Chapter 3

Challenges and Perspectives of the Polymer-Induced Liquid-Precursor Process: The Pathway from Liquid-Condensed Mineral Precursors to Mesocrystalline Products

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Crystallization and phase separation is a key topic in physical chemistry which greatly impacts a plurality of scientific disciplines, be they of applied or fundamental nature. Our increasing insight into realistic and non-idealized systems is clearly becoming manifest in the rising number of publications challenging the simplicity of classical theories. These developments recently condensed into the concept of so-called nonclassical crystallization which embraces all crystallization pathways taking place outside of the framework of classical nucleation theories (Cölfen et al. 2008; De Yoreo et al. 2017, Chap. 1; Lutsko 2017, Chap. 2; Andreassen and Lewis 2017, Chap. 7; Yang and Ter Horst 2017, Chap. 16). Research has revealed three important phenomena, all of which were observed in crystallizations in real systems and have profoundly altered our perception of crystallization in general: the pathway of oriented attachment, the existence of prenucleation clusters and the occurrence of a liquid-condensed mineral precursor.

Following the pathway of oriented attachment (Penn et al. 2017) to its final destination, we can end up with so-called mesocrystals (Cölfen et al. 2008; Rao and Cölfen 2017). In this chapter, we stick to the recently proposed definition of a mesocrystal given by Bergström et al. (2015) which states that it is a “nanostructured material with a defined long-range order on the atomic scale, which can be inferred

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from the existence of an essentially sharp wide angle diffraction pattern (with sharp Bragg peaks) together with clear evidence that the material consists of individual nanoparticle building units".

The notion of prenucleation clusters challenged classical nucleation theory profoundly (Gebauer et al. 2008). These clusters, formed from constituents of the mineral to be precipitated, are considered to be thermodynamically relatively stable as they exist even in non-saturated conditions. These highly dynamic entities exhibit solute character and are not thought to be a new phase. It was recently proposed that the phase separation of these solutes is accompanied/triggered by a distinct decrease in their dynamics and leads to the formation of a condensed liquid phase comprised of these dispersed nanoconstituents (Wallace et al. 2013; Gebauer et al. 2014); this behaviour may then be used to discriminate between solutes (i.e. prenucleation entities) and separate phases (i.e. post-nucleation entities). But as of yet, their impact on crystallization and phase separation remains to be understood.

The scope of this chapter is the last but not least of the aforementioned three phenomena, the formation of liquid-condensed phases (LCP) in supersaturated solutions of small, sparingly soluble, inorganic and mineral-forming compounds such as calcium carbonate. Laurie Gower and co-workers were the first to propose the occurrence of liquid-like amorphous intermediates during the precipitation of calcium carbonate in a set of seminal contributions from 1997 to 2000 (Gower 1997; Gower and Odom 2000). They reported formation of mineral tablets and other non-equilibrium-shaped yet crystalline bodies of calcium carbonate and explained their genesis by the precipitation and coalescence of highly hydrated and hence liquid-like colloids of a transient amorphous mineral precursor phase. The formation of this exceptional phase was assumed to be induced by the presence of tiny amounts of a small polyanionic polymer (e.g. polyaspartate) and, hence, was dubbed the polymer-induced liquid-precursor (PILP) process.

Due to the liquid-like state of the transient amorphous phase, a multitude of mineral morphologies can be generated, illustrating the outstanding potential of the PILP process for the morphosynthesis of non-facetted, non-equilibrium morphologies. If the transient droplets are kept dispersed while ripening, solid spheres with monomodal size distribution, hollow and hedgehog-like structures of crystalline calcium carbonate can be obtained (Faatz et al. 2004; Loges et al. 2006). Sedimentation and subsequent coalescence leads to the formation of films and tablets (Gower and Tirrell 1998; Gower and Odom 2000; Amos et al. 2007; Cantaert et al. 2012; Jiang et al. 2013). Employment of patterned self-assembled monolayers varying in hydrophilicity enables effective templating of the mineral film (Kim et al. 2007). If the PILP film is generated beneath Langmuir monolayers, single-crystalline aragonite patches of films can be produced which are similar in morphology (a half micron in thickness and several microns in width) to molluscan nacreous tablets (Amos et al. 2007) and which in fact were successfully used for producing multilayered and nacre-like structures by multiple Langmuir–Schäfer transfers (Volkmer et al. 2005). The liquid-like colloidal intermediates can be accumulated and molded into an array of non-equilibrium morphologies by templating approaches. This has been exemplified by the retrosynthesis of nacre

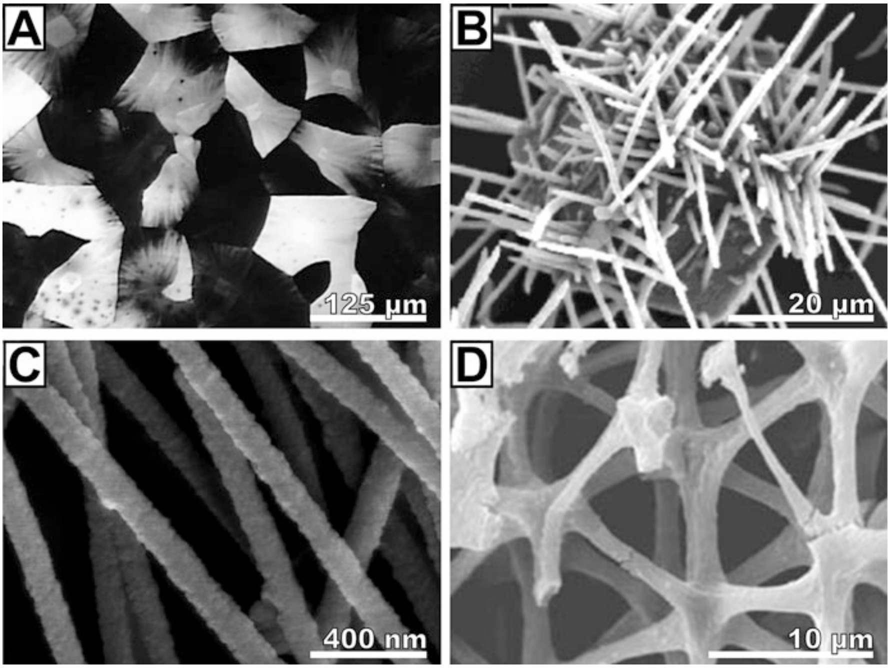

Fig. 3.1 Morphologies generated by means of the polymer-induced liquid-precursor (PILP) process: (**a**) a mosaic mesocrystalline film of calcium carbonate, (**b**) microfibrous calcite, (**c**) calcium carbonate fibres obtained by capillary infiltration into track-etch membranes and (**d**) bicontinuous calcium carbonate structure obtained by infiltration of a PHEMA hydrogel replica of a sea urchin spine. Subfigures are reprinted with permission from (**a**) Gower & Odom (Gower and Odom 2000), copyright 2000 Elsevier; (**b**) Olszta et al. (2004), copyright 2004 American Chemical Society; (**d**) Kim et al. (2011), copyright Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2011; (**e**) Cheng and Gower (2006), copyright 2006 American Chemical Society

or sea urchin spines (Volkmer et al. 2005; Gehrke et al. 2005), by infiltration of PILP phase in small volumes (Olszta et al. 2003a), coating of scaffolds (Sommerdijk et al. 2007) or exploitation of capillarity effects to produce, for example, nanowires (see Fig. 3.1) (Gower 2008; Kim et al. 2011). Fibrous mineral structures can be readily synthesized not only by infiltration but also in a self-organized fashion (see Fig. 3.1). This process was originally proposed to follow a solution–precursor–solid (SPS) mechanism starting from a seed crystal analogous to the established vapour–liquid–solid (VLS) and solution–liquid–solid (SLS) mechanisms (Olszta et al. 2004; Homeijer et al. 2010). Interestingly, Gower's group observed via time-lapse video that fibrous calcite can also be formed in the absence of seed crystals. In these experiments, amorphous fibres seem to be "extruded" from cracks in a gelatinous globule of precursor (Olszta et al. 2003b). Several hypotheses have been proposed to explain the formation of these fibrous morphologies, such as oriented attachment (although the fibres were stated as being formed from amorphous nanoparticles) or polymer coating of the sides of the fibre to allow for attachment of particles solely at the growth tip (Cantaert et al. 2013). The finding of Gower et al. that seed crystals are not required showed that fibres can also form by an apparently different mechanism. Therefore, the hypothesis was modified to include a mechanism by which an autocatalytic assembly of nanodroplets or nanoparticles at high energy surface protrusions could provide the "seed" (site-specific growth center) necessary for generating one-dimensional growth of fibers (Homeijer et al. 2010). The pronounced nanogranular texture of the generated fibres seems to indicate that the precursor particles are not always purely liquid-like, even though they coalesce fairly well to form single-crystalline fibres (if a singular nucleation event drives the crystallization across the accumulated amorphous phase). With regard to the variable stability of the precursor colloids, it seems clear that multiple formation mechanisms exist which all allow for initiating or collecting the colloids at a site-specific growth centre.

