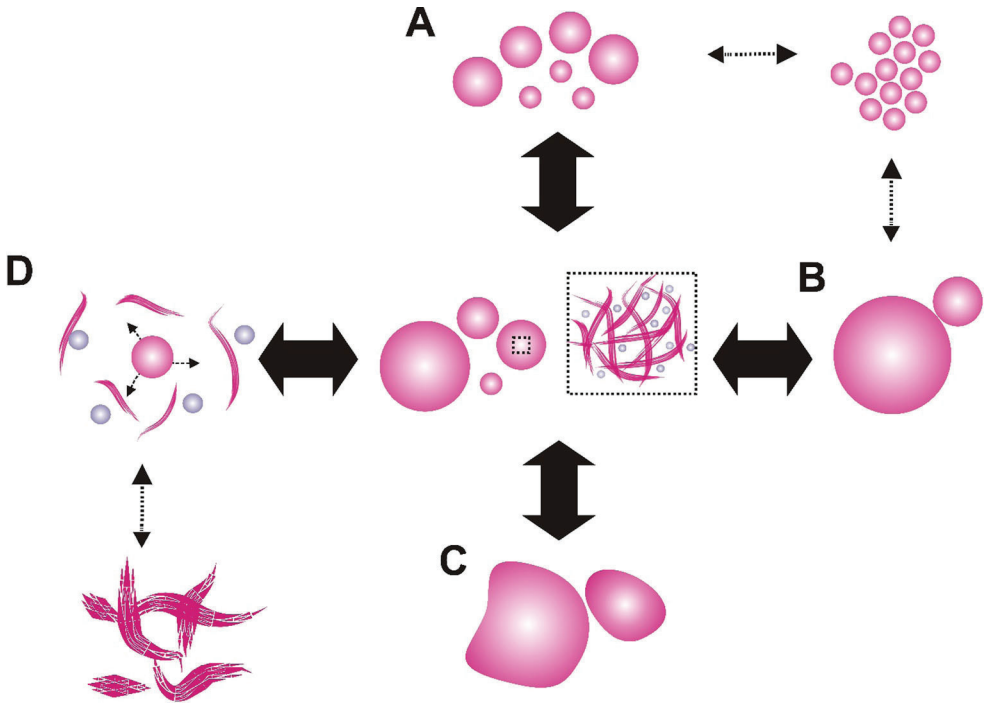

Fig. 10 Schematic representation of the multiple fates of organic liquid like phases during mineralization involving **a**, **b** dynamic coalescence, division and packing of droplets. A destabilization effect towards liquid like phases can lead to **c** change in liquid behavior such as cohesiveness or **d** release of droplet constituents as aqueous solute monomers or products of self association that serve distinct biochemical functions

otherwise, and are provided to explore the consequences of physico-chemical non-ideality operating during biomineralization.

Conclusions

The biological means for mineralization are elegant and sophisticated in comparison to the synthetic counterparts. In this review, we highlight novel features of nucleation and crystal growth phenomena emerging in non-ideal conditions specifically molecular crowding, confinement at distinct length scales and the phase behavior of biomolecules. To summarize:

1 Macromolecular crowding tunes pre- and post-nucleation regimes of mineralization. Prior to nucleation, the stability of mineral precursors and induction periods for nucleation are tuned by crowding agents. During particle growth, crowded solutions significantly affect the size, polymorph behavior and structure of mineral particles. For instance, PEG-crowding leads to destabilization of pre-nucleation clusters and the formation of metastable mineral forms. In contrast, crowded environments of charged macromolecules such as proteins and PSS lead to relative cluster stabilization and suppression of metastable forms. These effects emerge from the direct consequences of crowded environments on mineralization reactions as well as deviation in the ideal behavior of mineralization additives affecting self-association and conformational stability. Thus, crowded environments can tune mineral growth and form based on the physicochemical nature of nucleation and crystallization additives.

2 Matrices, gels and vesicles represent confinement-based modulation of mineralization at different length scales. By providing tuned nucleation conditions, critical size constraints towards mineral precursors and distinct solvent environments from the bulk aqueous phase, confinement modulates the formation and stability of mineral precursors. Based on conditions of confinement, possible consequences can include stabilization of amorphous mineral precursors and the emergence of single-crystalline, mesocrystalline or polycrystalline structural forms. Since the true complexity of biological niches is represented by concomitantly operating confinement and crowding induced by heterogeneous biomolecules, it emerges that a competition for space between the maturing mineral and organic matrix can tune nucleation and crystal growth (in addition to the direct interactions between inorganic precursors and nucleation or crystallization additives).

3 Organic PILPs or liquid-like organic phases affect the formation and morphology of mineral products. Under mineralization conditions, the composition, viscosity and stability of organic PILP phases play important roles in regulating mineralization in terms of structure, size and shape. The synergism of liquid-like forms of mineralization additives and inorganic precursors can offer a sophisticated regulation over mineralization via functional roles for the liquid–liquid interface during crystallization and prospects for recycling mineralization additives. The phase behavior of nucleation and crystallization additives as aqueous

solutes, insoluble assemblies, coacervates or organic PILPs in course of material formation may be advantageous in regulating transitions of the mineral phase and directing mineral form and structure.

