Urol Res (2010) 38:281–292
DOI 10.1007/s00240-010-0288-z

SYMPOSIUM PAPER

Mineralogical signatures of stone formation mechanisms

Laurie B. Gower · Fairland F. Amos · Saeed R. Khan

Received: 23 June 2010 / Accepted: 24 June 2010 / Published online: 13 July 2010

Abstract The mechanisms involved in biomineralization are modulated through interactions with organic matrix. In the case of stone formation, the role of the organic macromolecules in the complex urinary environment is not clear, but the presence of mineralogical 'signatures' suggests that some aspects of stone formation may result from a non-classical crystallization process that is induced by acidic proteins. An amorphous precursor has been detected in many biologically controlled mineralization reactions, which is thought to be regulated by non-specific interactions between soluble acidic proteins and mineral ions. Using in vitro model systems, we find that a liquid-phase amorphous mineral precursor induced by acidic polypeptides can lead to crystal textures that resemble those found in Randall's plaque and kidney stones. This polymer-induced liquid-precursor process leads to agglomerates of coalesced mineral spherules, dense-packed spherulites with concentric laminations, mineral coatings and 'cements', and collagen-associated mineralization. Through the use of in vitro model systems, the mechanisms involved in the formation of these crystallographic features may be resolved, enhancing our understanding of the potential role(s) that proteins play in stone formation.

Proceedings paper from the third international urolithiasis research symposium, Indianapolis, IN, USA, December 3–4, 2009.

L. B. Gower (✉) · F. F. Amos
Department of Materials Science and Engineering,
University of Florida, Gainesville, FL, USA
e-mail: lgowe@mse.ufl.edu

S. R. Khan
Department of Pathology, Center for the Study of Lithiasis,
College of Medicine, University of Florida, Gainesville, FL, USA

Keywords Nephrolithiasis · Biomineralization mechanisms · Randall's plaque · Acidic proteins

Abbreviations

PILP	Polymer-induced liquid-precursor
$CaCO_3$	Calcium carbonate
CaP	Calcium phosphate
CaOx	Calcium oxalate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
POM	Polarized optical microscopy

Introduction

Biologically formed hard tissues, referred to as biominerals, have intrigued the materials engineering community for years because of the high degree of crystallographic control that is exerted during the precipitation of the bioinorganic crystals. While there are precise control mechanisms in place for desirable biominerals, such as in bones and teeth, there is an unfortunate lack of control in pathological deposits, such as mineral plaque and stones associated with nephrolithiasis. There are, however, some commonalities among the processes involved in both biologically controlled and pathological biomineralization, and one predominant factor is the presence of organic matter. These include the stimulatory or inhibitory influence of biopolymers on crystal nucleation [1–7], growth [8–11], and/or aggregation [12–14]. In the case of stone formation, it is presumed that macromolecules secreted into the urine are 'designed' to inhibit one or all of these aspects of crystallization; thus, much stone research has focused on the inhibitory potential of various biopolymers. The most potent crystallization inhibitors are typically proteins enriched

with acidic residues, such as aspartic or glutamic acid [15], phosphorylated serine or threonine [6, 16, 17], or carboxylate or sulfate containing glycose units [18, 19].

Our research deals with investigating both biologically controlled and pathological biomineralization because we are interested in the role that acidic biopolymers play in modulating crystal growth [20]. The approach we use is based on biomimetic model systems which allow for the influence of various factors to be examined in a less complex reaction medium than the physiological environment, which is particularly the case for urine. Using this approach, one can identify features that appear relevant to those found in the biological realm, and use the in vitro model system to decipher the underlying crystallochemical mechanism(s). With a better understanding of how these types of crystal aggregates are formed in stones, one is better armed with the knowledge of how to prevent those mechanisms from occurring in stone formers.

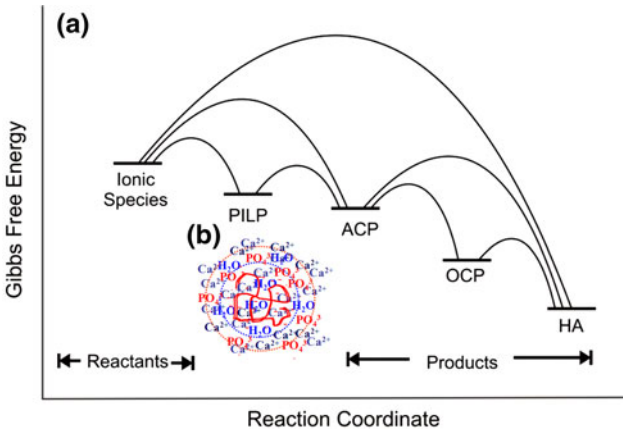

Fig. 1 Multi-step crystallization pathway. **a** According to Ostwald's rule, less stable phases may precipitate out first before transforming to the most thermodynamically stable product. This can include the amorphous phase (e.g. ACP) as well as a PILP phase. **b** Schematic representation of how a PILP droplet might be formed, with a polyanionic polypeptide (*orange random coil*) that has sequestered calcium and phosphate ions along with their associated hydration

Non-classical crystallization processes

Kidney stones are polycrystalline aggregates often composed of some combination of calcium oxalate (CaOx) and/or calcium phosphate (CaP), along with a considerable amount of organic matter from the urine that either becomes entrapped, or plays a role, during the formation of the stone [21–25]. Given that crystal growth in pathological deposits is not regulated by highly specific protein–crystal interactions (as is considered the case for biologically controlled mineralizations), there is not likely just *one* fundamental mechanism for all the different stone types that are formed. The biopolymers could impact the crystallization process during the nucleation stage, or during crystal growth, and/or during aggregation. Thus, nephrolithiasis is a highly complex problem. Nevertheless, we have found that there are several features of kidney stones that can be reproduced in the beaker, and many of these rely on the transformation of the reaction to a non-classical crystallization process. Rather than the well-recognized conventional crystal nucleation and growth processes, the presence of acidic macromolecules can induce (or stabilize) an amorphous precursor to the mineral [20, 26–30]. According to Ostwald's rule [31, 32], if the supersaturation is high enough for multiple crystal phases to precipitate, the first occurring phase is often the least stable and closest in free energy to the mother phase; thus, the first phase to form may be an amorphous phase (Fig. 1). The amorphous phase is generally short-lived, but polymers can prolong its transformation, which depending on the relative heights of subsequent energy barriers [32], will eventually transform into one of the more thermodynamically stable phases [e.g., hydroxyapatite (Hap) from amorphous calcium phosphate (ACP)]. The final more stable phase will typically be what is observed in the crystal products (such as in a stone), but this multistep crystallization pathway has important consequences with respect to the growth and aggregation behavior of the crystals, as discussed below.

Transient amorphous precursors have been identified in the biologically controlled biominerals of several species, including invertebrates which tend to use calcium carbonate ($CaCO_3$) in their exoskeletal elements [33–37], as well as vertebrates, which use calcium phosphate (CaP) in their bones and teeth [38, 39]. This paradigm shift toward recognizing the importance of an amorphous precursor has only become well accepted by the biomineralization community in recent years [20, 40, 41].

Our group has discovered that an additional step can occur in the multi-step crystallization pathway induced by acidic polypeptides (e.g. polyaspartic acid), where it seems that an excess of hydration waters become entrapped in the amorphous precursor such that it has fluidic character [20, 42, 43]. We call this a polymer-induced liquid-precursor (PILP) phase (Fig. 1b) [43] to distinguish it from the more traditional, solid amorphous particles. Although the exact molecular mechanism of the PILP process is still being investigated, it appears as though the anionic polymer (polyaspartic acid is deprotonated at neutral pH) sequesters the positively charged calcium ions, which then sequester oppositely charged counterions (e.g., carbonate, phosphate, oxalate), until the solution undergoes liquid–liquid phase separation as nanodroplets of the precursor phase condense within the crystallizing solution [44]. The fluidic character of this newly identified phase leads to interesting features in the mineral products, and some of these features resemble

features found in kidney stones. Therefore, our group has proposed the following hypothesis.