The PILP process was recently applied to calcium phosphates in order to establish a new route to biomimetic bone graft substitutes. In these studies, collagenous tissues were mineralized with calcium phosphate under PILP-generating conditions. The obtained fibres mineralized with hydroxyapatite show remarkable similarities to native bone as they feature intrafibrillar and uniaxially oriented crystals which are oriented roughly parallel to the fibril axis, generating diffraction patterns similar to those of native bone (Olszta et al. 2007; Jee et al. 2010a); such diffraction patterns cannot be generated by standard in vitro mineralization approaches. Olszta et al. proposed that the liquid mineral phase is drawn into the gap regions of collagen fibrils by capillary effects. A cryogenic transmission electron microscopy (cryoTEM) study by Nudelman et al. corroborated the existence of an amorphous precursor which selectively binds to and enters the fibres and proposed further that a net negative surface charge of the transient intermediate of the mineral may be crucial, as it may allow for the infiltration of the precursor complexes and their interaction with positively charged regions within the collagen fibril (Nudelman et al. 2010). In addition to these remarkable achievements, the PILP system has also been successfully employed to reconstitute partially demineralized human dentin resulting in a partial recovery of its mechanical properties (Burwell et al. 2012).

All of these studies exemplify the key feature of the PILP process: due to the presence of the polymer, the process of crystallization undergoes "conversion from a solution crystallization process to a solidification process" (Gower and Odom 2000). This is in sharp contrast to classical crystallization in which crystal morphology is dominated by crystal facets that result from the slowest growing crystal faces with lowest surface energy; crystallinity emerges concomitantly to the initial liquid/solid phase separation. In the PILP process, crystallization appears to proceed as a gel or solid/solid transformation, which is *after* phase separation and thus downstream to the formation of the transient liquid-amorphous intermediate and its solidification. This union of a solidification process with a final pseudomorphic transformation to crystallinity paves the way for the multitude of morphologies presented above.

Because the formation of the transient liquid-condensed mineral precursor phase is in contradiction with classical theories of nucleation and growth, its physicochemical basis was enigmatic from the beginning and still remains unclear.

Initially, it was merely attributed to the action of the polyanionic polymer additive and the assumption that the polymer induces and stabilizes the liquid precursor by sequestering ions and water and thereby triggering the unusual liquid/liquid phase separation. However, the formation of a liquid-amorphous calcium carbonate phase in the absence of additives – thus a liquid/liquid phase separation of a pure solution of a mineral (i.e. water/calcium carbonate) – was first proposed by Faatz et al. (2004). They speculatively explained the formation of spherical amorphous calcium carbonate (ACC) with monomodal particle size distribution by a binodal liquid/liquid phase separation leading to the formation of a transient liquid phase.[1] However, they based their speculation only on the temperature-dependent monodispersity of the ACC spheres and did not provide any proof for their phase diagram, let alone for the claimed liquid-like state of the early mineralization stages. A few years later, Rieger et al. provided in a cryoTEM study first indications for liquid/liquid phase separation (Rieger et al. 2007): 100 milliseconds after turbulent mixing of the two educts sodium carbonate and calcium chloride at $\mathrm{pH} = 11$, they observed the formation of a hydrated liquid-like phase resembling the initial bicontinuous state of a spinodal phase. This experiment has been interpreted several times as an indication of spinodal decomposition which is reasonable for this particular experiment given the rapidity of ascending supersaturation. However, we believe caution should be exercised in interpreting these findings and extrapolating them to all liquid phase precursors. First, the educts were mixed under turbulent conditions which can lead easily to vortex formation and thus to an initial inhomogeneous mixing of educts. The droplet-like structures are then an artificial result of the solution's inhomogeneous supersaturation, the precipitation reaction starts heterogeneously at the interfaces of the intermingled educt solutions (Haberkorn et al. 2003; see also the Faraday Discussion transcripts 2007; Rieger et al. 2007).[2] Second, the cryogenic sampling was conducted with a guillotine-like device which shoots the TEM grid through a free jet of mother solution into the cryogen. The intermittent sheer stress during such a sampling can lead to coalescence of droplets and might feign by this a bicontinuous thus spinodal state. In the face of these problems, Wolf et al. devised a contact-free crystallization setup based on acoustical levitation, by which the formation of calcium carbonate could be studied under purely diffusion-controlled conditions. This approach provides reliable homogenous crystallization conditions so that artefacts arising from foreign interfaces (except the air/liquid interface) can be ruled out, be they due to reaction vessels or due to turbulent mixing. These experiments further substantiated the occurrence of a liquid-like mineral phase at the onset of the homogenous formation of calcium carbonate in

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[1] Although often misinterpreted, the contribution of Faatz et al. does not claim spinodal decomposition. According to them, spinodal decomposition would lead to "ill-defined morphologies" whereas binodal decomposition to the observed monodisperse spheres. In subsequent contributions, they state their speculation about the binodal nature of this phase separation process much more clearly (Faatz 2005; Faatz et al. 2005).

[2] Rieger pointed out that one should not interpret his findings as a claim for a spinodal demixing (see Faraday Discussion transcripts 2007).

the absence of any additives (Wolf et al. 2008). The initial liquid-condensed phase (LCP) consisted of highly hydrated amorphous calcium carbonate droplets which were emulsion-like in appearance (Wolf et al. 2008; Wolf et al. 2011b). However, a direct evidence for their liquid state in solution was still pending. Later, Bewernitz et al. further corroborated the liquid state of this transient mineral phase by ^{13}C T_2 relaxation and ^{13}C PFG-STE diffusion NMR measurements (Bewernitz et al. 2012). However, there is still a missing link connecting the pure LCP mineral precursor and the PILP process; only the latter has thus far been shown to be suitable for morphosynthesis.

So far it is only clear that the polymer is not literally *inducing* the liquid-amorphous mineral phase, as it was evidenced that the LCP can also form in the absence of polymer. However, far more of this phase is produced in the presence of polymer (>16× the amount of bound calcium, see Bewernitz et al. 2012), so the polymer therefore enhances the stability of the LCP increasing the quantity formed. Without polymer, the concentration of the LCP was too low to be detected by conventional light scattering. In principle, this effect can be basically understood if one considers the generic effect of inhibiting additives: the additive raises the barrier(s) for nucleation and crystal growth, and thus the nucleation rate of a crystalline phase drops. Amorphous phases are usually thermodynamically unstable but have a lower surface energy due to their structural disorder or the presence of hydration waters. Because the nucleation rate is a sensitive function of the surface energy, amorphous phases thus typically have a higher rate of nucleation than the crystalline phases, especially in the presence of an inhibiting polymer. In later stages of ripening, amorphous phases have to compete against crystalline phases and dissolve in favour of the crystalline phases according to the Ostwald–Lussac rule of stages, feeding the growth of the crystalline phase due to the lower solubility product of the amorphous phases. The presence of inhibiting polymer abrogates or at least diminishes this "life-threatening" competition between the amorphous and crystalline phases and abets the formation of amorphous phases and prolongs their lifetime. These fundamental considerations already show that it is the polymer which takes the essential kinetic role in transforming an amorphous phase from a mere short-lived or unstable species to the principal agent of the precipitation. However, this is not a sufficient explanation for the emergence of a liquid-condensed phase instead of a solid amorphous phase and for the phenomenon of the PILP process and its capability to produce crystalline bodies with non-equilibrium morphologies. Throughout this chapter, we will provide several less expected actions of the polymeric additive. All of these actions are crucial for inducing a PILP process and will underline the fact that the polymer acts in the PILP system not only as an additive affecting the structure and morphology of the forming material, but it does so as a major process-directing agent (Olszta et al. 2007).

In further consideration of this perplexing system, we know various additives like magnesium, phosphate, silicate or citric acid were shown to do a similar job in favouring the formation of an amorphous phase (Aizenberg et al. 1996; Loste et al. 2003; Addadi et al. 2003; Gower 2008; Kellermeier et al. 2010; Tobler et al. 2015;

Kababya et al. 2015); but none of them was suitable for generating non-equilibrium morphologies like those that can be achieved with a PILP system – so what is the special and pivotal twist the polymer gives to the PILP process, turning it into a versatile route for morphosynthesis? Recent contributions of Wolf et al. gave a first insight into this and provided the missing link between the emergence of a pure liquid-condensed mineral phase and the PILP process. They give clear evidence for classical emulsion-like behaviour of the liquid-condensed phase at early stages of mineralization (see Fig. 3.2) (Wolf et al. 2011a, b, 2012). Therefore, we have to realize that the stage of mineralization which is critical for morphosynthesis and which follows directly the initial liquid/liquid phase separation is clearly governed by laws of colloid science (as is true for any other colloidal and dispersed system) and that all morphogenetic phenomena in the PILP process can only be thoroughly understood if the colloidal character of the key players, the emulsified droplets and their interaction with the different entities in the reaction volume – such as surfaces, crystal, ions or polymers – are fully taken into account. The contributions of Wolf et al. revealed that the emulsified state is electrostatically stabilized by the negative surface charge of the mineral droplets and is strongly affected by additives, in full accordance with the DLVO theory. As predicted by this classic theory, the addition of "innocent" spectator ions like sodium and chloride, which are intrinsically present in all standard metathesis precipitations (e.g. in Faatz et al. 2004; Pontoni et al. 2004), leads to aggregation and slight coalescence of the emulsion (see Fig. 3.2) (Wolf et al. 2011b). Consequently, polymers, be they basic or acidic, can have a pronounced effect on the emulsified state of the transient LCP mineral precursor. As we know from colloid science, the actual impact of an additive on dispersions can be diverse and may vary with the molecular charge, size and concentration of the employed additive; for a short summary of these interactions, please see the excursus at the end of this chapter. In the case of the PILP process, it was evidenced that addition of small amounts of a polyanionic polymer can lead to a collapse of the emulsion, whereas a larger amount of polyelectrolytes can lead to stabilization of the emulsion (Wolf et al. 2011a, see also the excursus at the end of this chapter; Wolf et al. 2012). This shows clearly that the response of the system (stabilization vs. demulsification) is a complex function of additive concentration but also of surface charge of the dispersed colloid, the interparticle distance and, therefore, of the particle number density of the dispersed phase (which is markedly enhanced with polymer). Applied to the PILP system, we can infer that coalescence and sedimentation of the PILP phase droplets is thus not triggered until a sufficient and critical amount of droplets are formed. Thus, one may conceptualize the PILP process to a fundamental yet Janus-faced action of the polymeric additive: first, stabilization and accumulation of an ion-enriched liquid-condensed phase (LCP) by inhibiting classical nucleation in the early stages of mineralization and, second, destabilization of the subsequent emulsified state, allowing for its final coalescence at later stages of the reaction (Wolf et al. 2011a, b).

