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On the biophysical regulation of mineral growth: Standing out from the crowd

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ABSTRACT

Biogenic mineralization processes are generally regulated by soluble additives and insoluble matrices. This endows precise control over the different stages of mineralization such as the uptake, transport of mineral precursors as well as the subsequent deposition of the mineral phases with consistent compositions and morphologies. Programmed in the interactions of organic molecules with different precursor species and the fine modulation of the niche environments, a formative elegance is reflected in the biological means for crystal formation in comparison to the synthetic counterparts. In order to spotlight the role of prevalent biophysical environments in the emergence of fascinating materials, we revisit biologically modulated mineralization to describe nucleation and crystallization under physicochemical highly non-ideal conditions on account of macromolecular crowding and the gel-like nature of cellular matrices.

Biomineralization addresses the formation and properties of inorganic materials deposited in living organisms (Bäuerlein et al., 2007; Lowenstam and Weiner, 1989; Mann, 2001). In the past decades, the field of biomineralization has emerged from vital efforts in accessing the fascinating biodiversity of minerals in terms of composition and morphology to present times where the research focus is towards the formation and structure-property relations of these fascinating biomaterials (Gebauer et al., 2008; Gong et al., 2012; Lowenstam and Weiner, 1989; Pouget et al., 2009; Seto et al., 2012; Vidavsky et al., 2014). This transition has been accentuated by the multidisciplinary nature of the field that permeates through disciplines such as crystallography, evolutionary biology, material sciences, physical chemistry, medicine and oceanography. Despite numerous investigations addressing biomineralization processes, important questions regarding the extent of biological control over the stability of precursors, early nucleation events, phase transformations, polymorph selection and precise control over mineral morphology remain debated. Therefore through this article, we seek an overview for mineralization in confined environments that are typically rich in biomolecules and other additives. Although certain biomineralization processes are attributed to 'active' processes (eg. enzymatic catalysis, self-assembly of biomolecules) especially in composite biominerals, the impact of the concomitant physico-chemical parameters such as crowded environments, pH, gel-like environments and surface wettability is not well-addressed. As we discuss these phenomena, the biomineralization process emerges to be an intricate synergy between the organic and mineral phase during which each phase influences the maturation of the biomineral, the typical consequence of which is the amalgamation of inorganic and organic components in a material exhibiting extraordinary properties.

Biomineralization and molecular crowding

In biological media, a significant fraction of volume is occupied by macromolecules (~20–30%) resulting in molecular crowding (Ellis, 2001; Minton, 2006). Such environments can lead to non-ideal behaviour of molecules, affecting reaction rates and equilibria of molecular interactions as well as imparting non-specific steric repulsion due to the excluded volume (Ellis, 2001; Ellis and Minton, 2003; Fulton, 1982; Minton, 1981, 1998). For example, bacterial cytoplasm and blood plasma have macromolecular concentrations of about 350 and 80 mg/ml, respectively (Chebotareva et al., 2004; Zimmerman and Trach, 1991). The non-ideal behaviour of molecules is very significant in such crowded environments and even simple processes such as

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transport by diffusion are significantly slowed down resulting in different timescales in crowded and non-crowded environments. Tracer proteins in concentrated polymer and protein solutions are determined to be sub-diffusive, reflecting the anomalous diffusion behaviour of cellular constituents *in vivo* (Banks and Fradin, 2005). Even the transport of small ions is significantly slowed down in crowded or gel environments, which results in a complete change of the physicochemical conditions for a mineralization event. For instance, the diffusion coefficient of small monovalent ions is reduced in the extracellular environments (Nicholson and Phillips, 1981). This natural crowded environment for mineralization is, however, only very badly reflected by most of the mineralization experiments in presence of additives, which we use to study additive controlled mineralization and which apply diluted conditions, where even small additive amounts already can have huge effects on the outcome of a mineralization reaction (Meldrum and Cölfen, 2008; Song and Cölfen, 2011).