Hypothesis An amorphous mineral precursor may be induced by acidic biopolymers in the urinary environment, thus playing a key role in kidney stone formation through several possible routes:

1. Accumulation of calcium phosphate PILP droplets that solidify can lead to agglomerates of ACP that serves as a nidus for CaOx deposition.
2. Dense-packed spherulites can grow from amorphous globules, and diffusion-limited exclusion of entrapped organic impurities leads to concentrically laminated spherules.
3. A fluidic mineral precursor can seep into cracks and crevices via capillary action, forming a mineral 'cement' throughout the crystals and macromolecular aggregate.
4. When PILP nanodroplets adsorb onto surfaces, the fluidic character enables them to coalesce into smooth and continuous films and coatings; sequential depositions of films could lead to 'growth ring' types of laminations.
5. Nanodroplets of PILP phase can promote the mineralization of collagen, such that collagen in the basement membrane and interstitium may serve as a substrate for Randall's plaque.

Materials and methods

The data presented here is from a collection of projects dealing with the PILP process, with detailed methods presented in the references indicated. In general, the crystals are grown in aqueous solutions containing micromolar quantities of poly-L-aspartic acid-sodium salt (10–100 μg/ml). The polyaspartate molecular weight is typically in the range of 5–33 kDa, depending on the reaction of interest (e.g., lower MW favors $CaCO_3$ film deposition [43, 45, 46] while higher MW favors collagen mineralization). CaP spherulites and composite 'stones' are described in prior reports [47, 48]. In general, the reactions are typically performed at moderately high supersaturation and without stirring, and can take hours to run when at room temperature. The collagen mineralization reaction is done in a tris buffer with stirring at 37°C, and can take up 3–16 days, depending on the reaction conditions [49–53].

Results and discussion

Our group's initial studies dealt with mimicking biologically controlled mineralization reactions, but when side products of crystal aggregates kept forming in the reactions, our attention turned to the stone field. In the presence of polyaspartate, aggregates form with CaOx (Fig. 2) [20], CaP (Fig. 3), and $CaCO_3$ (Fig. 4) [54]. We believe these aggregates result from accumulation of PILP droplets, as evidenced by PILP nanodroplets that grow large enough to observe on an optical microscope before they solidify and crystallize. For example, Fig. 2 shows micron-sized spherules of CaOx which had formed and coalesced into films (Fig. 2a), along with 'dumbbells' (Fig. 2b), spherulites (Fig. 2c), and agglomerates (Fig. 2d). Agglomerates of partially coalesced CaP particles are shown in Fig. 3a. Note the similarity between the CaP agglomerates and that seen in the stone shown in Fig. 3b [24]. The central nidus of this stone was CaP, which is shown at higher magnification in Fig. 3c. The agglomerate appears to be formed from partially coalesced amorphous spherules; thus, a PILP-like precursor with fluidic character may have been involved.

Mechanisms of formation of polycrystalline aggregates

It should be noted that there are two pathways that can lead to polycrystalline aggregates that are derived from distinctly different mechanisms (Fig. 5) [55]. The polycrystalline aggregate of a spherulite is formed from radial growth of polycrystals from a centralized nucleation point. The radial growth leads to a 'Maltese Cross' pattern in cross-polarized light, which can be seen in the large centralized spherulite of Fig. 2c. As the radial polycrystals are growing, they can form branches which help to fill space, particularly when there is a preference for twinning or splay (Fig. 5b). A partially formed spherulite resembles the shape of a dumbbell (Fig. 5b), which is often the morphology of crystals found in urine [56]. Continued growth will lead to a spherulite, which has a radial texture of the polycrystals (Fig. 5c). If the crystals are growing from within an amorphous globule, the space surrounding the forming crystallites is already filled with mineral precursor, and will eventually crystallize as well, leading to a dense-packed spherulite. On the other hand, if the crystals are growing from solution by the conventional crystallization process, there is likely to be more space between the crystallites as they adopt their needle or platy crystal habits in the solution without being surrounded by amorphous phase to fill in the spaces.

In contrast to the polycrystalline aggregates formed by the well-defined spherulitic *growth process*, true *aggregation* occurs when pre-formed crystals come together (Fig. 5d). Such an aggregate can then be reinforced if the crystals become cemented together either by an organic 'glue' or a mineral 'cement' (see for example Figs. 2d, 8c). The PILP droplets have a tendency to aggregate together and with other crystals, as seen in Fig. 2c, where the precursor

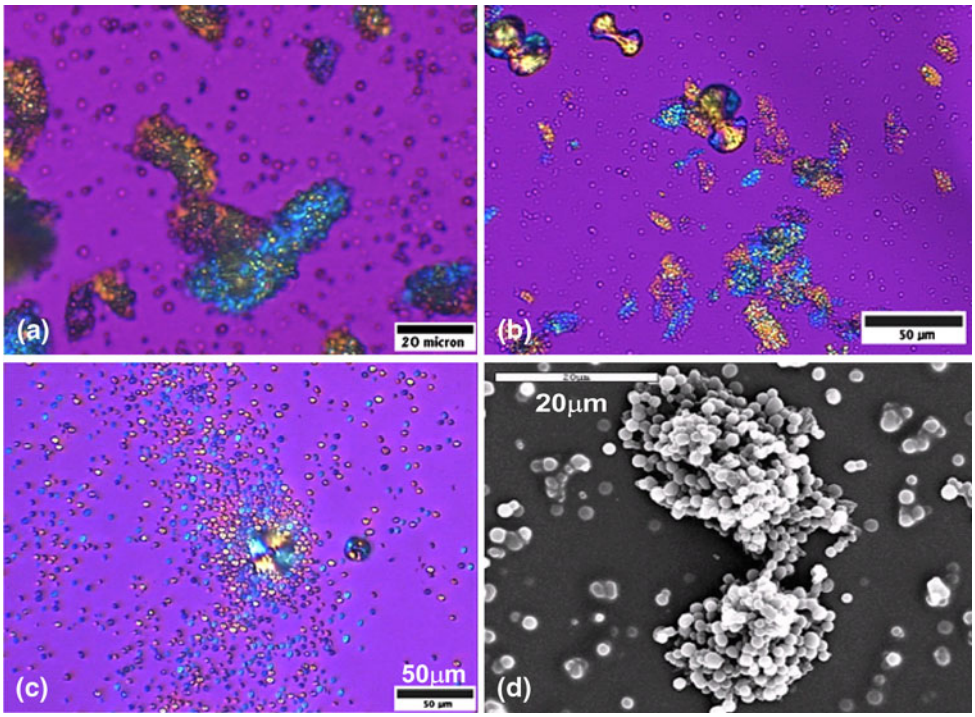

Fig. 2 Evidence of CaOx PILP phase. The polarized optical micrographs were taken using a first-order red λ-plate so that amorphous particles can be seen because isotopic materials exhibit a *magenta* background (including the isotropic glass substrate). **a** *Small round crystals* are from an amorphous precursor, and the birefringent films (*orange* and *blue patches*) are from coalesced precursor droplets. **b** Many film patches are seen at lower magnification, along with 'dumbbell' partial spherulites. **c** Precursor particles appear to have aggregated onto a larger spherulite. The birefringent colors indicate that the spherules are now crystalline, and the Maltese Cross is indicative of a spherulite. **d** SEM shows an agglomerate of CaOx particles cemented to a CaOx film. The *darker gray particles* are partially embedded in the film. (Reproduced with permission from Amos et al. [55]. Copyright 2006, John Wiley & Sons Limited)

droplets appear to have been adsorbing onto a larger spherulite. We often observe that these types of aggregates become 'cemented' together (Fig. 2d), which presumably arises from the partial liquid-like character of a PILP precursor. Depending on the degree of fluidity of the amorphous precursor, one can imagine that a liquid-like phase could adsorb onto a forming stone and seep into the cracks and crevices via capillary action, thus increasing the contact area and fortifying the mineral cement.