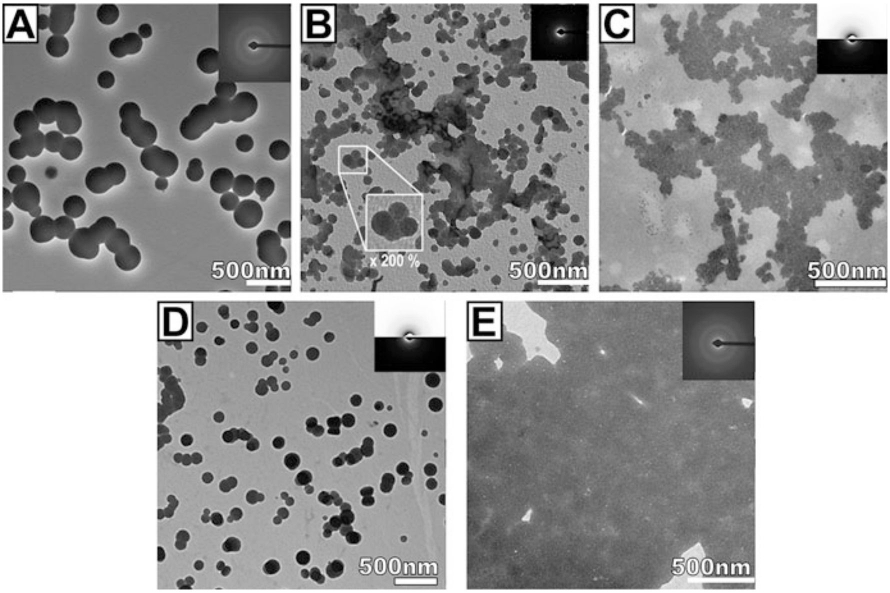


Fig. 3.2 Micrographs of liquid-condensed phases (LCPs) of calcium carbonate obtained under levitation; the insets show selected area electron diffractograms that indicate the amorphous state of the droplets. (**a**) LCP after 400 s of evaporation of a 4 μl droplet of saturated calcium bicarbonate solution. (**b**) LCP after 400 s, under addition of sodium chloride: aggregation and coalescence occurs due to screening of the particle surface charge. (**c**) LCP after 400 s obtained under influence of basic lysozyme (pI = 11.35): the droplets have coalesced and form a mineral film. (**d**) Under addition of the unusual acidic protein ovalbumin from the hen eggshell (pI = 4.6), the LCP is stabilized and still exists after 1000 s. (**e**) If only small amounts of an acidic protein is added (here 100 μg/mL of the aspartic-rich intracrystalline protein caspartin extracted the prismatic layer of *Pinna nobilis*), a well-developed mineral film is observed Subfigures (**a**) resp. (**c**) and (**d**) reprinted with permission from Wolf et al. (2008; resp. 2011a). Copyright 2008 resp. 2011 American Chemical Society. Subfigure (**b**) resp. (**e**) reproduced from Wolf et al. (2011b; resp. 2012) with permission from The Royal Society of Chemistry

3.1 Mechanistical Aspects

After this synopsis on our current knowledge of the PILP process, we will now dive deeper into various mechanistic aspects of the PILP process. First, we will start with a brief reprise of pertinent models of phase separation processes. Then, we will discuss each stage of the liquid/liquid phase separation process separately: pre-critical, critical – i.e. the phase separation itself – and the post-critical pseudomorphic phase transformation process. In each of these stages, we will discuss important aspects of how the polymer impacts on the crystallization process and how it abets the formation of a liquid-condensed mineral precursor phase. We will put a special focus on questions yet to be answered. Please note the distinction between liquid-condensed phase in the absence of additives (LCP) and the polymer-induced liquid-precursor (PILP) phase, which is stabilized and only formed in the presence of polymer. In the majority of studies which address or employ the PILP process, the mineral system of calcium carbonate served as the model system, mainly due to its high relevance in biomineralization systems and its apparent simplicity. Accordingly, we will focus in this review on the calcium carbonate system, although the PILP process has been expanded to non-calcium-based carbonates (Homeijer et al. 2008, 2010; Wolf et al. 2011b) and organic compounds like amino acids (Wohlrab et al. 2005; Jiang et al. 2011) and applied additionally to calcium phosphate (Jee et al. 2010b, 2011) and oxalate (Gower 2008), both of which are relevant to kidney stones (Amos et al. 2009).

3.2 Fundamental Concepts of Liquid/Liquid Phase Separations

Following the fundamental treatments of Gibbs (Gibbs 1877), for multicomponent systems to be stable against phase separation, they must resist two distinct scenarios: (α) they may not separate if a density fluctuation of high amplitude and finite size occurs; (β) they have to remain stable against small density fluctuations over a large volume. The first case (α) represents a typical metastable system whose curvature of the free energy of mixing is positive ($\partial^2 f/\partial c^2 > 0$). In the phase diagram, this region is delimited towards the stable region by the coexistence line, the so-called binodal (see Fig. 3.3a). By crossing this line, the system becomes metastable against spontaneous local density fluctuations, and separation *may* take place if these fluctuations are of sufficiently high amplitude. This case is subject to the classical nucleation theory (CNT) which describes the genesis of a new phase by the stochastic formation of nascent clusters (i.e. density fluctuations) which redissolve below a critical particle radius (Vekilov 2010). Once the cluster's surface energy is outbalanced by its bulk energy (i.e. the fluctuation reaches a sufficient amplitude), nucleation occurs, where the metastable and so-called critical nucleation cluster becomes stabilized by further growth that now lowers its free energy, and thus represents a new phase which – if the cluster does not redissolve – will grow in an ion-by-ion-mediated fashion as long as the supersaturation prevails, i.e. the system again becomes stable by reaching the binodal. The zone of metastability, which is governed by this first scenario, is separated from the zone of instability by the spinodal ($\partial^2 f/\partial c^2 < 0$). Beyond this line, the system becomes unstable even to infinitesimally small density fluctuations. This represents the second scenario (β), the thermodynamic background of which was elucidated by the pertinent works of Cahn and Hilliard (1959). In this regime of instability, phase separation is solely controlled by diffusion as there is no thermodynamic barrier against phase separation. Due to the curvature of the free energy of mixing, uphill diffusion against the concentration gradient takes place, leading to an increase in the concentration of the new phase with time, rather than size. In these stages, the density fluctuations

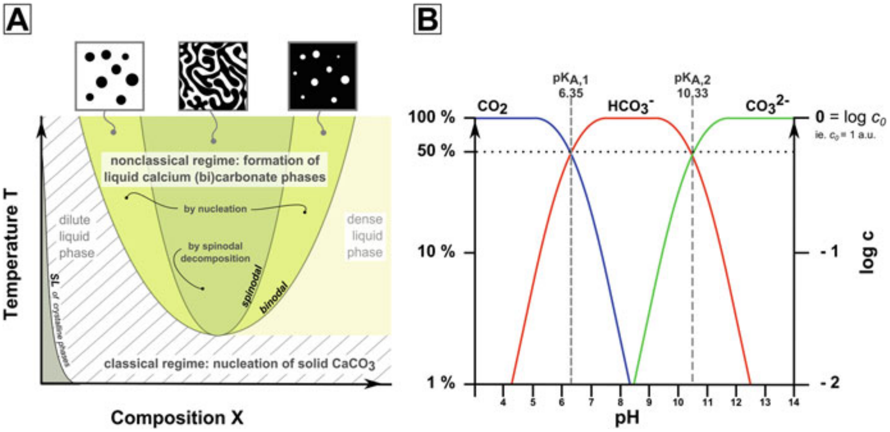


Fig. 3.3 (**a**) Hypothetical phase diagram of calcium (bi)carbonate solutions based on the works of Hilliard and Cahn. The *dashed region* represents the region of the phase diagram in which classical nucleation occurs. The *yellow* inset is the region in which a liquid-amorphous phase of calcium carbonate can form. If we assume a superposition of two phase diagrams of calcium carbonate and calcium bicarbonate, the SL line of the crystalline phases would represent the binodal line of the calcium carbonate system, and the *inset* would belong to the phase diagram of calcium bicarbonate. (**b**) Hägg diagram of a carbonate solution illustrating the ion speciation as a function of pH. Please note that in solution, virtually, no free carbonic acid exists but rapidly converts to solvated carbon dioxide. At $pH = pK_{A,2}$ 10.33, 50 % of the carbonate ions are protonated, i.e. HCO_3^-. At physiological relevant pH = 7, virtually no deprotonated carbonate is present: the solution contains 80 % bicarbonates, 20 % solvated carbon dioxide and only less than 0.4 ‰ deprotonated carbonates

gradually intensify and the solution's microstructure is distinctly fine and uniform. It may pass through a characteristic bicontinuous stage; this happens typically at a composition of 50:50 which is often where the spinodal curve is breached (see Fig. 3.3a). The system eventually ripens into a stage of spherical monomodal colloids and can resemble the morphology of colloids formed binodally.