Overall, non-ideal physico-chemistry of biochemical spaces provides an unparalleled regulation of nucleation and crystal growth. Evolutionary optimized biomineralization processes are an inspiration for synthetic approaches in providing precise structure, form and functionality to materials at different length scales. Further investigations in this direction will certainly yield key insights into the regulation of nucleation and crystallization phenomena in 'Nature's forge' as well as enhance biomimetic materials design.

Acknowledgment AR thanks the Freiburg Institute for Advanced Studies for its kind support.

Compliance with ethical standards

Conflict of interests Ashit Rao declares that he has no conflicts of interest. Helmut Cölfen declares that he has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Addadi L, Raz S, Weiner S (2003) Taking advantage of disorder: amor phous calcium carbonate and its roles in biomineralization. Adv Mater 15:959 970

Arakawa T, Timasheff SN (1985) Mechanism of polyethylene glycol interaction with proteins. Biochemistry 24:6756 6762

Arias JL, Fernández MAS (2008) Polysaccharides and proteoglycans in calcium carbonate based biomineralization. Chem Rev 108:4475 4482

Behera RK, Theil EC (2014) Moving Fe2+ from ferritin ion channels to catalytic OH centers depends on conserved protein cage carboxyl ates. Proc Natl Acad Sci U S A 111:7925 7930

Beinert H, Holm RH, Münck E (1997) Iron sulfur clusters: nature's mod ular, multipurpose structures. Science 277:653 659

Bentov S, Weil S, Glazer L, Sagi A, Berman A (2010) Stabilization of amorphous calcium carbonate by phosphate rich organic matrix pro teins and by single phosphoamino acids. J Struct Biol 171:207 215

Berg JK, Jordan T, Binder Y, Börner HG, Gebauer D (2013) Mg2+ tunes the wettability of liquid precursors of CaCO3: toward controlling mineralization sites in hybrid materials. J Am Chem Soc 135: 12512 12515

Bertinetti L, Masic A, Schuetz R, Barbetta A, Seidt B, Wagermaier W, Fratzl P (2015) Osmotically driven tensile stress in collagen based mineralized tissues. J Mech Behav Biomed Mater 52:14 21

Bewernitz MA, Gebauer D, Long J, Cölfen H, Gower LB (2012) A metastable liquid precursor phase of calcium carbonate and its inter actions with polyaspartate. Faraday Discuss 159:291 312

Bolen D (2004) Effects of naturally occurring osmolytes on protein sta bility and solubility: issues important in protein crystallization. Methods 34:312 322

Brangwynne CP, Eckmann CR, Courson DS, Rybarska A, Hoege C, Gharakhani J, Jülicher F, Hyman AA (2009) Germline P granules are liquid droplets that localize by controlled dissolution/condensa tion. Science 324:1729 1732

Brangwynne CP, Mitchison TJ, Hyman AA (2011) Active liquid like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes. Proc Natl Acad Sci U S A 108:4334 4339

Brangwynne CP, Tompa P, Pappu RV (2015) Polymer physics of intra cellular phase transitions. Nat Phys 11:899 904

Brubach J B, Mermet A, Filabozzi A, Gerschel A, Lairez D, Krafft M, Roy P (2001) Dependence of water dynamics upon confinement size. J Phys Chem B 105:430 435

Brunner E, Lutz K, Sumper M (2004) Biomimetic synthesis of silica nanospheres depends on the aggregation and phase separation of polyamines in aqueous solution. Phys Chem Chem Phys 6:854 857

Butler MF, Glaser N, Weaver AC, Kirkland M, Heppenstall Butler M (2006) Calcium carbonate crystallization in the presence of biopoly mers. Cryst Growth Des 6:781 794

Cartwright JH, Checa AG, Escribano B, Sainz Díaz CI (2009) Spiral and target patterns in bivalve nacre manifest a natural excitable medium from layer growth of a biological liquid crystal. Proc Natl Acad Sci U S A 106:10499 10504

Cartwright JH, Checa AG, Gale JD, Gebauer D, Sainz-Díaz CI (2012) Calcium carbonate polyamorphism and its role in biomineralization: how many amorphous calcium carbonates are there? Angew Chem Int Ed 51:11960 11970

Cartwright JH, Checa AG, Rousseau M (2013) Pearls are self organized natural ratchets. Langmuir 29:8370 8376

Chang EP, Williamson G, Evans JS (2015) Focused ion beam tomogra phy reveals the presence of micro , meso , and macroporous intracrystalline regions introduced into calcite crystals by the gastro pod nacre protein AP7. Cryst Growth Des 15:1577 1582

Chang EP, Perovic I, Rao A, Cölfen H, Evans JS (2016a) In vitro glyco sylation and its impact on the functionality of a recombinant intracrystalline nacre protein, AP24. Biochemistry 55(7):1024 1035

Chang EP, Roncal Herrero T, Morgan T, Dunn KE, Rao A, Kunitake JA, Lui S, Bilton M, Estroff LA, Kröger R (2016b) Synergistic biomin eralization phenomena created by a combinatorial nacre protein model system. Biochemistry 55(16):2401 2410