CaP and CaOx spherulites

In the case of the CaOx crystals in stones, it seems likely that polycrystalline aggregates may form by both processes, where the randomly arranged and more faceted crystals appear to be true aggregates (Fig. 5d), while the spherical deposits with radial texture appear to be dense-packed spherulites (Fig. 3b). Spherulites are commonly seen in the in vitro model of CaOx, as shown in Figs. 2 and 5c.

In a simple model system for CaP with acidic polypeptides, it was also found that polyaspartate promotes the formation of dense-packed spherulites (Fig. 6a) [20, 47]. As mentioned above, the densely packed structures are thought to arise from radial growth from within a preformed amorphous phase. In the example shown in Fig. 6b–d, it appears that the space-filling property arises from polycrystal growth from within an ion-enriched gelatinous globule. For example, the early stage spherulites of CaP shown in Fig. 6b are rather faint in birefringence, and they flattened when deposited on a substrate (Fig. 6c). But as they crystallized, the birefringence became brighter until the spherulites had fully densified and could no longer transmit light (when they become 10 s of microns in diameter), where they finally appear dark brown (Fig. 6d). Needle-like crystals can also form from the globules (Fig. 6d), and in this case, seem to form from a surface dissolution–recrystallization reaction (since they are not as densely packed).

There is another crystallographic texture observed in these CaP spherulites formed in vitro that may also be relevant to certain features observed in Randall's plaque, which has taken the spotlight in recent years.

Randall's plaque and concentrically laminated spherules

Recent evidence by Evan and coworkers [57–61] implicates Randall's plaque as playing an important role in

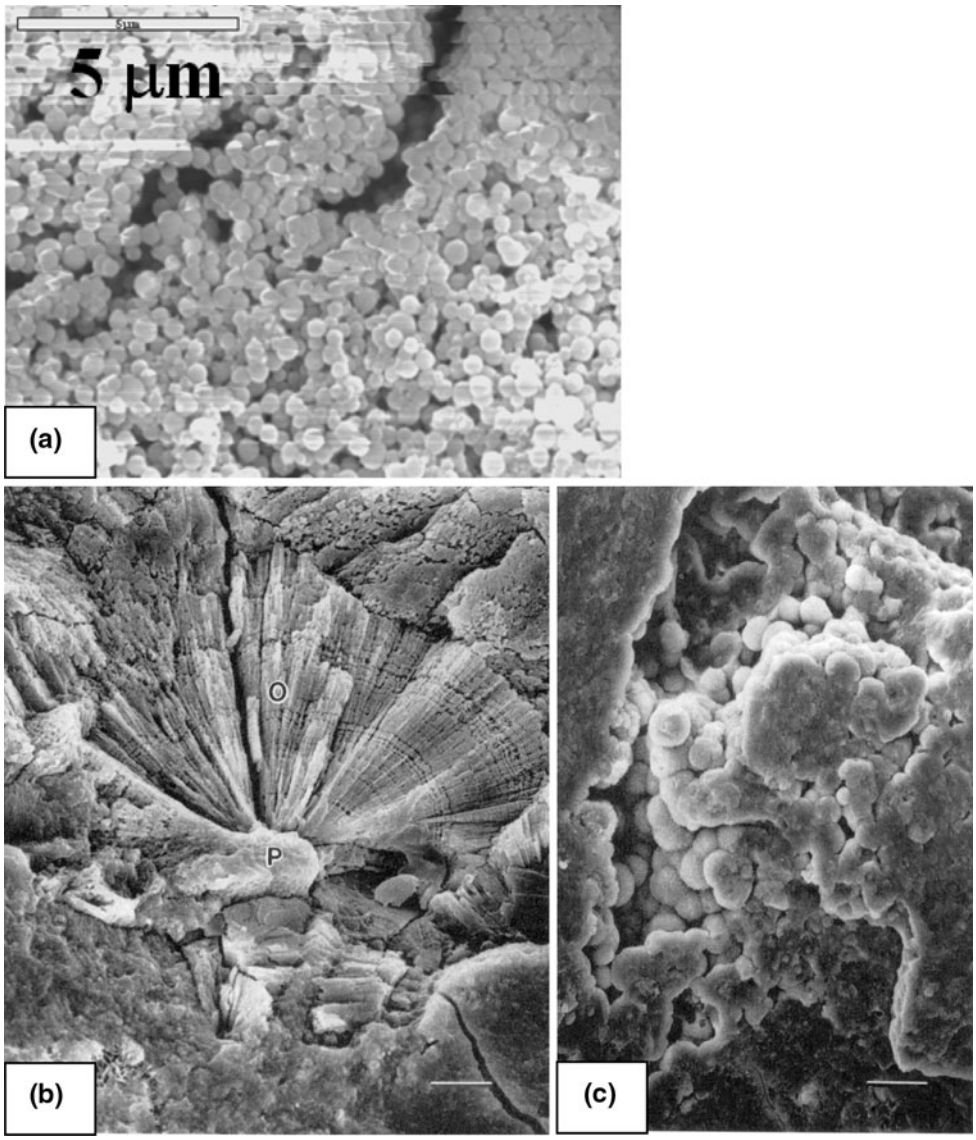

Fig. 3 Evidence of CaP PILP phase and comparison to a stone nidus. **a** SEM of an agglomerate of CaP spherules. They have partially coalesced in the *top right corner*. **b** Composite stone composed of a CaP central core (labeled *P*) and a CaOx outer layer (labeled *O*). The CaOx region appears to have a radial spherulitic texture and exhibits some concentric laminations. *Bar* 5 µm. **d** Higher magnification view of the CaP core, which shows an agglomerate of partially coalesced particles. *Bar* 1 µm (Reprinted with permission from Khan [24]. Copyright 1997, American Urological Association Inc.)

idiopathic calcium oxalate (CaOx) stone formation (Fig. 7) [58]. As originally described by Randall [62], and further examined in greater detail by the Evan team, this pathological mineralization starts with the deposition of calcium phosphate (CaP). The initial deposits appear as small electron-dense multilaminated spherules (roughly 50 nm in diameter) that form first at the basement membrane of the thin loops of Henle (Fig. 7a) [61], and then spread into the nearby interstitial space (Fig. 7b), ultimately extending beyond the urothelial lining into urinary space, where the spherules somehow stimulate attachment and/or nucleation of the CaOx crystals that grow into a large stone.

The spherules in Randall's plaque are quite small, but even full-sized stones often exhibit concentric laminations, and these often occur in the CaOx regions containing a radial sub-texture indicative of spherulites. The concentric layers in these larger multi-laminated structures are typically attributed to daily (or dietary) growth rings, which are thought to form when the urinary environment, which is normally metastable, becomes excessively supersaturated (such as by dehydration, or hyperoxaluria). Our in vitro studies may provide an alternative hypothesis for how such multi-laminated spherical deposits may be formed. For example, multi-laminated spherulites can be generated with an amorphous precursor (Fig. 6), but in this case, the laminations do not occur by sequential deposition of mineral layers. These synthetic spherulites are much larger than the plaque spherules, but commensurate with the laminated spherulitic texture seen in many stones, such as the one shown in Fig. 3b. Our 2D model system of spherulitic $CaCO_3$ films (Fig. 4) shows that the concentric laminations result from diffusion-limited exclusion of the polymeric impurity, which leads to polymer-enriched layers, as seen by the fluorescently labeled polyaspartate (Fig. 4b) [63]. In