3.3 The Pre-critical Stage in Real Systems

According to CNT, the formation of nuclei is a consequence of stochastic density fluctuations constrained to the metastable region. In the non-saturated regime, these clusters should be inherently unstable and thus dissolve and not be detectable. But in 2008, the existence of so-called prenucleation clusters (PNCs) was proclaimed by Gebauer et al. (2008). By means of analytical ultracentrifugation in combination with Ca^{2+}-activity measurements, they revealed the existence of calcium carbonate clusters of distinct size even in non-saturated solutions. PNCs are considered by some to be thermodynamically stable solutes; in the case of calcium carbonate, it was found that these PNCs are 7 k_BT more stable than the single, hydrated ions in solution. Furthermore, the stability of PNCs was shown to be dependent on the pH; their formation seems to be favoured at lower pH (Gebauer et al. 2008; Bewernitz et al. 2012). As of yet, detailed information regarding the structural composition at the molecular level of PNCs is not available. In order to fill this gap, computer simulations were undertaken in which PNCs were found to be hydrated and highly fluctuating oligomers of calcium carbonate ion pairs featuring (partially branched) chain- or ringlike motifs. The characteristic 1:1 ion ratio of this "dynamically ordered liquid-like oxyanion polymer" (DOLLOP) was corroborated by recent ESI-MS analysis (Wolf et al. 2011b; Demichelis et al. 2011). Other molecular dynamic simulations have also predicted the formation of a dense liquid phase through liquid/liquid separation within the concentration range in which clusters are observed (Wallace et al. 2013).

So far, the actual role and impact of PNCs on the mineralization process is still enigmatic and stirs disputes (see Faraday Discussion transcripts 2012). Do they provide a low-barrier pathway for nucleation? Or do they – due to their pronounced stability – pose an additional barrier for classical nucleation of a *crystalline* phase and allow, due to their structural disorder, the formation of a liquid-amorphous phase? Then the system's rate of nucleation is lowered so distinctly that the system may safely traverse the region of classical nucleation of the phase diagram and eventually enter the region of liquid/liquid phase separation (Wallace et al. 2013); or the prenucleation clusters start to segregate and consequently provide a low-energy barrier pathway for liquid/liquid phase separation (Wolf et al. 2011b; Gebauer et al. 2014). Their structural disorder and their high degree of hydration may then create lower surface energy than a crystalline nucleus, thereby favouring the formation of a liquid-amorphous phase. In the latter scenario, PNCs would represent the initial building blocks of the liquid-amorphous precursor phase. Initial validation for this assumption seems to be found in a recent report which showed that the distinct proto-crystalline features of ACC are related to different pH-dependent variants of PNCs (Gebauer et al. 2010). Further substantiation for this postulate is provided by the fact that in all existing systems which have been reported to exhibit liquid-condensed phases, prenucleation clusters have additionally been found: in a variety of bivalent carbonates (Wolf et al. 2011b), amino acids (Wohlrab et al. 2005; Jiang et al. 2011; Kellermeier et al. 2012) and calcium phosphate (Jee et al. 2010a). An extensive study of the early stages of calcium phosphate formation bringing together a set of different techniques suggested an ion-association process of prenucleation species, the Posner clusters, which assemble to polymeric entities of nanometre size. However a liquid state of the transient intermediate was not reported in this study (Habraken et al. 2013); the Posner cluster (aggregates) may be incapable of binding a sufficient amount of water to reach a liquid-like state. Yet in the presence of polymer, the precursor species are able to infiltrate the nanoscopic gap zones, while the conventional ACP phase does not; thus the polymer may provide additional hydration. In summary, while evidenced in a number of systems, further experimental proof is yet to be furnished demonstrating the potential role of PNCs as the structural unit of the liquid-amorphous phase.

3.4 The Critical Stage: Liquid/Liquid Phase Separation

It has often been rejected that the liquid/liquid phase separation in aqueous mineral systems is akin to that of polymer and protein systems (Petsev et al. 2003; see Faraday Discussion transcripts 2012). But now, the occurrence of a liquid/liquid phase separation in supersaturated solutions of minerals is an apparent and settled fact (Wolf et al. 2008; Bewernitz et al. 2012), albeit one in which the underlying mechanism is still enigmatic. The computational results suggesting that PNCs feature a polymer-like molecular structure underlines the fact that the calcium carbonate system may have more in common with polymer systems than initially assumed. Many thermodynamic features of polymers are dominated by entropic considerations, and the same is true of water in aqueous-based systems, so we believe close attention needs to be paid to the behaviour of ions in the presence of both.

Currently, we can only speculate under which conditions a binodal or spinodal decomposition may occur, and if a spinodal decomposition in mineral solutions is possible at all. We should keep in mind that, although often consulted, morphology of a ripe stage is rarely a good proxy for assessing a formation mechanism. A binodal process was put forward by Faatz et al. (2004), whereas the work of Rieger et al. (2007) was sporadically seen as a corroboration of a spinodal decomposition (see Faraday Discussion transcripts 2012; Gebauer et al. 2014). Also, the contribution of Wolf et al. (2008) was occasionally interpreted as evidence for binodal decomposition, but we clearly refrain from such speculation (as in Gebauer et al. 2014) as we did not probe the moment of phase separation, but a later stage. It is worth pointing out that the formation of monodispersed solid spheres were repeatedly interpreted as an indication of a spinodal *and* for a binodal mechanism (Pontoni et al. 2003; Faatz et al. 2004). However, such an observation points neither in the binodal nor in the spinodal direction; both pathways can yield monodispersed spheres. It is not even an indication for a liquid-like intermediate state because it can similarly be explained by the pertinent model of LaMer in full agreement with the notions of classical nucleation of a solid phase (LaMer and Dinegar 1950; Navrotsky 2004). Thus, without monitoring the initial stages of formation in real time, one should not employ morphological observations as an evidence of mechanism, only as an indicator.

Faatz et al. (2004) were the first to apply the generic model of Cahn and Hilliard (1959) to the calcium carbonate system. They proposed a schematic but nevertheless instructive phase diagram of the calcium carbonate system which was recently revisited by Wallace et al. (2013) in a slightly revised form (see Fig. 3.3). Beyond the solubility line(s) (SL) of solid calcium carbonate, formation of crystalline calcium carbonate may take place by the classical nucleation process, i.e. by binodal solid/liquid phase separation. The liquid/liquid coexistence line represents the border of the metastable region in which a liquid phase of calcium carbonate may form via a binodal route. Once the system crosses the coexistence line, we can expect that the rate of nucleation for a solid drops (Galkin and Vekilov 2000a).

Pushing the system even further beyond the liquid/liquid instability line, spinodal decomposition will take place. The schematic phase diagram, as given in Fig. 3.3, reflects the now established fact that a multitude of different pathways can occur in one system (please note that binodal solid/liquid demixing is still possible beyond the liquid/liquid coexistence line) and accounts for the formation of a liquid-condensed phase of calcium carbonate (Nielsen et al. 2014).

Another crucial question is still without reply. Do we know which kind of chemical species actually undergoes liquid/liquid phase separation? Commonly seen as a phase separation of the ionic constituents of calcium carbonate, we cannot rule out the option that only PNCs of calcium carbonate separate. If we review the pertinent literature, it seems even reasonable to predict that we are actually observing the liquid/liquid phase separation of a calcium *bi*carbonate(-biased) phase. Bicarbonate is virtually omnipresent in carbonate solutions – even at $pH = pK_{A,2} = 10.33$, half of the carbonates are protonated (in form of bicarbonate, HCO_3^-; see Fig. 3.3-b). At neutral pH, the solution contains virtually only bicarbonates and solvated carbon dioxide ($CO_{2\,(aq)}$); only a fraction (less than 0.4 ‰) are fully deprotonated carbonates (CO_3^{2-}). Thus, we have to realize that only minor amounts of free deprotonated carbonate are present under standard conditions; the majority of the carbonate ions are present in a protonated state. The idea that we observe the liquid/liquid phase separation of a calcium *bi*carbonate(-biased) phase finds corroboration in the fact that the formation/stability of a liquid-amorphous mineral phase is facilitated/enhanced at near neutral pH (Wolf et al. 2008, 2011b; Bewernitz et al. 2012). In situ NMR studies of Bewernitz et al. (2012) support this concept even further as the peak position indicated that the liquid-condensed phase is bicarbonate-biased. We could thus draw the conclusion that the complex behaviour of the calcium carbonate system is owed to an intrinsic competition between two different phase separation pathways of two closely related species in chemical equilibrium, i.e. the binodal solid/liquid phase separation of calcium carbonate and the liquid/liquid phase separation of calcium bicarbonate. Treating the different protonation states individually, we can see the phase diagram simply as a superposition of two independent phase diagrams, one of calcium carbonate overlaid with that of calcium bicarbonate. In other words, the nonclassical behaviour of the calcium carbonate system could then be traced back to the fundamental acid–base properties of the carbonate anion, i.e. its multiproticity paired with a relatively high $pK_{A,2}$. Consequently, liquid-amorphous intermediates are not only a feature of the extensively studied calcium carbonate system, but could be shown to be characteristic of a considerable set of different carbonate minerals (Wolf et al. 2011b).