Chebotareva NA, Harding SE, Winzor DJ (2001) Ultracentrifugal studies of the effect of molecular crowding by trimethylamine N-oxide on the self-association of muscle glycogen phosphorylase b. Eur J Biochem 268:506 513

Checa AG, Cartwright JH, Willinger M G (2011) Mineral bridges in nacre. J Struct Biol 176:330 339

Checa AG, Macías Sánchez E, Harper EM, Cartwright JH (2016) Organic membranes determine the pattern of the columnar prismatic layer of mollusc shells. Proc R Soc Lond B 283:20160032

Cölfen H (2010) Biomineralization: a crystal clear view. Nat Mater 9: 960 961

Cölfen H, Antonietti M (2008) Mesocrystals and nonclassical crystalliza tion. Wiley, Chichester

Cölfen H, Winzor DJ (1997) A computer program based on the psi func tion for model independent analysis of sedimentation equilibrium distributions reflecting macromolecular interactions. In: Jaenicke R, Durchschlag H (eds) Analytical ultracentrifugation IV. Springer, Berlin, pp 36 42

Cölfen H, Harding SE, Vårum KM, Winzor DJ (1996) A study by ana lytical ultracentrifugation on the interaction between lysozyme and extensively deacetylated chitin (chitosan). Carbohydr Polym 30:45 53

Cölfen H, Harding SE, Wilson EK, Scrutton NS, Winzor DJ (1997) Low temperature solution behaviour of Methylophilus methylotrophus electron transferring flavoprotein: a study by analytical ultracentri fugation. Eur Biophys J 25:411 416

Davis Searles PR, Saunders AJ, Erie DA, Winzor DJ, Pielak GJ (2001) Interpreting the effects of small uncharged solutes on protein folding equilibria. Annu Rev Biophys Biomol Struct 30(1):271 306

de Nooijer LJ, Toyofuku T, Kitazato H (2009) Foraminifera promote calcification by elevating their intracellular pH. Proc Natl Acad Sci U S A 106:15374 15378

De Yoreo JJ, Gilbert PU, Sommerdijk NA, Penn RL, Whitelam S, Joester D, Zhang H, Rimer JD, Navrotsky A, Banfield JF (2015) Crystallization by particle attachment in synthetic, biogenic, and geologic environments. Science 349:aaa6760

Demichelis R, Raiteri P, Gale JD, Quigley D, Gebauer D (2011) Stable prenucleation mineral clusters are liquid like ionic polymers. Nat Commun 2:590

Derganc J, Čopič A (2016) Membrane bending by protein crowding is affected by protein lateral confinement. Biochim Biophys Acta Biomembr 1858:1152 1159

Deszczynski M, Harding SE, Winzor DJ (2006) Negative second virial coefficients as predictors of protein crystal growth: evidence from sedimentation equilibrium studies that refutes the designation of those light scattering parameters as osmotic virial coefficients. Biophys Chem 120:106 113

Dewavrin J Y, Hamzavi N, Shim V, Raghunath M (2014) Tuning the architecture of three dimensional collagen hydrogels by physiolog ical macromolecular crowding. Acta Biomater 10:4351 4359

Dey A, Bomans PH, Müller FA, Will J, Frederik PM, de With G, Sommerdijk NA (2010) The role of prenucleation clusters in surface induced calcium phosphate crystallization. Nat Mater 9: 1010 1014

Dill KA, Ghosh K, Schmit JD (2011) Physical limits of cells and proteomes. Proc Natl Acad Sci U S A 108:17876 17882

DiMasi E, Olszta MJ, Patel VM, Gower LB (2003) When is template directed mineralization really template directed? CrystEngComm 5: 346 350

Edidin M (2003) The state of lipid rafts: from model membranes to cells. Annu Rev Biophys Biomol Struct 32:257 283

Ellis RJ (2001) Macromolecular crowding: obvious but underappreciat ed. Trends Biochem Sci 26:597 604

Ellis RJ, Minton AP (2003) Cell biology: join the crowd. Nature 425:27 28

Erickson HP (2009) Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron mi croscopy. Biol Proced Online 11:32

Erlkamp M, Marion J, Martinez N, Czeslik C, Peters J, Winter R (2015) Influence of pressure and crowding on the sub nanosecond dynam ics of globular proteins. J Phys Chem B 119:4842 4848

Evans JS (2008) “Tuning in” to mollusk shell nacre and prismatic asso ciated protein terminal sequences. Implications for biomineraliza tion and the construction of high performance inorganic organic composites. Chem Rev 108:4455 4462

Falini G, Fermani S, Gazzano M, Ripamonti A (2000) Polymorphism and architectural crystal assembly of calcium carbonate in biologically inspired polymeric matrices. J Chem Soc Dalton Trans :3983 3987

Fan W, Li C, Li S, Feng Q, Xie L, Zhang R (2007) Cloning, characteri zation, and expression patterns of three sarco/endoplasmic reticulum Ca2 + ATPase isoforms from pearl oyster (Pinctada fucata). Acta Biochim Biophys Sin 39:722 730