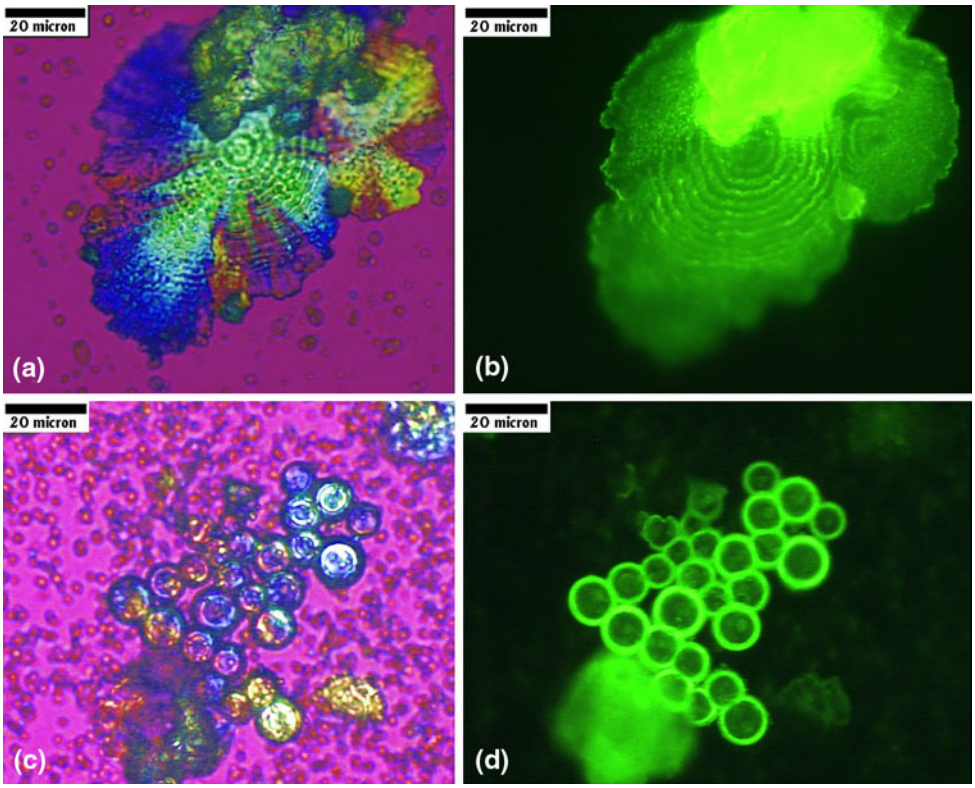

Fig. 4 Concentric laminations in $CaCO_3$ PILP products. **a** Polarized micrograph of a $CaCO_3$ thin film (~500 nm thick). The film has a spherulitic texture and developed concentric laminations as the amorphous film crystallized. **b** FITC-labeled polyaspartate demonstrates that the laminations are from polymer that became entrapped in diffusion-limited exclusion zones as the polymeric impurities were excluded ahead of the crystallization front. In the case of a radial crystal growth pattern that occurs in spherulite formation, the periodic zones are arranged in a concentric fashion orthogonal to the growth front. **c** Large spherules of $CaCO_3$ (~10 μm) formed along with the smaller less birefringent solidified PILP droplets (~3 μm). **d** These spherules contained a polymer-enriched shell (Reproduced with permission from Amos et al. [55]. Copyright 2006, John Wiley & Sons Limited)

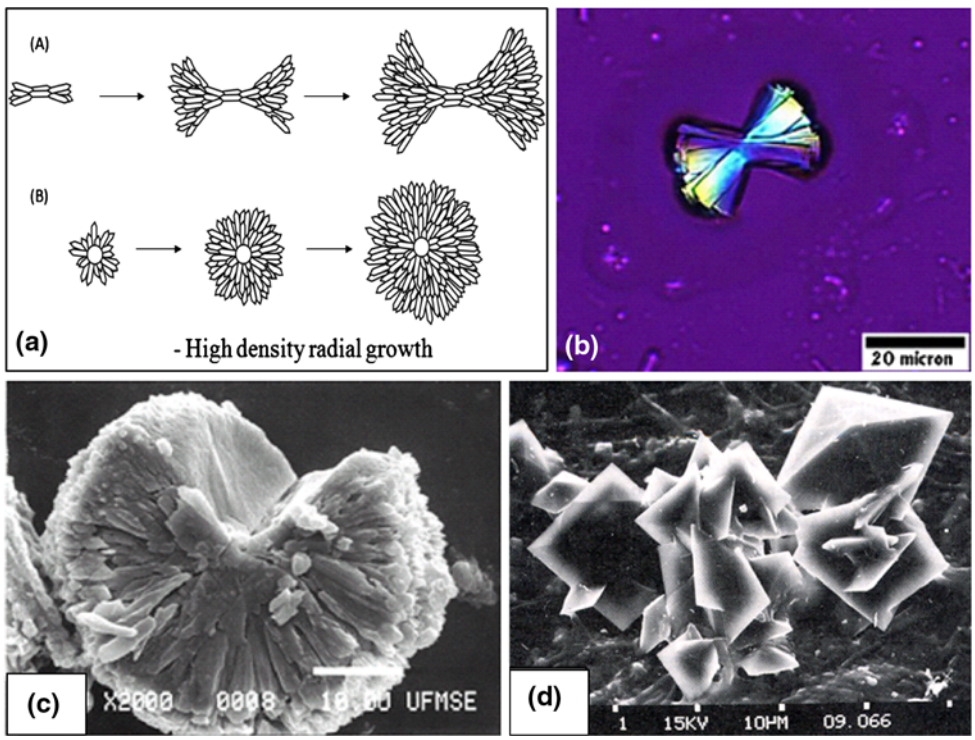

Fig. 5 Polycrystalline aggregate formation via two different pathways. **a** Schematic representation of the radial growth patterns that occur in spherulite formation. **b** A partial spherulite (under polarized light), which appears to branch outward due to twinning, has a 'dumbbell' morphology (analogous to the *top schematic* of **a**). **c** This spherulite has a radial texture, indicating that it is polycrystalline aggregate formed by radial growth of the thin CaOx crystals (analogous to the *bottom schematic* of **a**). **d** An aggregate of CaOx crystals with the traditional faceted crystal habit represents a true aggregation process consisting of preformed and intergrown crystals (Reproduced with permission from Amos et al. [55]. Copyright 2006, John Wiley & Sons Limited)