Why should such a bicarbonate-biased system favour the formation of a liquid phase? First, the protonation can be seen as the simplest form of chemical protective groups (as used in organic synthesis), acting as protection against crystallization by raising the barrier of nucleation and crystal growth by introducing an additional step of deprotonation, along with dehydration. The ion-enriched phase would presumably be highly supersaturated if it contained mainly fully deprotonated carbonate species and thus tend to nucleate a crystalline phase. As shown by

Bewernitz et al. (2012), the liquid-condensed phase produced at pH 8.5 is enriched with bicarbonate ions. The presence of hydroxyl groups, i.e. the protonated $^{-}O_2C$–*OH* functional group in bicarbonate ions, would deliver an effective means for binding water via hydrogen bonding and thus facilitate the formation of a highly hydrated and thus liquid phase (Wolf et al. 2008). Therefore, the system of calcium carbonate at relatively low pH could be seen as a system which is – due to the high amount of protonated carbonate – frustrated with regard to crystallization and thus prone to the formation of highly hydrated and amorphous phases via liquid/liquid phase separation (Wolf et al. 2008).

The locus and shape of the liquid/liquid coexistence line would be an ideal parameter to characterize PILP systems, irrespective of the species which actually separate. It is well known from other systems that the presence of (even simple) additives can distinctly shift the locus and the shape of the coexistence line as well as the position or shape of the spinodal and the binodal (Galkin and Vekilov 2000b). In the case of the calcium carbonate system, it is reasonable to assume that the locus is strongly affected by pH, which may render the region of liquid/liquid phase separation more accessible at low, near neutral pH. Another important additive may be magnesium, which has been reported several times to facilitate the formation of a PILP phase (Kim et al. 2007; Cheng et al. 2007; Schenk et al. 2012; Cantaert et al. 2013). Polymeric additives may be able to shift the locus. Besides their pertinent inhibition effects on classical crystallizations which promote the formation of a (liquid) amorphous phase (see above), Bewernitz et al. (2012) showed recently that polyelectrolytes may change the pH profile of a precipitation process considerably. At early stages of a mineralization, this can diminish the pH slope which hampers "classical" nucleation of a crystalline phase by reducing supersaturation and, on the other hand, provides more bicarbonate ions for the formation of the bicarbonate-biased PILP phase. This behaviour is remarkable since the concentrations of polyelectrolytes in PILP reactions are typically "homeopathically" low (e.g. 20 μg ml^{-1}), yet can cause a pH shift of about 0.5 in late stages of precipitation (Bewernitz et al. 2012). However, the mechanism by which the additive so strongly affects pH evolution has not yet been revealed.

3.5 The Post-critical Stage, Part I: The Emulsified Stage of the Liquid-Condensed Phase

Once the liquid/liquid phase separation has taken place, the subsequent (additive-free) two-phased state behaves like a classical emulsion, as already described in the introductory synopsis. The droplets are electrostatically stabilized by a negative surface charge (Wolf et al. 2011a). Sheering of the emulsified liquid-condensed phase can lead to coalescence, and even the addition of the simplest additives may have a pronounced effect on the mineral emulsion (Wolf et al. 2011b). Sodium chloride leads to aggregation and slight coalescence of the emulsion (see Fig 3.2) due to screening of repulsive electrostatic forces by increasing the ionic strength of the solution (Wolf et al. 2011b). As outlined above, polymeric additives, be they basic or acidic, can also have a pronounced effect on emulsions (Wolf et al. 2011b). In the case of calcium carbonate, the actual impact depends on the molecular charge density and molecular weight, as well as initial concentration of the employed additive, and fits expectations based on the DLVO theory. Basic (bio)polymers lead to a demulsification by surface charge neutralization, whereas acidic polymers, if employed at sufficiently high concentrations, stabilize the emulsified state (see Fig 3.2) (Wolf et al. 2011a). But if the initial concentration of the acidic (bio)polymer is reduced considerably, the system enters the regime of depletion destabilization, and a prominent change from stabilization to destabilization occurs: the emulsified state of the PILP phase collapses and the droplets sediment and coalesce to a mineral film, the hallmark of the $CaCO_3$ PILP process (see Fig 3.2) (Wolf et al. 2012). To resume, *one* essential task of the polymer is that it allows for coalescence by inducing the collapse of the initial emulsified state of the liquid-condensed precursor phase (e.g. once a critical particle number density is reached). But this demulsification action is only one of the crucial steps in transforming a classical crystallization process into a pseudomorphic solidification process. Besides this colloido-chemical effect, the polymer additionally exerts numerous effects on the precipitation reaction which are essential for turning it into a PILP process. Initially, it allows for the accumulation of large amounts of LCP since it is able to sequester and to stabilize more ions, PNCs and/or LCP. By this, and by its preferential binding to active crystal growth sites, the polymer represses classical crystal nucleation and growth which leads, in turn, to the important effect that already formed amorphous material is not redissolved, as the Ostwald–Lussac rule of stages would suggest. The reverse case is impressively visualized by a recent liquid cell TEM study in which the formation of, what appeared to be, a liquid-condensed phase and aragonite was observed side by side. While the aragonite crystal grows, the liquid-condensed phase seems to be “slurped up”, thus simply serving as an ion supply as the abutting crystal grows into its characteristic twinned crystal habit (Nielsen et al. 2014). This system did not contain polymer, so even though a liquid-condensed phase may have been present, the classic PILP pseudomorphic transformation did not occur, consistent with many other studies that cannot achieve this without polymer. Ultimately, the second foundation of the PILP process thus relies on the polymer’s influence on the stability and transformation kinetics of the various mineral phases.

To this point, a considerable variety of acidic (bio)polymers have been shown to be capable of inducing PILP formation: polyaspartate (Gower and Tirrell 1998; Gower and Odom 2000; Jee et al. 2010a), polyacrylic acid (Balz et al. 2005; Harris et al. 2015), various block copolymers (Kim et al. 2009; Zhu et al. 2009), intracrystalline proteins from biominerals like ovalbumin from the hen eggshell (Wolf et al. 2011a) or caspartin and calprismin from the prismatic layer of *Pinna nobilis* (Wolf et al. 2012) and osteopontin from bovine milk (Rodriguez et al. 2014). Even short synthetic peptides (Sugawara et al. 2006; Dai et al. 2008a), DNA

(Sommerdijk et al. 2007) or polysaccharides (Hardikar and Matijevic 2001; Zhong and Chu 2009) have been shown to be employable in the PILP process. Additionally, Wolf et al. (2011a) were the first to show that basic polymers are capable of inducing a PILP phase: they evidenced that the PILP process can be induced by the addition of the basic protein lysozyme, which proceeds presumably by interconnection of the mineral precursor droplets. Recently, Cantaert et al. (2012; 2013) and Schenk et al. (2012; 2014) extended this observation to cationic polymers such as poly(allylamine) for the morphosynthesis of calcium carbonate films and fibres. This finding is of particular importance since polycationic polymers, like lysozyme or poly(allylamine), are not able to sequester cations from solution, in contrast to polyanionic polymers like polyaspartate (Wolf et al. 2011a); nevertheless, they were shown to be suitable for morphosynthesis (Schenk et al. 2012; Cantaert et al. 2012, 2013; Schenk et al. 2014). This clearly suggests that the impact of the polymer on the precipitation is much more complex than simply affecting calcium or carbonate ions and rather involves interactions with (pre)nucleation clusters or the liquid-condensed phase.

In the following we try to give a short account of the different, beneficial effects of PILP-active polymers. A polymer suitable for PILP may show all of these traits, like polyaspartate, or may lack some of them, like poly(allylamine). It is still not fully understood which traits are indispensable for a polymer to qualify as an additive for the PILP process.

1. At first glance, one may expect that the polymer reduces the overall solution's supersaturation by sequestering calcium ions (depending on the polymer's molecular charge), a behaviour demonstrated for acidic polyelectrolytes, e.g. proteins (Pipich et al. 2008), at higher concentration. However, some results show that calcium activity is *not* affected by the presence of acidic polyelectrolytes such as PAA if concentrations are used which are characteristic for PILP processes (Gebauer et al. 2009).
2. It inhibits nucleation and growth of a crystalline phase of calcium carbonate (Kim et al. 2009; Gebauer et al. 2009; Wolf et al. 2011a). None or only minor amounts of a crystalline and thermodynamically more stable phase of calcium carbonate is formed if the conditions are ideally stabilized. As stated above, this has two far-reaching and crucial consequences. First, the amorphous phases need not to compete for ions during growth, and thus more LCP can form until supersaturation ceases. Second, no Ostwald step ripening occurs and thus saturation with respect to the amorphous phases prevails. No crystalline phase grows in solution at the expense of the amorphous phases. This considerably increases the lifetime of the thermodynamically unstable amorphous phases.
3. It affects the pH profile of the mineralization since it acts as a typical buffering agent. Depending on the polymer concentration, the asymptote of the pH development may be attuned to the polymer's pK_A value, which can increase the supply of bicarbonate, which in turn can abet the formation of an amorphous phase or shift the solution's composition in favour of a bicarbonate-biased liquid phase (Pipich et al. 2008; Bewernitz et al. 2012).