Feng J, Wu G, Qing C (2016) Biomimetic synthesis of hollow calcium carbonate with the existence of the agar matrix and bovine serum albumin. Mater Sci Eng C 58:409 411

Fernandez-Martinez A, Kalkan B, Clark SM, Waychunas GA (2013) Pressure induced polyamorphism and formation of ‘Aragonitic’Amorphous calcium carbonate. Angew Chem Int Ed 52:8354 8357

Frenkel J (1939) Statistical theory of condensation phenomena. J Chem Phys 7:200 201

Gebauer D, Cölfen H (2011) Prenucleation clusters and non classical nucleation. Nano Today 6:564 584

Gebauer D, Völkel A, Cölfen H (2008) Stable prenucleation calcium carbonate clusters. Science 322:1819 1822

Gebauer D, Cölfen H, Verch A, Antonietti M (2009) The multiple roles of additives in CaCO3 crystallization: a quantitative case study. Adv Mater 21:435 439

Gebauer D, Kellermeier M, Gale JD, Bergström L, Cölfen H (2014) Pre nucleation clusters as solute precursors in crystallisation. Chem Soc Rev 43:2348 2371

Gong H, Yang Y, Pluntke M, Marti O, Majer Z, Sewald N, Volkmer D (2014) Calcium carbonate crystal growth beneath Langmuir mono layers of acidic β hairpin peptides. Dalton Trans 43:16857 16871

Goswami N, Giri A, Bootharaju MS, Xavier PL, Pradeep T, Pal SK (2011) Copper quantum clusters in protein matrix: potential sensor of Pb2+ Ion. Anal Chem 83:9676 9680

Gower LB (2008) Biomimetic model systems for investigating the amor phous precursor pathway and its role in biomineralization. Chem Rev 108:4551 4627

Gower LB, Odom DJ (2000) Deposition of calcium carbonate films by a polymer induced liquid precursor (PILP) process. J Cryst Growth 210:719 734

Guilak F, Erickson GR, Ting Beall HP (2002) The effects of osmotic stress on the viscoelastic and physical properties of articular chondrocytes. Biophys J 82:720 727

Guo C, Irudayaraj J (2011) Fluorescent Ag clusters via a protein directed approach as a Hg(II) ion sensor. Anal Chem 83:2883 2889

Ha J M, Wolf JH, Hillmyer MA, Ward MD (2004) Polymorph selectivity under nanoscopic confinement. J Am Chem Soc 126:3382 3383

Hall DR, Jacobsen MP, Winzor DJ (1995) Stabilizing effect of sucrose against irreversible denaturation of rabbit muscle lactate dehydroge nase. Biophys Chem 57(1):47 54

Hamilton BD, Hillmyer MA, Ward MD (2008) Glycine polymorphism in nanoscale crystallization chambers. Cryst Growth Des 8:3368 3375

Hamley IW (2010) Liquid crystal phase formation by biopolymers. Soft Matter 6:1863 1871

Heiss A, Jahnen Dechent W, Endo H, Schwahn D (2007) Structural dy namics of a colloidal protein mineral complex bestowing on calci um phosphate a high solubility in biological fluids. Biointerphases 2:16 20

Heiss A, Pipich V, Jahnen Dechent W, Schwahn D (2010) Fetuin A is a mineral carrier protein: small angle neutron scattering provides new insight on Fetuin A controlled calcification inhibition. Biophys J 99: 3986 3995

Hyman AA, Brangwynne CP (2011) Beyond stereospecificity: liquids and mesoscale organization of cytoplasm. Dev Cell 21:14 16

Hyman AA, Weber CA, Jülicher F (2014) Liquid liquid phase separation in biology. Annu Rev Cell Dev Biol 30:39 58

Ianeselli L, Zhang F, Skoda MWA, Jacobs RMJ, Martin RA, Callow S, Prévost S, Schreiber F (2010) Protein protein interactions in oval bumin solutions studied by small angle scattering: effect of ionic strength and the chemical nature of cations. J Phys Chem B 114: 3776 3783

Jackson DJ, McDougall C, Woodcroft B, Moase P, Rose RA, Kube M, Reinhardt R, Rokhsar DS, Montagnani C, Joubert C (2010) Parallel evolution of nacre building gene sets in molluscs. Mol Biol Evol 27: 591 608

Jain G, Pendola M, Rao A, Cölfen H, Evans JS (2016) A model sea urchin spicule matrix protein self associates to form mineral modifying protein hydrogels. Biochemistry 55(31):4410 4421

Jolivet J P, Tronc E, Chanéac C (2006) Iron oxides: from molecular clusters to solid. A nice example of chemical versatility. Compt Rendus Geosci 338:488 497

Knight DP, Vollrath F (2002) Biological liquid crystal elastomers. Philos Trans R Soc Lond B 357:155 163

Knothe TM (2003) “Whither flows the fluid in bone?” An osteocyte’s perspective. J Biomech 36:1409 1424

Konno H, Taylor LS (2006) Influence of different polymers on the crys tallization tendency of molecularly dispersed amorphous felodipine. J Pharm Sci 95:2692 2705