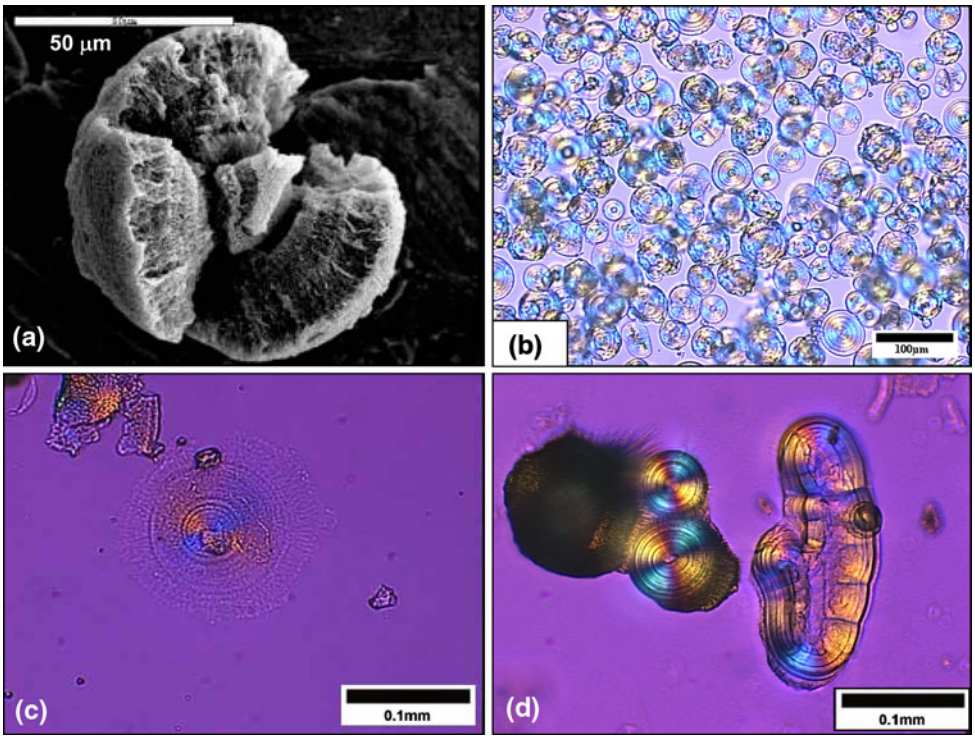

Fig. 6 Formation of CaP spherulites from amorphous globules. **a** SEM of a dense-packed spherulite with a denser shell and concentric laminations. **b** Early stage globules contain a faint birefringence as they are just starting to crystallize. **c** Upon drying, some spherulites collapsed, and appeared to be composed of a slightly rigid gelatinous globule. **d** Maturing spherulites became highly birefringent, and ultimately became *brown* as they densified and blocked light transmission. The *particle on the right* appears to be composed of coalesced globules of spherulites. The *middle* spherulite is starting to form a *brown crust* as it undergoes dissolution–recrystallization. The spherulite to the *left* shows needles that grow from solution, as opposed to the dense-packed smooth-shelled spherulite in **a**. All of the spherulites exhibit pronounced concentric laminations (Reproduced with permission from Amos et al. [55]. Copyright 2006, John Wiley & Sons Limited)

the case of 3D spherules (Fig. 4c, d), the polyaspartate became particularly enriched in an outer shell that had formed (Fig. 4d). When stained, the spherules of Randall's plaque show the presence of organic matter in concentric layers (Fig. 7c, d), particularly at the outer border. Evan et al. [58] also used immunogold labeling to show an enrichment of osteopontin at the interface between organic and mineral layers in the spherules. In the urinary environment, there are many proteins, lipids, and muco-polysaccharides, all of which could become entrapped in the amorphous precursor, and therefore would be expected to generate pronounced laminations as they are excluded during the crystallization process.

Ryall [64] has put together an interesting collage of multi-laminated spherules found in a wide variety of organisms, ranging from 'nanobacteria', to the electron-dense concretions in the midgut of various insects, and calcifying granules in the hepatopancreas of snails and so on; all of which bear a strikingly resemblance to the multilaminated spherules found in Randall's plaque. She draws the conclusion that "it is likely that the granules in human kidneys fulfill analogous functions to those in other organisms—particularly in calcium homeostasis". While the function of the spherules (often times amorphous) in many of these organisms may be for ion storage, their formation does not appear to require a high degree of crystal modulation. Therefore, one could argue that this similarity in morphology and texture is simply a result of how an amorphous mineral precipitates, and particularly with entrapped organics that succumb to diffusion-limited exclusion, leading to concentric laminations. In support of this, the calcium phosphate in plaque has been described as containing amorphous calcium phosphate (ACP), as well as 'biological' apatite similar to bone [65]. In the case of CaOx, Ryall found that protease-treated CaOx monohydrate crystals extracted from urine contained sub-crystalline ovoid nanoparticles (50–100 nm in size). Similar features were observed in the CaOx crystals of plant raphides [64]. Nanogranular textures have been observed in numerous $CaCO_3$ and CaP biominerals as well [66–71], many of which have since been shown to form from an amorphous precursor. AFM and TEM of our PILP-formed films and crystals show a remnant nano-colloidal texture as well [46, 72]. Thus, this nanogranular texture may simply be a 'mineralogical signature' of an amorphous precursor in the CaOx biominerals of stones as well.

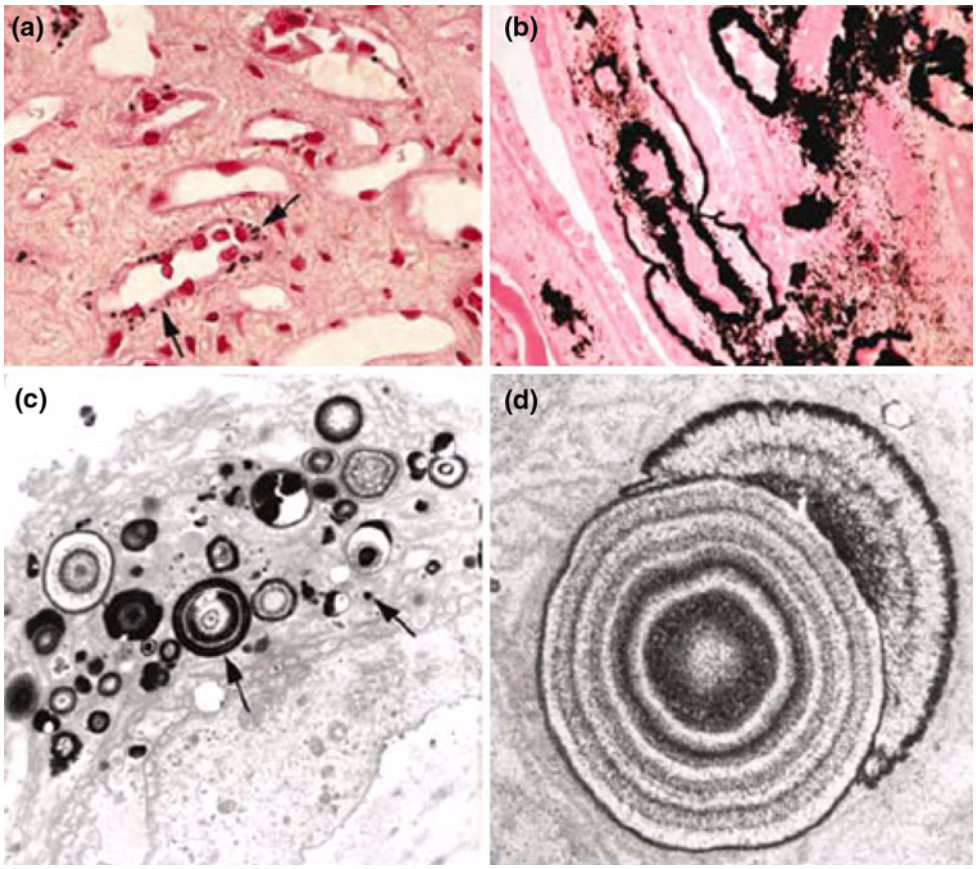

Fig. 7 Evan's work on idiopathic CaOx stone forming patients shows the location and morphology of newly forming precipitates found in Randall's plaque. **a** Histologic optical image (Yasue stained) of a papillary biopsy section with minimal plaque showing spherically shaped, brown-black deposits only in the basement membranes of the thin loops of Henle (*arrows*) (×900). **b** A denser region of plaque shows an accumulation of such deposits forming a continuous layer of calcified matrix (×500). **c** TEM image of deposits first seen in the basement membrane of the thin loops of Henle (×25,000). **d** High magnification image of one of the deposits (×70,000), which are typically multilaminated spherules containing alternating light and dark electron dense rings (Reprinted with permission from Evan et al. [58]. Copyright 2006, American Institute of Physics)

CaP–CaOx composite stones

An important consequence of the Randall's plaque is that it provides an anchored mineralization site from which the CaP deposits apparently stimulate CaOx crystal formation into the urinary space [58, 65]. We hypothesize that the CaP minerals that form the nidus of some stones might be generated by an accretion of CaP precursor phase. For example, the spherules of partially coalesced CaP droplets/particles in Fig. 3a appear similar to the partially coalesced CaP spherules seen in the multiphase composite stone of Fig. 3c [24]. It is thought that the differences in pH between the urine and tissue could be responsible for the change in mineral composition because HA forms in a more basic pH, while the lower pH of urine favors CaOx. We have done some in vitro studies on the overgrowth of calcium oxalate on calcium phosphate spherulites, where both minerals were formed in the presence of polyaspartate (Fig. 8) [55]. The CaP precipitates grew from accumulation of PILP droplets that transformed into amorphous globules, which then solidified into dense-packed spherulites, as shown in Fig. 8a. CaOx was then precipitated in the presence of these pre-formed spherulites, and with the polyaspartate additive, it was found that the amorphous CaOx PILP droplets liked to adsorb to the preformed CaP spherulites, and ultimately formed a large spherulitic structure on top of the CaP nidus (Fig. 8b).