4. It may change the actual composition of the droplets: the water content is increased, calcium is sequestered hyper-stoichiometrically in the early stages, and binding of bicarbonate may be favoured over carbonate ions (Dai et al. 2008b; Bewernitz et al. 2012).
5. It apparently influences the interactions of the droplets/phase with functionalized surfaces (e.g. patterned SAMs or collagen fibrils).

3.6 The Post-critical Stage, Part II: Pseudomorphic Transformation Amorphous to (Meso)crystalline Material

How does, as the final step, the material transform into a crystalline material? Apparently, there are several possible ways. One possibility may be the formation of a crystal by classical nucleation within one of the still liquid precursor droplets. Such a process has already been reported for protein crystallization in which the supersaturation of protein inside the droplets was shown to be higher than in the initial solution (Vekilov 2012). As a matter of fact, this mode has actually not yet been documented for liquid-condensed phases of minerals. In the absence of additives and under sufficient saturation, the liquid-condensed phase can densify, dehydrate and solidify over the course of time forming (assumingly monohydrated) ACC, as is the case in the Faatz method (Faatz et al. 2004). The final transformation to crystalline material proceeds typically via redissolution/recrystallization, i.e. Ostwald–Lussac ripening, and may occur well before solidification of the droplets – as impressively imaged by Nielsen et al. (2014) – or when the droplets reached a dry state (Wolf et al. 2008; Ihli et al. 2014). The presence of solid ACC seems to abet this transformation in solution by a template action of the ACC spheres (Zhang and Liu 2007; Wolf et al. 2008; Freeman 2014) and can result in interesting morphologies like polycrystalline hedgehog spheres (Shen et al. 2006; Loges et al. 2006). Under essentially dry conditions, ACC is remarkably stable owing to the fact that the first transformation to crystalline material can preferably occur via a dissolution/reprecipitation pathway. It is probable that this proceeds in localized, small domains, be it on the ACC surface or in small intergranular pockets entrapped within ACC (Ihli et al. 2014).

In the presence of polymeric additives, the mechanism described above gives way to a pseudomorphic transformation, i.e. the transformation of the amorphous material to a crystalline state with preservation of its morphology. Thus a combination of these critical stages provides a morphosynthetic route to non-equilibrium-shaped crystals by a mechanism we refer to as colloid assembly and transformation (CAT).

If we pool various facts and observations concerning ripening and crystallization of amorphous calcium carbonate intermediates obtained both from calcareous biominerals and biomimetic processes, we can draft a relatively detailed working model describing the process of crystallization in PILP-based systems (see Fig. 3.4):

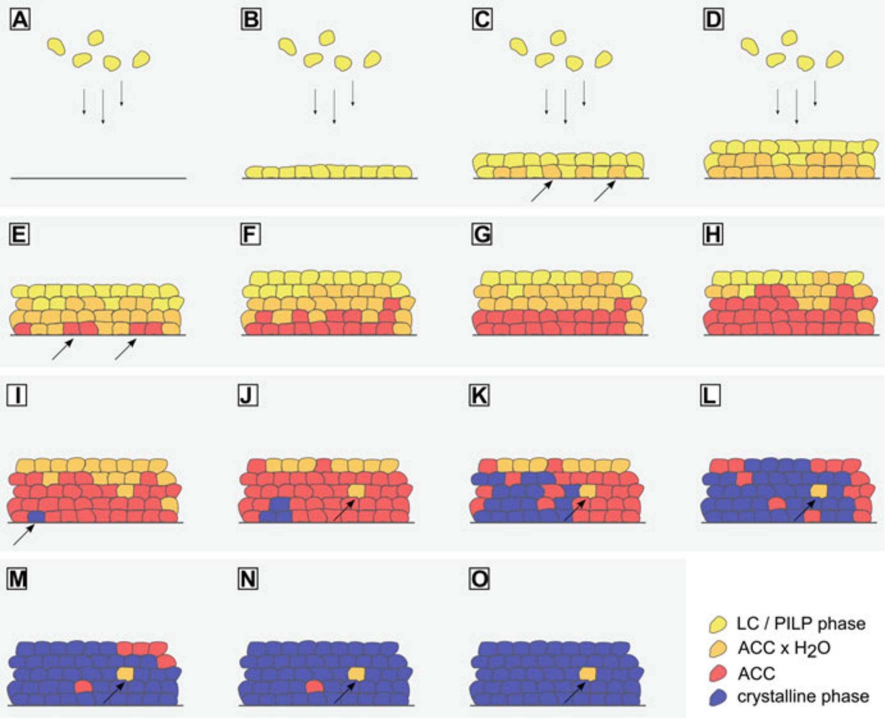


Fig. 3.4 Generic model of mineralization by colloid assembly and transformation (CAT), i.e. the formation of mesocrystals *via* a PILP intermediate; crucial details in each step are indicated by *diagonal arrows*. Stages **a**–**d**: Sedimentation of still liquid-like or viscous droplets. Stages **b**–**d**: The droplets accumulate in a space-filling fashion. Stage **c**: First steps of solidification, i.e. first dehydration from liquid-like ACC, respectively, PILP, to ACC × H_2O. Stages **e**–**h**: Second stage of dehydration, from ACC × H_2O to ACC. Stage **i**: Initial nucleation and solid–solid transformation to crystalline material. Stage **j**: Propagation of crystallinity throughout nearly the entire mineral volume via isoepitaxy across the interconnected and coalesced ACC particles. Stages **n**–**o**: Singular granules may remain amorphous throughout the process; their transformation may be impeded if the composition does not meet the requirements, e.g. it contains too much water, foreign ions like magnesium, polymeric impurities or improper stoichiometry

(a) The highly hydrated and thus still liquid-like or viscous ACC phase is formed. If the polymer inhibits classical crystal nucleation and growth in the neighbouring solution, the PILP phase is not redissolved for feeding the growth of a crystalline phase, as the Ostwald step rule would predict. At a critical point, e.g. when a critical droplet number density is reached, demulsification is induced by the polymeric additive via the mechanism of depletion destabilization; the initially stable emulsion collapses and the hydrated PILP phase starts to sediment (Fig 3.4, stages A to D) (Gower and Odom 2000; Wolf et al. 2011a).

(b) The droplets sediment on the substrate/mould. One of the key actions of the polymeric additive is to prolong the hydrated and thus liquid-like state of

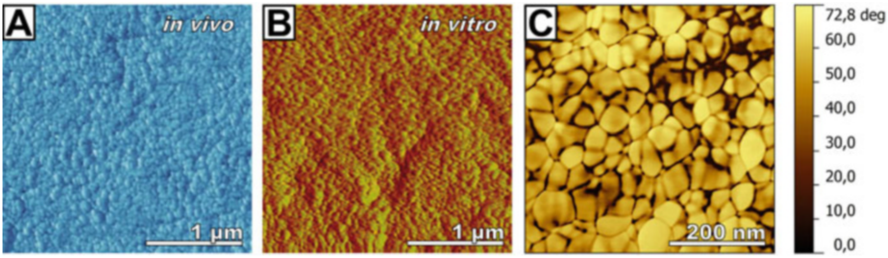


Fig. 3.5 Atomic force microscopy micrographs of the nanogranular texture from (**a**) a transverse and mirror-polished section through a calcite prism from the shell of *Pinna nobilis* and (**b**) a mineral film generated by the PILP process. (**c**) A typical phase image of the biogenic calcium carbonate from the calcite prisms extracted from the shell of *Pinna nobilis.* The phase contrast indicates organic material which ensheathes the individual nanogranules. Subfigure (**b**) is reprinted with permission from Kim et al. (2007), copyright 2007 American Chemical Society. Subfigure (**c**) reproduced from Wolf et al. (2012) with permission from The Royal Society of Chemistry, copyright 2012

the droplets, so that the droplets accumulate in a space-filling fashion (Yang et al. 2011); each droplet adopts its shape to its individual mould/surrounding. Although they appear to coalesce fully, some "memory" of their individual colloidal shapes is retained, which is sometimes described as a nanocolloidal or nanogranular texture when observed in the final product by AFM measurements (see Fig. 3.5, A to C) (Kim et al. 2007). However, as hydration waters are also exiting the PILP phase with time (Dai et al. 2008b), the coalescence ability is reduced as the droplets become more gel or solid-like particles. For this reason, morphologies such as film formation are mainly found in reaction vessels with short transport distances. The dehydration kinetics is likely influenced by the polymer characteristics (such as molecular weight and charge density) and seems to be markedly influenced by interaction of the colloids with substrates and templates. For instance, it was recently exemplified that one can tune the wettability of the PILP phase for a given organic surface functionalization just by adjusting the magnesium content of the LCP mineral precursor (Berg et al. 2013).