Kory N, Thiam A R, Farese RV, Walther TC (2015) Protein crowding is a determinant of lipid droplet protein composition. Dev Cell 34:351 363

Kosanović C, Falini G, Kralj D (2010) Mineralization of calcium carbon ates in gelling media. Cryst Growth Des 11:269 277

Lareu RR, Subramhanya KH, Peng Y, Benny P, Chen C, Wang Z, Rajagopalan R, Raghunath M (2007) Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged mac romolecules: the biological relevance of the excluded volume effect. FEBS Lett 581:2709 2714

Li H, Estroff LA (2009) Calcite growth in hydrogels: assessing the mech anism of polymer network incorporation into single crystals. Adv Mater 21:470 473

Lin Y, Protter DS, Rosen MK, Parker R (2015) Formation and maturation of phase separated liquid droplets by RNA binding proteins. Mol Cell 60:208 219

Lingwood D, Simons K (2010) Lipid rafts as a membrane organizing principle. Science 327:46 50

Liu B, Zeng HC (2005) Symmetric and asymmetric Ostwald ripening in the fabrication of homogeneous core shell semiconductors. Small 1: 566 571

Lowenstam HA, Weiner S (1989) On biomineralization. Oxford University Press, Oxford; on demand

Mackinder L, Wheeler G, Schroeder D, von Dassow P, Riebesell U, Brownlee C (2011) Expression of biomineralization related ion transport genes in Emiliania huxleyi. Environ Microbiol 13:3250 3265

Mahamid J, Sharir A, Gur D, Zelzer E, Addadi L, Weiner S (2011) Bone mineralization proceeds through intracellular calcium phosphate loaded vesicles: a cryo electron microscopy study. J Struct Biol 174:527 535

Mann S (2001) Biomineralization: principles and concepts in bioinorganic materials chemistry, vol 5. Oxford University Press, Oxford, on demand

Mann K, Maček B, Olsen JV (2006) Proteomic analysis of the acid soluble organic matrix of the chicken calcified eggshell layer. Proteomics 6:3801 3810

Mann K, Wilt FH, Poustka AJ (2010) Proteomic analysis of sea urchin (Strongylocentrotus purpuratus) spicule matrix. Proteome Sci 8:1

Marie B, Marie A, Jackson DJ, Dubost L, Degnan BM, Milet C, Marin F (2010) Proteomic analysis of the organic matrix of the abalone Haliotis asinina calcified shell. Proteome Sci 8:1

Meldrum FC, Cölfen H (2008) Controlling mineral morphologies and structures in biological and synthetic systems. Chem Rev 108: 4332 4432

Minton AP (1992) Confinement as a determinant of macromolecular structure and reactivity. Biophys J 63:1090

Minton AP (1995) Confinement as a determinant of macromolecular structure and reactivity. II. Effects of weakly attractive interactions between confined macrosolutes and confining structures. Biophys J 68:1311

Minton AP (2001) The influence of macromolecular crowding and mac romolecular confinement on biochemical reactions in physiological media. J Biol Chem 276:10577 10580

Minton AP (2006) Macromolecular crowding. Curr Biol 16:R269 R271

Moilanen DE, Levinger NE, Spry D, Fayer M (2007) Confinement or the nature of the interface? Dynamics of nanoscopic water. J Am Chem Soc 129:14311 14318

Molliex A, Temirov J, Lee J, Coughlin M, Kanagaraj AP, Kim HJ, Mittag T, Taylor JP (2015) Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. Cell 163:123 133

Morissette SL, Almarsson Ö, Peterson ML, Remenar JF, Read MJ, Lemmo AV, Ellis S, Cima MJ, Gardner CR (2004) High throughput crystallization: polymorphs, salts, co crystals and sol vates of pharmaceutical solids. Adv Drug Deliv Rev 56:275 300

Murr MM, Thakur GS, Li Y, Tsuruta H, Mezic I, Morse DE (2009) New pathway for self assembly and emergent properties. Nano Today 4: 116 124

Nakahara H (1991) Nacre formation in bivalve and gastropod molluscs. In: Suga S, Nakahara H (eds) Mechanisms and phylogeny of min eralization in biological systems. Springer, Berlin, pp 343 350

Navrotsky A (2004) Energetic clues to pathways to biomineralization: precursors, clusters, and nanoparticles. Proc Natl Acad Sci U S A 101:12096 12101

Niederberger M, Cölfen H (2006) Oriented attachment and mesocrystals: non classical crystallization mechanisms based on nanoparticle as sembly. Phys Chem Chem Phys 8:3271 3287

Nikolov S, Petrov M, Lymperakis L, Friák M, Sachs C, Fabritius HO, Raabe D, Neugebauer J (2010) Revealing the design principles of high performance biological composites using Ab initio and multiscale simulations: the example of lobster cuticle. Adv Mater 22:519 526

Nudelman F, Pieterse K, George A, Bomans PH, Friedrich H, Brylka LJ, Hilbers PA, de With G, Sommerdijk NA (2010) The role of collagen in bone apatite formation in the presence of hydroxyapatite nucle ation inhibitors. Nat Mater 9:1004 1009