Depending on the reaction conditions, in some cases the CaOx PILP droplets coalesced into a partial mineral coating (Fig. 8c) [55]. This type of deposition, if performed repeatedly, might be expected to lead to a 'growth ring' type of structure, analogous to the kidney stones that appear to have continuous, film-like multilayered coatings (Fig. 8d) [55]. Mineral films and coatings are typical of mineral formed by the PILP process (e.g., Figs. 2, 4), which is what led to the initial discovery of this non-classical crystallization process in the $CaCO_3$ system [43, 45]. From this study of biomimetic composite 'stones', one can see that an amorphous precursor can lead to layered textures by two different pathways: (1) diffusion-limited exclusion of organics, or (2) sequential deposition of mineral coatings.

'Inhibitory' proteins

The urinary tract contains many acidic glycoproteins (as well as lipids) which could conceivably induce the amorphous precursor pathway. A more in-depth review on this subject is provided in our chapter of the book on *Biomineralization- Medical Aspects of Solubility* [55].

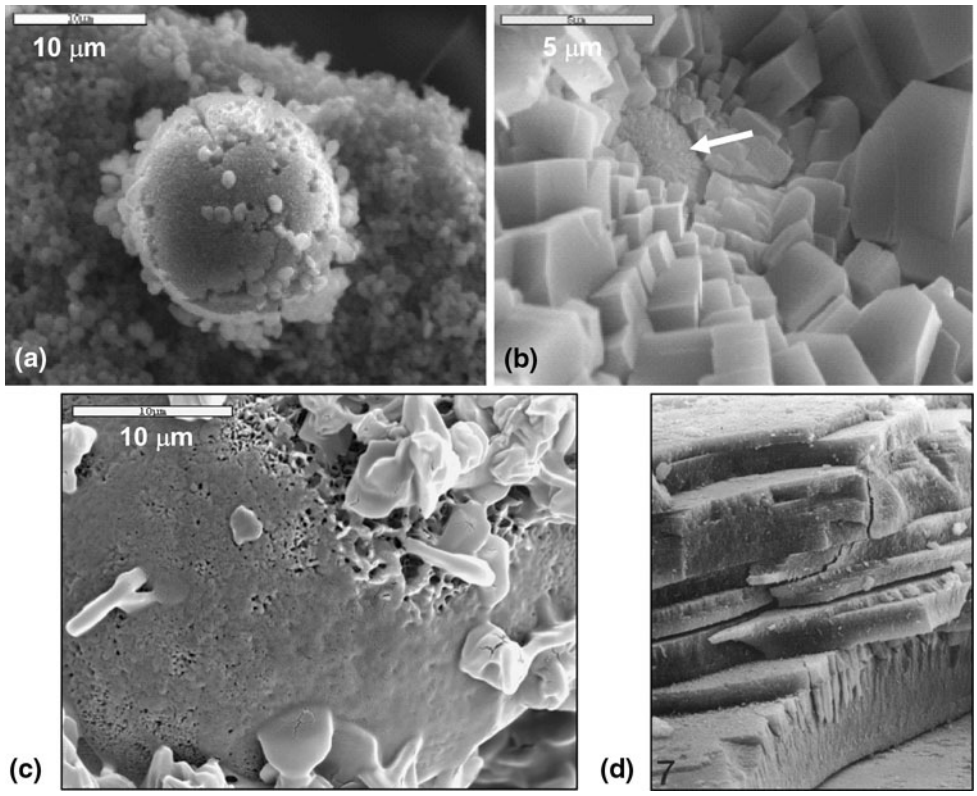

Fig. 8 CaP–CaOx composite particles. **a** SEM of a dense-packed spherulite formed from agglomeration of CaP precursor particles. **b** CaOx overgrowth on a CaP spherulitic core (marked with an *arrow*). There is a radial texture, but the CaOx polycrystals have grown large enough to see their facets. **c** A large CaP spherulite as the core with a smooth thin coating of CaOx. Other poorly identified crystals appear to have aggregated and become cemented onto the particle by the mineral coating. **d** A whitlockite stone showing numerous well-defined mineral layers. Notably, the mineral layers in this stone are about the same thickness as the majority of films deposited with the PILP process (about half a micron). It is not clear whether this stone has a spherulitic texture, but the relatively separated structure of the laminations suggests they are caused by a sequential deposition of mineral coatings (Reproduced with permission from Amos et al. [55]. Copyright 2006, John Wiley & Sons Limited)

Many in vitro models of stone formation consider the acidic proteins as having an inhibitory action, which is therefore assumed to provide a protective action against stone formation. However, it should be noted that it is the acidic "inhibitory" polymers (e.g., polyaspartate) that tend to generate the amorphous precursor pathway when a high enough supersaturation is reached. Thus, even though the function of these proteins may well be to protect against urolithiasis, it is quite possible that this inhibitory action could be overwhelmed by the supersaturation or other factors in the reaction environment, and thus lead to the amorphous precursor pathway. In other words, a reaction medium that has inhibitory additives can eventually 'crash out' with spherulites and/or droplets of a precursor phase. Spherulitic growth is rapid and can lead to relatively large structures in a short time; likewise, the aggregation tendency of crystals in a solution containing precursor droplets can also generate large structures quickly; so both of these aggregation phenomena could contribute to crystal retention in the renal tubules and augment stone formation.

Osteopontin, which is a prominent protein associated with mineral in the plaque [73] (as well as in bone), is a highly acidic protein which could conceivably induce an amorphous phase in the biological environment. Such acidic proteins are generally considered to be inhibitors of crystal nucleation [15, 74, 75], and indeed, this is true of polyaspartate, our simple model protein, as well. Yet, we find that the inhibitory action of polyaspartate promotes the mineralization of collagen [50], which may be relevant to the initial stages of plaque formation in the basement membrane and interstitium. In that project, we are interested in the mechanisms involved in bone formation [50]. We have shown that intrafibrillar mineralization of collagen can be achieved using the PILP process [50–52], and have proposed that the precursor phase is drawn into the interstices of collagen fibrils via capillary action [50]. In the control reaction, which did not include polyaspartate, it was found that the collagen fibrils are rather poor at promoting mineralization. This work was done with type-I collagen; thus, more studies are needed to determine if this might be the

case with the type-IV collagen found in the basement membrane, and if mineralization of such tissues with the PILP process might lead to structures that resemble Randall's plaque.

It seems reasonable to postulate that osteopontin, which is prevalent in plaque, may be there for the purpose of inhibiting mineralization within the tissue if the cells sense an excessive ion concentration in the papillary tissue. However, if the inhibitory potential of such proteins is overwhelmed (such as by a dehydration rise in supersaturation, or hypercalciuria or hyperoxaluria), a metastable amorphous phase could be formed, which could instigate this unfortunate set of consequences.