(c) The forming mineral body initially remains amorphous since its chemical state is not yet ready for crystallization, which may require exclusion of the polymer, dehydration, deprotonation of bicarbonate or balancing of ion stoichiometry (Fig 3.4, stages A to H) (Politi et al. 2004; Dai et al. 2008b; Wolf et al. 2012; Gong et al. 2012; Ihli et al. 2014). If the chemical state for crystallization is not reached, singular granules may stay amorphous even if they are surrounded by crystallinity (as indicated by an arrow in stage J) (Wolf et al. 2012; Gong et al. 2012). Note that it has been suggested that only anhydrous ACC readily transforms to calcite (Politi et al. 2008; Gong et al. 2012; Ihli et al. 2014); this seems counterintuitive since hydration of water could provide more mobility to the ions during assembly.

(d) From the time when the droplets have formed, they start to dehydrate and subsequently solidify and form anhydrous ACC (e.g. stages C and E, transition from yellow via orange to red). This dehydration occurs concomitantly with an exclusion of the polymer (Dai et al. 2008b). The process of syneresis can exert a strain on the dehydrating bulk phase which sometimes leads to marked lattice strain, or jointing and crack formation; similar processes lead to prism formation in basalt or starch (Gower and Odom 2000; Toramaru and Matsumoto 2004; Gilman 2009). For PILP systems, photoelasticity could be observed (Leupold and Wolf 2015), and regular cracking was reported in large-scale PILP films of amino acids (Jiang et al. 2013).

(e) Nucleation occurs somewhere in the dehydrating amorphous bulk mineral, e.g. by heterogeneous nucleation on the surrounding matrix (stage I, the first crystallizing granule is indicated by an arrow, transition from red to blue) (Wolf et al. 2012).

(f) From this point on, the crystallinity percolates throughout the bulk material, sometimes following a stochastic and tortuous path; one dehydrated granule after the other crystallizes by solid/solid transformation induced by isoepitaxial nucleation (stages I to O) (Killian et al. 2009; Seto et al. 2011; Wolf et al. 2012; Gal et al. 2014). This process, because of the initial colloidal assembly of colloidal droplets/particles, yields a mesocrystalline material: the crystalline nanogranules are all highly co-oriented since the crystal orientation originates from a singular nucleation event, with neighbouring granule transformations inheriting the same orientation by isoepitaxy (Wolf et al. 2012; Gong et al. 2012; Gal et al. 2013, 2014). In some cases, crystallinity may proceed across the amorphous body following more “traditional” trails, such as dislocation defects that lead to spiral growth hillocks as the crystallinity slowly propagates within the amorphous film. This is evidenced by transition bars with crystallographic symmetry indicative of a lattice directed organization of species; the transition bars can be seen midway in the amorphous-to-crystalline transformation, either as faceted rhombs resulting from the calcite symmetry or radial patterns emanating from spherulitic growth (Dai et al. 2008a).

A recent study which reported the formation of thin mosaic and mesocrystalline thin films of DL-lysine by a PILP process provided initial evidence that the above model is essentially correct and valid as well for non-carbonate-based organic systems (Jiang et al. 2013).

3.7 Relevance to Biomineralization Systems

The idea that the PILP process might be of high relevance in biomineralization has been championed by Gower since its initial discovery in 1998 (Gower and Tirrell 1998). Interestingly, the discovery of the PILP phenomenon coincided with a paradigmatic change in our understanding of biomineralization processes. On the one side, key contributions in the analysis of biominerals from the Weizman institute showed that amorphous calcium carbonate plays a ubiquitous role in biogenic crystallization (Aizenberg et al. 1996; Weiss et al. 2002; Levi-Kalisman et al. 2002; Addadi et al. 2003; Politi et al. 2004, 2006, 2008). On the other side, the in vitro studies of the PILP process clearly demonstrated the immense impact minor amounts of polyanionic additives can have in transforming the mineralization to a precursor process. Polyaspartate, which was employed as an additive in the first experiments evidencing the PILP process, is a clear analogue to intracrystalline proteins found in biominerals. The PILP process evidenced for the first time that these biopolymers, which are typically unusually acidic due to a high fraction of aspartic acid in the primary sequence or post-translational modifications, are able to shift gears towards a crystallization pathway which proceeds in a particle-mediated route via an amorphous intermediate, akin to the processes observed in calcareous biominerals. Moreover, the strikingly similar nanogranular appearance of PILP materials and calcareous biominerals renders Gower's assumption very appealing: both calcareous biominerals, like mollusc shells (Li et al. 2011; Wolf et al. 2012) or sea urchin spines (Killian et al. 2009; Seto et al. 2011), and PILP-generated materials are composed from individual nanogranules which are about 100 nm in diameter and (partially) enwrapped in an organic ensheathment (Jacob et al. 2011; Wolf et al. 2012); see Fig. 3.5. This nanogranular texture occurs in most types of calcareous biominerals, be it in calcareous sponge spicules (Sethmann 2005), in nacre (Mutvei 1978; Rousseau et al. 2005; Jacob et al. 2011; Wolf et al. 2015), in prismatic and lamellar layers of bivalves (Dauphin 2008; Li et al. 2011; Wolf et al. 2012, 2015), in brachiopod shells (Dauphin 2008) or even in urchin spines (Seto et al. 2011) and coccoliths (Henriksen et al. 2004). Nanogranularity is also observed in calcareous fossils or non-calcareous biominerals (for an exhaustive review on its occurence, see Wolf et al. 2016). Since it strongly affects numerous properties, be it crack propagation, nanoplasticity or self-healing, it represents a fundamental and ubiquitous process-structure-property relationship of biominerals (Wolf et al. 2016). The nanogranular unity of biominerals and PILP-generated materials may portend that both form via the same pathway involving at least some form of liquid-like colloidal amorphous intermediate (Wolf et al. 2012). But this is not the only aspect indicating a PILP-like process in vivo. Besides the distinct nanogranular texture, calcareous biominerals are typically space-filling mesocrystals formed via a colloid-mediated crystallization pathway (Yang et al. 2011; Seto et al. 2011; Wolf et al. 2012; Hovden et al. 2015). Furthermore, the unexpected finding of intracrystalline preservation of nanoscale amorphous regions can be easily explained with the described ripening model simply by keeping in mind that individual nanogranules may not always crystallize (see Fig. 3.4, stages N to O) (Seto et al. 2011; Wolf et al. 2012; Gong et al. 2012), particularly when they contain too much water or polymer and have the wrong stoichiometry or when they have only insufficient contact points with the surrounding mineral so that isoepitaxial nucleation is not possible. Since dehydration is inherent to a PILP process, effects associated with syneresis should be also traceable in biominerals. The formation of prismatic calcite in mollusc shells could be a syneresis phenomenon similar to columnar jointing in basalt or starch (Toramaru and Matsumoto 2004; Gilman 2009). However this speculation still awaits its verification. Growth processes in vivo occur by a layer-wise deposition mode and proceed at a very slow speed so that syneresis effects may only occur in thick- or fast-growing biominerals, if at all.

It is obvious that all calcifying species have to cope with the same physicochemical "quirks" of the calcium carbonate system. As we have seen, this mineral system is not the simple and well-behaved model system as was initially thought. One quirk of the calcium carbonate system is clearly the occurrence of liquid-amorphous phases both in the presence of acidic (bio)polymers or even without, especially at near neutral/physiological conditions. Nature seems to suppress nucleation virtually at all costs, mainly by means of unusually acidic proteins. It seems that this was the basic requirement that promoted biomineral evolution in the first place, and recent studies revealed that such unusual acidic "inhibitory" proteins like caspartin extracted from *Pinna nobilis* in fact abet the formation of an LC/PILP phase (Wolf et al. 2012). Why shouldn't calcifying species exploit then the exact quirks that they have to face for their biogenic production of calcium carbonate when they are ultimately rewarded with a protective shell and other evolutionary advantages? Given this, it seems reasonable that different phylogenetic species developed individual but nevertheless comparable sets of molecular and physicochemical tweaks to deal with the calcium carbonate system. With millions of years of evolution, today these tweaks are highly sophisticated, employing elegantly self-organizing entities like liquid crystal formation of collagen and chitin templates, or aggregation-prone intrinsically disordered proteins (Evans 2013). Notably though, these more sophisticated features may be more related to the organic matrices and scaffolds, while the actual mineralization process can seemingly be accomplished with less sophisticated proteins (that can be effectively emulated with simple polyelectrolytes). Of course there may be minor tweaks in the proteins involved in controlling crystal phase, orientation and texture; but the overriding morphological control may be simpler than was long perceived. This may be pertinent with respect to phylogenetic and evolutionary analysis of various biomineralizing organisms. From an engineering perspective, these concepts may provide a great deal of inspiration for material synthesis via the PILP process.