Oaki Y, Hayashi S, Imai H (2007) A hierarchical self similar structure of oriented calcite with association of an agar gel matrix: inheritance of crystal habit from nanoscale. Chem Commun 27:2841 2843

Ogston AG, Winzor DJ (1975) Treatment of thermodynamic nonideality in equilibrium studies on associating solutes. J Phys Chem 79:2496 2500

Olszta MJ, Odom DJ, Douglas EP, Gower LB (2009) A new paradigm for biomineral formation: mineralization via an amorphous liquid phase precursor. Connect Tissue Res 44(Suppl 1):326 334

Oparin AI (1938) The origin of life. Macmillan, New York

Panheleux M, Bain M, Fernandez M, Morales I, Gautron J, Arias J, Solomon S, Hincke M, Nys Y (1999) Organic matrix composition and ultrastructure of eggshell: a comparative study. Br Poult Sci 40: 240 252

Patel CN, Noble SM, Weatherly GT, Tripathy A, Winzor DJ, Pielak GJ (2002) Effects of molecular crowding by saccharides on α-chymo trypsin dimerization. Protein Sci 11(5):997 1003

Pereira Mouriès L, Almeida MJ, Ribeiro C, Peduzzi J, Barthélemy M, Milet C, Lopez E (2002) Soluble silk like organic matrix in the nacreous layer of the bivalve Pinctada maxima. Eur J Biochem 269:4994 5003

Perovic I, Chang EP, Lui M, Rao A, Cölfen H, Evans JS (2014) A nacre protein, n16. 3, self assembles to form protein oligomers that dimen sionally limit and organize mineral deposits. Biochemistry 53:2739 2748

Poon J, Bailey M, Winzor DJ, Davidson BE, Sawyer WH (1997) Effects of molecular crowding on the interaction between DNA and the Escherichia coli regulatory protein TyrR. Biophys J 73:3257 3264

Prajapati S, Tao J, Ruan Q, De Yoreo JJ, Moradian Oldak J (2016) Matrix metalloproteinase 20 mediates dental enamel biomineralization by preventing protein occlusion inside apatite crystals. Biomaterials 75: 260 270

Qin C, Baba O, Butler W (2004) Post translational modifications of sib ling proteins and their roles in osteogenesis and dentinogenesis. Crit Rev Oral Biol Med 15:126 136

Raabe D, Romano P, Sachs C, Fabritius H, Al Sawalmih A, Yi S B, Servos G, Hartwig H (2006) Microstructure and crystallographic texture of the chitin protein network in the biological composite