Conclusion

In the case of pathological biomineralization, many of the proteins extracted from stones are known to be modulators of crystallization in vitro, which could be active during crystal nucleation, growth, or aggregation. Stones form in a complex urinary environment and are composed of a heterogeneous population of mineral products, so some or all of these stages may be important in the formation or retention of the stone. One might assume that since there are many crystal types and crystal faces present, that highly specific interactions are not involved in the inhibitory and blocking potential of these proteins; instead, more generic crystal-binding domains, such as domains of acidic functionality, could be useful for inhibiting both crystal nucleation and growth, as well as aggregation (such as via steric repulsion of a protein of appropriate size). It is possible that these non-specific inhibitory proteins may be secreted by the renal epithelial cells, most often resulting in successful inhibition of the stone; but under certain conditions, the desired inhibitory action of the protein may be overcome by the supersaturation or other environmental factors, leading to amorphous precursors. This seems to be evidenced by some of the mineralogical 'signatures' found in stones, such as agglomerates and compact spherulites, core-overgrowth structures, concentric laminations, nanospherical subunits, and generalized aggregation tendencies and cementatious properties. Thus, while an amorphous precursor might be very useful in biologically controlled mineralization, it could have detrimental effects in contributing to pathological biomineralization.

Acknowledgments This research was supported primarily by the National Institutes of Health grant RO1 DE13492—Bioengineering Research Partnership (work by Fairland Amos) although some observations were a result of studies funded by grants from the National Science Foundation DMR-0094209—Relevance of a PILP process to biomineralization, BES-0404000—Nanoscale Interdisciplinary Research Team, and NASA NRA 00-OSS-01-043—Exobiology Program.

References

1. Khan SR, Kok DJ (2004) Modulators of urinary stone formation. Front Biosci 9:1450–1482
2. Grases F, Isern B, Perello J, Costa-Bauza A (2004) The role of glycoproteins in calcium oxalate crystal development. BJU Int 94:177–181
3. Beshensky AM, Wesson JA, Worcester EM, Sorokina EJ, Snyder CJ, Kleinman JG (2001) Effects of urinary macromolecules on hydroxyapatite crystal formation. J Am Soc Nephrol 12:2108–2116
4. Mo L, Liaw L, Evan AP, Sommer AJ, Lieske JC, Wu XR (2007) Renal calcinosis and stone formation in mice lacking osteopontin, Tamm-Horsfall protein, or both. Am J Physiol Renal Fluid Electrolyte Physiol 293:F1935–F1943
5. Atmani F, Khan SR (1999) Role of urinary bikunin in the inhibition of calcium oxalate crystallization. J Am Soc Nephrol 10:S385–S388
6. George A, Veis A (2008) Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. Chem Rev 108:4670–4693
7. Tsortos A, Nancollas GH (2002) The role of polycarboxylic acids in calcium phosphate mineralization. J Colloid Interface Sci 250:159–167
8. Weaver ML, Qiu SR, Hoyer JR, Casey WH, Nancollas GH, De Yoreo JJ (2009) Surface aggregation of urinary proteins and aspartic acid-rich peptides on the faces of calcium oxalate monohydrate investigated by in situ force microscopy. Calcif Tissue Int 84:462–473
9. Qiu SR, Wierzbicki A, Orme CA, Cody AM, Hoyer JR, Nancollas GH, Zepeda S, Yoreo JJD (2004) Molecular modulation of calcium oxalate crystallization by osteopontin and citrate. Proc Natl Acad Sci 101(7):1811–1815
10. Akyol E, O¨ner M (2007) Inhibition of calcium oxalate monohydrate crystal growth using polyelectrolytes. J Cryst Growth 307:137–144
11. Langdon A, Wignall GR, Rogers K, Sorensen ES, Denstedt J, Grohe B, Goldberg HA, Hunter GK (2009) Kinetics of calcium oxalate crystal growth in the presence of osteopontin isoforms: an analysis by scanning confocal interference microcopy. Calcif Tissue Int 84:240–248
12. Sheng XX, Jung TS, Wesson JA, Ward MD (2005) Adhesion at calcium oxalate crystal surfaces and the effect of urinary constituents. Proc Natl Acad Sci USA 102:267–272
13. Wesson JA, Ward MD (2007) Pathological biomineralization of kidney stones. Elements 3:415–421
14. Konya E, Umekawa T, Iguchi M, Kurita T (2003) The role of osteopontin on calcium oxalate crystal formation. Eur Urol 43:564–571
15. Weiner S, Addadi L (1991) Acidic macromolecules of mineralized tissues: the controllers of crystal formation. Trends Biochem Sci 16(7):252–256
16. Marsh ME (1989) Binding of calcium and phosphate ions to dentin phosphophoryn. Biochemistry 28:346–352
17. Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J (2002) Osteopontin—a molecule for all seasons. QJM 95:3–13
18. Arias JL, Neira-Carrillo An, Arias JI, Escobar C, Bodero M, David M, Fernández MaS (2004) Sulfated polymers in biological mineralization: a plausible source for bio-inspired engineering J Mater Chem 14:2154–2160
19. Marsh ME (1994) Polyanion mediated mineralisation-assembly and reorganisation of acidic polysaccharides in the Golgi system of a coccolithophorid alga during mineral deposition. Protoplasma 177:108–122