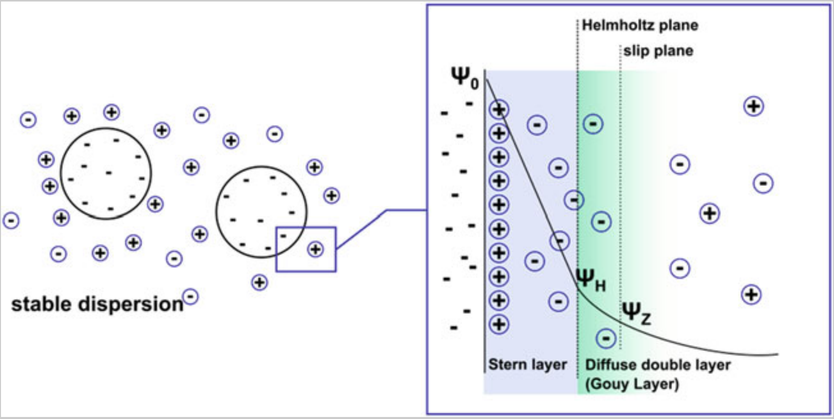
3.8 Conclusions and Outlook

Still being rather a curiosity than an accepted morphosynthetic approach, the full potential of the PILP mechanism for morphosynthesis is yet to be unlocked. However, the myriad of morphologies generated by the PILP process to date and the path-breaking contributions exemplifying the applicability of the PILP process for biomimetic bone graft substitutes clearly demonstrates the potential of the PILP process. Aside from this, currently, it is still essentially confined to relatively mundane and mainly carbonate-based minerals. Only for organic pigments was the PILP process successfully employed in the generation and property tuning of a functional material (Ma et al. 2009). This, and our limited mechanistic insight into all stages of this nonclassical crystallization process, severely narrows the applicability and transferability of the PILP process. However, its power for generating non-equilibrium shapes is already proven, and its potential role in biomineralization only affirms our need for more intensive research. The PILP process is one of the most prominent examples of nonclassical crystallization, although it may be not as nonclassical as first perceived: classical colloido-chemical scenarios intimately entangled with liquid/liquid phase separations come together in a kinetically steered mineral precipitation pathway. This said, the nonclassical PILP crystallization process may be a much more classical assembly pathway than currently assumed and thus be transferable to a wide range of compounds. This perspective is an appealing one because as of yet, a broadly applicable method which allows for the generation of nanoparticle superstructures and nanocomposite materials under morphological control does not exist. The relatively facile PILP process is currently one of the strongest candidates to fill this gap and thus clearly requires in-depth studies into its underlying mechanisms.

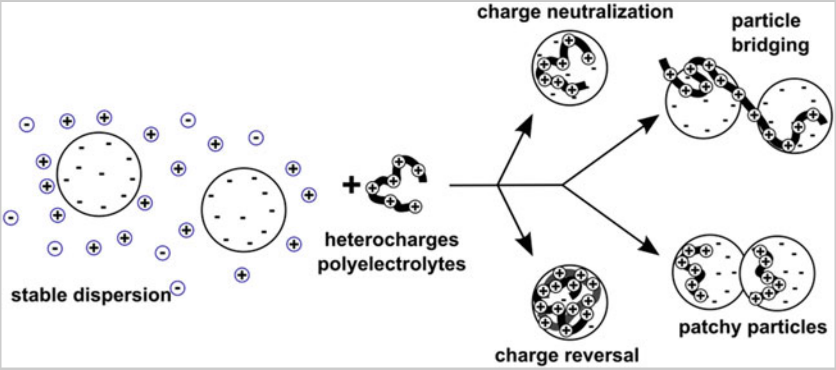
Excursus: Electrostatically Stabilized Dispersions and Their Interaction with Polyelectrolytes

Electrostatic Stabilization In the absence of (surface-modifying) additives, dispersions of colloids are only stable if the colloids carry a (near-) surface charge. As expounded in the models of Gouy–Chapman, Stern and Grahame (see Grahame (1947) and references therein), counterions form a diffuse ionic layer around the charged colloids from which the effective Coulombic repulsion arises. The interaction potential between the colloids results from a superposition of the attractive van der Waals' forces and the repulsive electrostatic interaction and features a potential barrier which may save the colloids from coagulation. The electrostatic interaction is strongly dependent on the electrolyte concentration in the continuous medium. The Debye screening length $1/k$ is a measure for the size of the diffusive double layer and represents the distance at which the electric potential drops to $1/e$ of its initial value. It drops with increasing ionic strength of the solution; thus, in relatively pure water with low amounts of a single charged electrolyte ($c_e = 10$ mmol l^{-1}), the diffusive double layer is roughly 500 nm thick; at higher values ($c_e = 0.1$ mol l^{-1}) the double layer shrinks to about 5 nm. At a critical concentration of c_e, the diffusive layer is so thin that the particles can approach so closely that van der Waals' forces dominate and coagulation can occur. Under orthokinetic conditions, i.e. the colloids perform not only Brownian movement but experience as well sheer forces due to stirring or shaking, the coagulation speed can increase by a factor of 10^4. Then, the hydrodynamic forces can easily push the particles across the potential barrier.

(continued)



Effects of Heterocharged Polymers The effect of a charged polymeric additive on a dispersion depends on its interaction with the colloids. If the polyelectrolyte is heterocharged with respect to the colloid, the polymer can adsorb and lead to bridging flocculation by interconnecting the colloids. In the case of highly charged polymers, charge neutralization is the most common mechanism for bridging flocculation because these polyelectrolytes tend to adsorb in a flat configuration. If the colloids have a rather low surface charge density, adsorption of highly charged polyelectrolytes can provide an overall charge neutralization but lead to a heterogeneous surface charge distribution, which enhances coagulation by the so-called electrostatic patch effect. At very high concentrations of surface-bounded polymer, re-stabilization of the dispersion may occur by surface charge reversal.



(continued)

Effects of Non-adsorbing Polymers Non-adsorbing polymers, e.g. homocharged polyelectrolytes, can cause so-called depletion effects which can occur in solutions in which the polymeric additive remains in the continuous medium and does not adsorb to the colloid's surface. The effect of depletion destabilization has been known since the early works of Traube in 1925 (Traube 1925); a theoretical explanation was first given by Asakura and Oosawa in 1958 (Asakura and Oosawa 1958). The driving force of depletion effects arises from the tendency of polymers to adopt the energetically most favoured conformation in solution, i.e. the minimization of the solution's free energy. If a free polymer experiences a confinement, e.g. by approaching closely an interface, a colloid or a second polymer, its conformational freedom is restrained which increases the free energy of the dispersion. Since this is unfavourable, each colloid is surrounded by a volume with a diameter δ from which non-adsorbing polymers are excluded, the so-called depletion layer. If the interparticle distance of two dispersed colloids becomes smaller than 2δ, – i.e. the depletion volumes overlap, e.g. due to an increase in the colloid's particle number density – the colloids force polymers to leave the interparticle space. The polymers diffuse out of the interparticle space and generate a polymer-excluded volume of pure solvent. This is energetically unfavourable: the solution's entropy is reduced by uphill diffusion, and the free energy of solvation arising from polymer-solvent interactions is diminished, especially if the liquid is a good solvent for the polymer. At low polymer concentration, polymer–polymer interactions are negligible, and the out-diffusion of the polymer and the concomitant gain in conformational freedom outbalance these costs. This results in a non-isotropic concentration gradient which creates a net osmotic pressure which pushes the colloids together and squeezes out the solvent: the dispersion experiences depletion destabilization and collapses. At high polymer concentrations, polymer–polymer interactions cannot be neglected anymore because the polymer's conformational freedom is already diminished by polymer–polymer interactions. The gain in conformational freedom of the polymer by out-diffusion cannot compensate for the cost of generating the polymer-excluded volume. Thus, the polymer stays in the interparticle space, enters the depletion volume and therefore exerts a counterforce on the colloidal particles which keeps them apart: the solution is stabilized by depletion stabilization.

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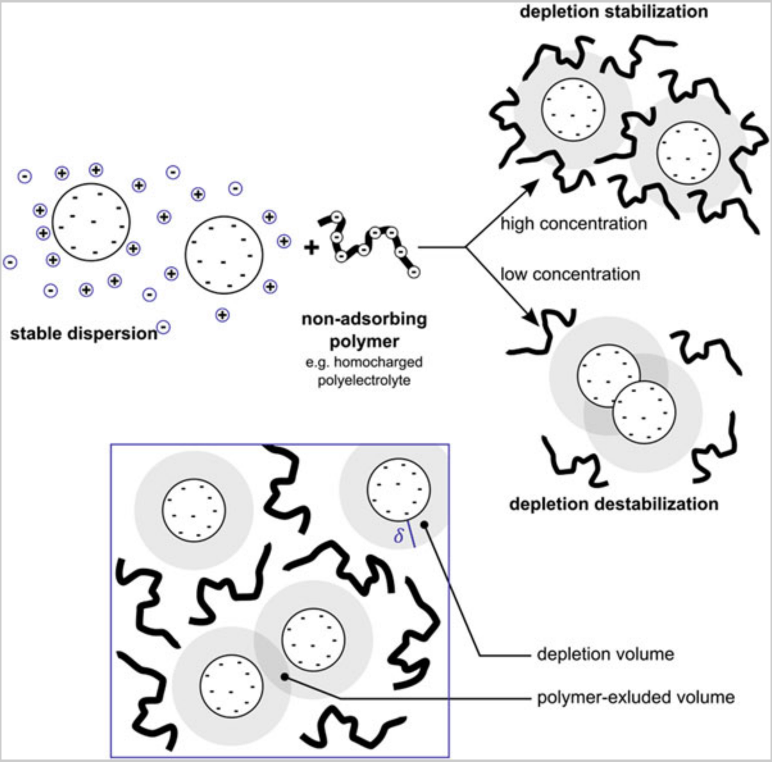

For further information, see Tadros (2013), Somasundaran (2006), Lagaly et al. (1997) or other pertinent monographies and reviews on colloido-chemistry and the DLVO theory.

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