material of the exoskeleton of the lobster Homarus americanus. Mater Sci Eng A 421:143 153
Raiteri P, Gale JD (2010) Water is the key to nonclassical nucleation of amorphous calcium carbonate. J Am Chem Soc 132:17623 17634
Rajapaksha A, Stanley CB, Todd BA (2015) Effects of macromolecular crowding on the structure of a protein complex: a small angle scat tering study of superoxide dismutase. Biophys J 108:967 974
Rao A, Cölfen H (2016) On the biophysical regulation of mineral growth: standing out from the crowd. J Struct Biol. doi:10.1016/j.jsb.2016.03.021
Rao A, Seto J, Berg JK, Kreft SG, Scheffner M, Cölfen H (2013) Roles of larval sea urchin spicule SM50 domains in organic matrix self assembly and calcium carbonate mineralization. J Struct Biol 183: 205 215
Rao A, Berg JK, Kellermeier M, Gebauer D (2014) Sweet on biomineralization: effects of carbohydrates on the early stages of calcium carbonate crystallization. Eur J Mineral 26:537 552
Rao A, Fernández MS, Cölfen H, Arias JL (2015) Distinct effects of avian egg derived anionic proteoglycans on the early stages of calcium carbonate mineralization. Cryst Growth Des 15:2052 2056
Rao A, Vásquez Quitral P, Fernández MS, Berg JK, Sánchez M, Drechsler M, Neira Carrillo A, Arias JL, Gebauer D, Cölfen H (2016) pH dependent schemes of calcium carbonate formation in the presence of alginates. Cryst Growth Des 16:1349 1359
Rivas G, Fernandez JA, Minton AP (1999) Direct observation of the self association of dilute proteins in the presence of inert macromolecules at high concentration via tracer sedimentation equilibrium: theory, experiment, and biological significance. Biochemistry 38: 9379 9388
Romano P, Fabritius H, Raabe D (2007) The exoskeleton of the lobster Homarus americanus as an example of a smart anisotropic biological material. Acta Biomater 3:301 309
Saeidi N, Karmelek KP, Paten JA, Zareian R, DiMasi E, Ruberti JW (2012) Molecular crowding of collagen: a pathway to produce highly organized collagenous structures. Biomaterials 33:7366 7374
Samiotakis A, Wittung Stafshede P, Cheung MS (2009) Folding, stability and shape of proteins in crowded environments: experimental and computational approaches. Int J Mol Sci 10:572 588
Schuck P (2003) On the analysis of protein self association by sedimentation velocity analytical ultracentrifugation. Anal Biochem 320: 104 124
Schwartz DK (2001) Mechanisms and kinetics of self assembled monolayer formation. Annu Rev Phys Chem 52:107 137
Scott DJ, Patel TR, Besong DM, Stetefeld J, Winzor DJ (2011) Examination of the discrepancy between size estimates for ovalbumin from small angle X ray scattering and other physicochemical measurements. J Phys Chem B 115:10725 10729
Seto J, Picker A, Chen Y, Rao A, Evans JS, Cölfen H (2014) Nacre protein sequence compartmentalizes mineral polymorphs in solution. Cryst Growth Des 14(4):1501 1505
Shearwin KE, Winzor DJ (1988) Effect of sucrose on the dimerization of α chymotrypsin allowance for thermodynamic nonideality arising from the presence of a small inert solute. Biophys Chem 31:287 294
Shearwin KE, Winzor DJ (1990a) Effect of calcium ion on the dimerization of α chymotrypsin. Biochim Biophys Acta 1038(1):136 138
Shearwin KE, Winzor DJ (1990b) Thermodynamic nonideality in macromolecular solutions. Eur J Biochem 190:523 529
Shtilerman MD, Ding TT, Lansbury PT (2002) Molecular crowding accelerates fibrillization of α synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? Biochemistry 41:3855 3860
Silvius JR (2003) Role of cholesterol in lipid raft formation: lessons from lipid model systems. Biochim Biophys Acta Biomembr 1610:174 183
Sottnik JL, Dai J, Zhang H, Campbell B, Keller ET (2015) Tumor induced pressure in the bone microenvironment causes osteocytes to promote the growth of prostate cancer bone metastases. Cancer Res 75:2151 2158
Spencer DS, Xu K, Logan TM, Zhou H X (2005) Effects of pH, salt, and macromolecular crowding on the stability of FK506 binding protein: an integrated experimental and theoretical study. J Mol Biol 351:219 232
Spitzer J, Poolman B (2009) The role of biomacromolecular crowding, ionic strength, and physicochemical gradients in the complexities of life's emergence. Microbiol Mol Biol Rev 73:371 388
Spitzer J, Pielak GJ, Poolman B (2015) Emergence of life: physical chemistry changes the paradigm. Biol Direct 10:33
Sudo S, Fujikawa T, Nagakura T, Ohkubo T, Sakaguchi K, Tanaka M, Nakashima K, Takahashi T (1997) Structures of mollusc shell framework proteins. Nature 387:563 564
Sumper M (2002) A phase separation model for the nanopatterning of diatom biosilica. Science 295:2430 2433
Sumper M (2004) Biomimetic patterning of silica by long chain polyamines. Angew Chem Int Ed 43:2251 2254
Sun J, Bhushan B (2012) Hierarchical structure and mechanical properties of nacre: a review. RSC Adv 2:7617 7632
Tagliabracci VS, Engel JL, Wen J, Wiley SE, Worby CA, Kinch LN, Xiao J, Grishin NV, Dixon JE (2012) Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. Science 336: 1150 1153
Tan BH, Tam KC, Lam YC, Tan CB (2005) Osmotic compressibility of soft colloidal systems. Langmuir 21:4283 4290
Tester CC, Brock RE, Wu C H, Krejci MR, Weigand S, Joester D (2011) In vitro synthesis and stabilization of amorphous calcium carbonate (ACC) nanoparticles within liposomes. CrystEngComm 13:3975 3978
Thula TT, Rodriguez DE, Lee MH, Pendi L, Podschun J, Gower LB (2011) In vitro mineralization of dense collagen substrates: a biomimetic approach toward the development of bone graft materials. Acta Biomater 7:3158 3169
Toroian D, Lim JE, Price PA (2007) The size exclusion characteristics of type I collagen implications for the role of noncollagenous bone constituents in mineralization. J Biol Chem 282:22437 22447
Verch A, Gebauer D, Antonietti M, Cölfen H (2011) How to control the scaling of CaCO 3: A "fingerprinting technique" to classify additives. Phys Chem Chem Phys 13:16811 16820
Vidavsky N, Addadi S, Mahamid J, Shimoni E, Ben Ezra D, Shpigel M, Weiner S, Addadi L (2014) Initial stages of calcium uptake and mineral deposition in sea urchin embryos. Proc Natl Acad Sci U S A 111:39 44
Volkmer D, Fricke M, Agena C, Mattay J (2004) Interfacial electrostatics guiding the crystallization of CaCO 3 underneath monolayers of calixarenes and resorcarenes. J Mater Chem 14:2249 2259
Wallace AF, Hedges LO, Fernandez Martinez A, Raiteri P, Gale JD, Waychunas GA, Whitelam S, Banfield JF, De Yoreo JJ (2013) Microscopic evidence for liquid liquid separation in supersaturated CaCO3 solutions. Science 341:885 889
Wang X, Sun H, Xia Y, Chen C, Xu H, Shan H, Lu JR (2009) Lysozyme mediated calcium carbonate mineralization. J Colloid Interface Sci 332:96 103
Wang X, Wu C, Tao K, Zhao K, Wang J, Xu H, Xia D, Shan H, Lu JR (2010) Influence of ovalbumin on CaCO3 precipitation during in vitro biomineralization. J Phys Chem B 114:5301 5308
Weiner S, Traub W (1980) X ray diffraction study of the insoluble organic matrix of mollusk shells. FEBS Lett 111:311 316
Wills PR, Winzor DJ (2005) van der Waals phase transition in protein solutions. Acta Crystallogr Sect D 61(6):832 836
Wills PR, Comper WD, Winzor DJ (1993) Thermodynamic nonideality in macromolecular solutions: interpretation of virial coefficients. Arch Biochem Biophys 300:206 212