20. Gower LB (2008) Biomimetic model systems for investigating the amorphous precursor pathway and its role in biomineralization. Chem Rev 108:4551–4627
21. Khan SR, Hackett RL (1987) Urolithogenesis of mixed foreign-body stones. J Urol 138:1321–1328
22. Daudon M, Jungers P, Bazin D (2008) Stone morphology: implication for pathogenesis. American Institute of Physics, pp 199–215
23. Khan SR, Glenton PA (1994) Association between calcium oxalate and calcium phosphate crystals in nephrolithiasis. J Urol 151:425A
24. Khan SR (1997) Calcium phosphate/calcium oxalate crystal association in urinary stones: implications for heterogeneous nucleation of calcium oxalate. J Urol 157:376–383
25. Evan AP (2010) Physiopathology and etiology of stone formation in the kidney and the urinary tract. Pediatr Nephrol 25(5):831–841
26. Politi Y, Mahamid J, Goldberg H, Weiner S, Addadi L (2007) Asprich mollusk shell protein: in vitro experiments aimed at elucidating function in $CaCO_3$ crystallization. CrystEngComm 9(12):1171–1177
27. Heiss A, DuChesne A, Denecke B, Grotzinger J, Yamamoto K, Renne T, Jahnen-Dechent W (2003) Structural basis of calcification inhibition by alpha(2)-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. J Biol Chem 278:13333–13341
28. Raz S, Hamilton PC, Wilt FH, Weiner S, Addadi L (2003) The transient phase of amorphous calcium carbonate in sea urchin larval spicules: the involvement of proteins and magnesium ions in its formation and stabilization. Adv Funct Mater 13:480–486
29. Aizenberg J, Lambert G, Addadi L, Weiner S (1996) Stabilization of amorphous calcium carbonate by specialized macromolecules in biological and synthetic precipitates. Adv Mater 8(3):222–226
30. Kwak SY, Wiedemann-Bidlack FB, Beniash E, Yamakoshi Y, Simmer JP, Litman A, Margolis HC (2009) Role of 20-kDa amelogenin (P148) phosphorylation in calcium phosphate formation in vitro. J Biol Chem 284:18972–18979
31. Ostwald W (1897) Studien uber die Bildung und Umwandlung fester Korper. Z Phys Chem 22:289–330
32. Wang L, Nancollas GH (2009) Pathways to biomineralization and biodemineralization of calcium phosphates: the thermodynamic and kinetic controls. Dalton Trans 15:2665–2672
33. Beniash E, Aizenberg J, Addadi L, Weiner S (1997) Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. Proc R Soc Lond B 264:461–465
34. Aizenberg J, Weiner S, Addadi L (2003) Coexistence of amorphous and crystalline calcium carbonate in skeletal tissues. Connect Tissue Res 44:20–25
35. Ma Y, Weiner S, Addadi L (2007) Mineral deposition and crystal growth in the continuously forming teeth of sea urchins. Adv Funct Mater 17:2693–2700
36. Nudelman F, Chen HH, Goldberg HA, Weiner S, Addadi L (2007) Spiers memorial lecture: lessons from biomineralization: comparing the growth strategies of mollusc shell prismatic and nacreous layers in *Atrina rigida*. Faraday Discuss 136:9–25
37. Nassif N, Pinna N, Gehrke N, Antonietti M, Jager C, Cölfen H (2005) Amorphous layer around aragonite platelets in nacre. Proc Natl Acad Sci USA 102:12653–12655
38. Mahamid J, Sharir A, Addadi L, Weiner S (2008) Amorphous calcium phosphate is a major component of the forming fin bones of zebrafish: indications for an amorphous precursor phase. Proc Natl Acad Sci USA 105:12748–12753
39. Beniash E, Metzler RA, Lam RSK, Gilbert P (2009) Transient amorphous calcium phosphate in forming enamel. J Struct Biol 166:133–143
40. Weiner S, Sagi I, Addadi L (2005) Choosing the crystallization path less traveled. Science 309:1027–1028
41. Weiner S (2006) Transient precursor strategy in mineral formation of bone. Bone 39:431–433
42. Dai L, Douglas EP, Gower LB (2008) Compositional analysis of a polymer-induced liquid-precursor (PILP) amorphous $CaCO_3$ phase. J Non-Cryst Solids 354:1845–1854
43. Gower LB, Odom DJ (2000) Deposition of calcium carbonate films by a polymer-induced liquid-precursor (PILP) process. J Crystal Growth 210(4):719–734
44. DiMasi E, Liu T, Olszta MJ, Gower LB (2005) Laser light scattering studies of a polymer-induced liquid-precursor (PILP) process for mineralization. In: Sandhage KH, Yang S, Douglas T, Parker AR, DiMasi E (eds) Biological and bio-inspired materials and devices. Materials Research Society proceedings, Warrendale, PA, pp K10.16.11–K10.16.17
45. Gower LA, Tirrell DA (1998) Calcium carbonate films and helices grown in solutions of poly(aspartate). J Crystal Growth 191(1-2):153–160
46. Kim Y-Y, Gower LB (2007) Patterning inorganic ($CaCO_3$) thin films via a polymer-induced-liquid-precursor (PILP) process. Langmuir 23(9):4862–4870
47. Amos FF, Dai L, Kumar R, Khan SR, Gower LB (2009) Mechanism of formation of concentrically laminated spherules: implication to Randall's plaque and stone formation. Urol Res 37(1):11–17
48. Amos FF (2005) Template-assisted mineral formation via an amorphous liquid phase precursor route. University of Florida, 257 pp
49. Jee SS, Culver L, Li Y, Douglas EP, Gower LB (2010) Biomimetic mineralization of collagen via an enzyme-aided PILP process. J Cryst Growth 312:1249–1256
50. Olszta MJ, Cheng X, Jee SS, Kumar R, Kim Y-Y, Kaufman MJ, Douglas EP, Gower LB (2007) Bone structure and formation: a new perspective. Mater Sci Eng R Rep 58:77–116
51. Olszta MJ, Douglas EP, Gower LB (2003) Scanning electron microscopic analysis of the mineralization of type I collagen via a polymer-induced liquid-precursor (PILP) process. Calcif Tissue Int 72:583–591
52. Olszta MJ, Odom DJ, Douglas EP, Gower LB (2003) A new paradigm for biomineral formation: mineralization via an amorphous liquid-phase precursor. Connect Tissue Res 44:326–334
53. Olszta MJ (2004) A new paradigm for biomineral formation: mineralization via an amorphous liquid-phase precursor process. University of Florida, 146 pp
54. Dai L, Cheng X, Gower LB (2008) Transition bars during transformation of an amorphous calcium carbonate precursor. Chem Mater 20(22):6917–6928
55. Amos FF, Olszta MJ, Khan SR, Gower LB (2006) Relevance of a polymer-induced liquid-precursor (PILP) mineralization process to normal and pathological biomineralization. In: Königsberger E, Königsberger L (eds) Biomineralization—medical aspects of solubility. Wiley, West Sussex, pp 125–217
56. Khan SR, Finlayson B, Hackett RL (1983) Experimental induction of crystalluria in rats using mini-osmotic pumps. Urol Res 11:199–205
57. Evan AP, Lingeman JE, Coe FL, Parks JH, Bledsoe SB, Shao YZ, Sommer AJ, Paterson RF, Kuo RL, Grynpas M (2003) Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. J Clin Invest 111:607–616
58. Evan A, Lingeman J, Coe FL, Worcester E (2006) Randall's plaque: pathogenesis and role in calcium oxalate nephrolithiasis. Kidney Int 69:1313–1318
59. Evan AP, Coe F, Lingeman JE (2007) Response to 'Randall's plaque and cell injury'. Kidney Int 71:83–84
60. Matlaga BR, Coe FL, Evan AP, Lingeman JE (2007) The role of Randall's plaques in the pathogenesis of calcium stones. J Urol 177:31–38

61. Evan AP (2006) Histopathology predicts the mechanism of stone formation. American Institute of Physics, pp 15–25
62. Randall A (1940) The etiology of primary renal calculus. Int Abst Surg 71:209–240
63. Dai L, Cheng X, Gower LB (2008) Transition bars during transformation of an amorphous calcium carbonate precursor. Chem Mat 20:6917–6928
64. Ryall RL (2008) The future of stone research: rummagings in the attic, Randall's plaque, nanobacteria, and lessons from phylogeny. Urol Res 36(2):77–97
65. Evan AP, Coe FL, Lingeman JE, Shao Y, Sommer AJ, Bledsoe SB, Anderson JC, Worcester EM (2007) Mechanism of formation of human calcium oxalate renal stones on Randall's plaque. Anat Rec Adv Integr Anat Evol Biol 290:1315–1323
66. Sethmann I, Putnis A, Grassmann O, Lobmann P (2005) Observation of nano-clustered calcite growth via a transient phase mediated by organic polyanions: a close match for biomineralization. Am Mineral 90:1213–1217
67. Sethmann I, Hinrichs R, Wörheide G, Putnis A (2006) Nano-cluster composite structure of calcitic sponge spicules—a case study of basic characteristics of biominerals. J Inorg Biochem 100:88–96
68. Sethmann I, Helbigb U, Wörheide G (2007) Octocoral sclerite ultrastructures and experimental approach to underlying biomineralisation principles. CrystEngComm 9(12):1262–1268
69. Dauphin Y (2001) Nanostructures of the nacreous layers in recent cephalopod shells. Palaeontologische Zeitschrift 75:113–122
70. Kirkham J, Brookes SJ, Shore RC, Bonass WA, Smith DA, Wallwork ML, Robinson C (1998) Atomic force microscopy studies of crystal surface topology during enamel development. Connect Tissue Res 38:91–100
71. Clode PL, Marshall AT (2003) Calcium associated with a fibrillar organic matrix in the scleractinian coral *Galaxea fascicularis*. Protoplasma 220:153–161
72. Amos FF, Sharbaugh DM, Talham DR, Gower LB, Fricke M, Volkmer D (2007) Formation of single-crystalline aragonite tablets/films via an amorphous precursor. Langmuir 23(4):1988–1994
73. Evan AP, Coe FL, Rittling SR, Bledsoe SM, Shao YZ, Lingeman JE, Worcester EM (2005) Apatite plaque particles in inner medulla of kidneys of calcium oxalate stone formers: Osteopontin localization. Kidney Int 68:145–154
74. Addadi L, Weiner S (1985) Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization. Proc Natl Acad Sci USA 82:4110–4114
75. Addadi L, Moradiam J, Shay E, Maroudas NG, Weiner S (1987) A chemical model for the cooperation of sulfates and carboxylates in calcite crystal nucleation: relevance to biomineralization. Proc Natl Acad Sci USA 84:2732–2736