Molecular crowding is highly relevant towards a realistic depiction of biomineralization. In certain mineralization processes, the prevailing biomolecular concentrations are significant especially at the organic-inorganic interface during the early stages of growth. In bone tissue, the organic content (primarily composed of proteins and polysaccharides) significantly varies between 3 and 28% depending on the developmental stage and specimen condition (Birchall and Thomas, 1983; Stack, 1955; Wise et al., 2007). Due to their negative charge and large hydrodynamic size, bone-associated proteoglycans are suggested to modulate the organization of the bone matrix (Mbuyi-Muamba et al., 1988). The role of collagen-associated acidic glycosaminoglycans abundant in bone tissue also cannot be underestimated (Wise et al., 2007). In such composite biominerals, the self-assembly of macromolecules is crucial and molecular crowding is highly essential for molecular organization and fibril formation (Lareu et al., 2007; Saeidi et al., 2012). Composed primarily of calcite, avian eggs shells can contain about 3.5% organics (Arias and Fernández, 2008; Nys et al., 2004, 1999; Soledad Fernandez et al., 2001). Varying protein and proteoglycan contents in the uterine fluid and egg-membrane are suggested to modulate egg mineralization (Gautron et al., 1997; Rao et al., 2015). Shells of certain mollusks can contain organic contents of about 20% (Krampitz et al., 1983). In the nascent stages of molar enamel maturation, the total protein content is as high as 12% (wet weight) (Termine et al., 1980). During initial stages of chiton teeth development i.e. the first 5–12 pairs at the posterior radula end, the teeth are colorless, indicative of a primarily organic composition. During the course of development, the teeth are gradually mineralized as shown by a color transition to reddish brown and black (Kirschvink and Lowenstam, 1979; Weaver et al., 2010). This is possibly one of the few biominerals, wherein the processes of organic scaffolding and mineralization are spatially well-distinct. Considering the high organic contents in these biominerals, extrapolation of *in vitro* studies on crystallization that generally utilize much lower concentrations can be potentially misleading. Moreover, it has been shown that a variation in the concentration of macromolecules such as lysozyme and PSS-*co*-PNIPAAM (a synthetic polymer) can alone induce polymorph selection (Wang et al., 2009; Xu et al., 2008).

In Nature, biomineral growth is achieved by the spatio-temporal regulation of processes such as ion/ion-cluster transport, nucleation, stabilization of intermediate amorphous mineral precursors and subsequent phase transformation. On the account of high macromolecular concentrations in biological environments, excluded volume effects can result in a deviation of apparent diffusion constants, reaction rates and equilibria depending on the shape, size and chemistry of the molecules (Banks and Fradin, 2005; Zimmerman and Minton, 1993). Therefore the experimental as well as in silico realization of biogenic mineralization processes may deviate from the actual scenario. Macromolecular crowding or volume exclusion can affect the chemical potential (μ) of solute species. (Ellis, 2001) In absence of solute-solute interactions and in dilute solutions, the ideal contribution is significant, given by

$$\mu^{ideal} = \mu^0 + RT\ln(c_I) \quad (1)$$

where μ^0 is the standard chemical potential and c_I is the molar concentration of the species *I*. However in a crowded environment, the chemical potential can exceed actual concentrations due to volume exclusion and is determined by a_I (thermodynamic activity i.e. effective concentration) and $a_I = \gamma_I c_I$ with the activity coefficient (γ_I) of the solute species.

$$\mu = \mu^0 + RT\ln(a_I) \quad (2)$$

Thus in ideal solutions ($\gamma_I = 1$), the chemical potential of the solutes is determined by concentration. However in biological environments, the volume excluded by macromolecules can result in solutes exhibiting chemical potential exceeding their concentrations. Molecular crowding has two important consequences considering biomolecules involved in mineralization. Firstly, molecular crowding affects reaction kinetics. For instance, enzymes such as T4 DNA ligases and polynucleotide kinase are activated by crowded conditions such as the presence of PEG (Zimmerman and Pheiffer, 1983; Zimmerman and Minton, 1993). Considering biomineral associated enzymes such as carbonic anhydrase and proteases, such interactions are quite intriguing because of the significant density changes occurring during mineral phase transformations and macromolecular self-assembly. For example, the activity of a hypothetical carbonic anhydrase can either depend on the association of the enzyme and [HCO_3^-] ions or the formation rate of an intermediate transition complex [enzyme·HCO_3^-]. In these respective cases, molecular crowding can lead to diffusion limitation or increase in thermodynamic activities and thus yield net reaction rates determined by these opposing effects. Concomitant mineral phase transitions to energetically downhill less hydrated, denser inorganic phases in presence of significant volume occupancy by macromolecules make enzymatic reactions more complex. Addressing biomolecular reactions in dynamic, crowded media is not only crucial for a comprehensive physiological understanding of nucleation and crystallization but also for technological applications (such as the role of collagenase in leather and gelatin production). For instance, matrix metalloproteases are suggested to guide the formation of biominerals such as the sea urchin skeletal elements (Ingersoll et al., 2003; Mann et al., 2008) and nacre (Yan et al., 2014). Investigating biomineral-associated proteolytic enzymes under non-ideal conditions will assist elucidating their regulatory functions as well as may help to identify potential denaturation-resistant enzymes for commercial purposes.

The second consequence of molecular crowding is the promotion of macromolecular assembly. Molecular crowding effects are augmented with an increase in macromolecular molar mass (Ellis, 2001). Thus the fate of biominerals is determined not only by the species affected by the excluded volume effects but also by the properties of the macromolecules inducing these effects. This is exemplified in case of larger extracellular matrix proteins such as collagen wherein self-assembly drives a decrease in the excluded volume and limits the configurational entropy associated with the molecular structure (Zeiger et al., 2012). Concomitant densification processes in an extracellular matrix-associated maturing mineral phase would lead to a pronounced decrease in excluded volume, further decreasing the free energy of the system. By modulating the available volume fraction, the activity coefficient of the dissolved proteins in equilibrium with aggregates can increase, thereby decreasing solubility and promoting

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