Wilson EK, Scrutton NS, Cölfen H, Harding SE, Jacobsen MP, Winzor DJ (1997) An ultracentrifugal approach to quantitative characterization of the molecular assembly of a physiological electron-transfer complex. Eur J Biochem 243:393 399

Wingender B, Bradley P, Saxena N, Ruberti JW, Gower L (2016) Biomimetic organization of collagen matrices to template bone like microstructures. Matrix Biol 52:384 396

Winzor DJ (1966) Detection of interaction in gluten extracts by gel filtration. Arch Biochem Biophys 113(2):421 426

Winzor DJ (2004) Determination of the net charge (valence) of a protein: a fundamental but elusive parameter. Anal Biochem 325:1 20

Winzor DJ, Wills PR (1986) Effects of thermodynamic nonideality on protein interactions: equivalence of interpretations based on excluded volume and preferential solvation. Biophys Chem 25:243 251

Winzor DJ, Wills PR (2006) Molecular crowding effects of linear polymers in protein solutions. Biophys Chem 119(2):186 195

Winzor CL, Winzor DJ, Paleg LG, Jones GP, Naidu BP (1992) Rationalization of the effects of compatible solutes on protein stability in terms of thermodynamic nonideality. Arch Biochem Biophys 296:102 107

Winzor DJ, Carrington LE, Deszczynski M, Harding SE (2004) Extent of charge screening in aqueous polysaccharide solutions. Biomacromolecules 5(6):2456 2460

Winzor DJ, Deszczynski M, Harding SE, Wills PR (2007) Nonequivalence of second virial coefficients from sedimentation equilibrium and static light scattering studies of protein solutions. Biophys Chem 128(1):46 55

Wohlrab S, Cölfen H, Antonietti M (2005) Crystalline, porous microspheres made from amino acids by using polymer induced liquid precursor phases. Angew Chem Int Ed 44:4087 4092

Wolf SE, Leiterer J, Pipich V, Barrea R, Emmerling F, Tremel W (2011) Strong stabilization of amorphous calcium carbonate emulsion by ovalbumin: gaining insight into the mechanism of 'polymer induced liquid precursor' processes. J Am Chem Soc 133:12642 12649

Woo E, Huh J, Jeong YG, Shin K (2007) From homogeneous to heterogeneous nucleation of chain molecules under nanoscopic cylindrical confinement. Phys Rev Lett 98:136103

Xia Y, Gu Y, Zhou X, Xu H, Zhao X, Yaseen M, Lu JR (2012) Controllable stabilization of poly (N isopropylacrylamide) based microgel films through biomimetic mineralization of calcium carbonate. Biomacromolecules 13:2299 2308

Xiang S, Kato M, Wu LC, Lin Y, Ding M, Zhang Y, Yu Y, McKnight SL (2015) The LC domain of hnRNPA2 adopts similar conformations in hydrogel polymers, liquid like droplets, and nuclei. Cell 163: 829 839

Xie AJ, Zhang CY, Shen YH, Qiu LG, Xiao PP, Hu ZY (2006) Morphologies of calcium carbonate crystallites grown from aqueous solutions containing polyethylene glycol. Cryst Res Technol 41: 967 971

Xie J, Zheng Y, Ying JY (2009) Protein directed synthesis of highly fluorescent gold nanoclusters. J Am Chem Soc 131:888 889

Xu G, Evans JS (1999) Model peptide studies of sequence repeats derived from the intracrystalline biomineralization protein, SM50. I. GVGGR and GMGGQ repeats. Biopolymers 49:303 312

Xu AW, Dong WF, Antonietti M, Cölfen H (2008) Polymorph switching of calcium carbonate crystals by polymer controlled crystallization. Adv Funct Mater 18:1307 1313

Xu Z, Hirtz M, Yuan S, Liu C, Chi L (2011) Selective deposition of organic molecules onto different densely packed self assembled monolayers: a molecular dynamics study. Chem Phys Lett 507: 138 143

Yang L, Killian CE, Kunz M, Tamura N, Gilbert P (2011) Biomineral nanoparticles are space filling. Nanoscale 3:603 609

Zhou H X, Dill KA (2001) Stabilization of proteins in confined spaces. Biochemistry 40:11289 11293

Zhou H X, Rivas G, Minton AP (2008) Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. Annu Rev Biophys 37:375

Zimmerman SB, Minton AP (1993) Macromolecular crowding: biochemical, biophysical, and physiological consequences. Annu Rev Biophys Biomol Struct 22:27 65

Zimmerman SB, Trach SO (1991) Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli. J Mol Biol 222:599 620