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Biomimetic Randall's Plaque as an *In Vitro* Model System for Studying the Role of Acidic Biopolymers in Idiopathic Stone Formation

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Abstract

Randall's plaque (RP) deposits seem to be consistent among the most common type of kidney stone formers, idiopathic calcium oxalate stone formers. This group forms calcium oxalate renal stones without any systemic symptoms, which contributes to the difficulty of understanding and treating this painful and recurring disease. Thus, the development of an *in vitro* model system to study idiopathic nephrolithiasis, beginning with RP pathogenesis, can help in identifying how plaques, and subsequently stones, form. One main theory of RP formation is that calcium phosphate deposits initially form in the basement membrane of the thin loops of Henle, which then fuse and spread into the interstitial tissue, and ultimately make their way across the urothelium, where upon exposure to the urine, the mineralized tissue serves as a nidus for overgrowth with calcium oxalate into a stone. Our group has found that many of the unusual morphologies found in RP and stones, such as concentrically-laminated spherulites and mineralized collagenous tissue, can be reproduced *in vitro* using a polymer-induced liquid-precursor (PILP) process, in which acidic polypeptides induce a liquid-phase amorphous precursor to the mineral, yielding non-equilibrium crystal morphologies. Given that there are many acidic proteins and polysaccharides present in the renal tissue and urine, we have put forth the hypothesis that the PILP system may be involved in urolithiasis. Therefore, our goal is to develop an *in vitro* model system of these two stages of composite stone formation in order to study the role that various acidic macromolecules may play. In our initial experiments presented here, the development of "biomimetic" RP was investigated, which will then serve as a nidus for calcium oxalate overgrowth studies. In order to mimic the tissue environment, MatriStem® (ACell, Inc.), a decellularized porcine urinary bladder matrix was used, because it has both an intact epithelial basement membrane surface and a tunica propria layer, thus providing the two types of matrix constituents found associated with mineral in the early stages of RP formation. We found that when using the PILP process to mineralize this tissue matrix, the two sides led to dramatically different mineral textures, and they bore a striking resemblance to native RP, which was not seen in the tissue mineralized via the classical crystal

Conflict of Interest

The authors declare that they have no conflict of interest.

nucleation and growth process. The interstitium side predominantly consisted of collagen associated mineral, while the luminal side had much less mineral, which appeared to be tiny spherules embedded within the basement membrane. Although these studies are only preliminary, they support our hypothesis that kidney stones may involve non-classical crystallization pathways induced by the large variety of macromolecular species in the urinary environment. We believe that mineralization of native tissue scaffolds is useful for developing a model system of stone formation, with the ultimate goal of developing strategies to avoid RP and its detrimental consequences in stone formation, or developing therapeutic treatments to prevent or cure the disease. Supported by NIDDK grant RO1DK092311.

Keywords

Randall's plaque; Nephrolithiasis; Biomineralization mechanisms; Acidic proteins; Osteopontin; calcium phosphate; PILP

Introduction

Randall's plaque (RP) was first discovered by Alexander Randall in the 1940s by examining over a thousand papillae from random cadaver kidneys [1, 2], and since then, these crystal deposits have been analyzed extensively to understand their role in kidney stone formation. From various studies on papillary biopsies [3–16], these plaques are described as whitish colored build-ups, and are found at or near the papillary tips and in the renal interstitium. Microscopic examination showed that the initial deposits are spherically-shaped and found in the basement membrane beneath cells of the thin loops of Henle [4, 6–10, 17]. RP was not observed in the tubular lumen, and signs of inflammation, cell injury, or interstitial fibrosis were rare. This is in contrast to other types of stone formers; however, this is not to say that cell damage has no role in RP [3, 6, 12–14, 18–22]. The spherules then appear to grow and fuse, eventually spreading throughout the interstitial spaces, with deposits that appear to be closely associated with the collagen bundles [6, 8, 10, 14, 23]. Eventually the plaque was shown to reach the suburothelial space and to penetrate the urothelium, where it becomes exposed to urinary fluid [4, 6–9, 14, 23, 24]. Using chemical analysis, the plaque was shown to contain calcium and phosphorus, thus indicative of calcium phosphate either in the amorphous or apatitic phase [4, 6–10, 12–18, 21–23, 25–29]. Electron diffraction of the plaque crystals confirmed their identity as biological apatite with various sites on the deposits showing different levels of crystallinity; crystalline, semicrystalline, to almost amorphous[14].

These studies led to the RP theory of stone formation, as suggested by Evan and his colleagues (their illustration is shown in Figure 1) [4]. They propose that the plaque initially forms in the basement membrane of the thin loops of Henle (Figure 2a) [4, 10]. However, others have suggested that the blood vessels are the origin, via a process resembling that of atherosclerotic plaque formation [22], or that crystal-induced kidney cell injury is the cause [14, 27, 30]. These mineral plaques then spread into the interstitial tissue (Figure 2b), and as it grows it reaches and breaks through the papillary surface epithelium. It is then exposed to the urine, where the pH and ionic constituents differ, such that a new mineral phase of

calcium oxalate is deposited on this plaque, resulting in stone formation [3, 4, 6–10, 12, 14, 18, 19, 24, 31–33].

Despite years of scientific research into the mechanisms of plaque formation and stone growth, the progression of the disease is still not fully understood [4, 6, 8, 15, 21, 22, 34–36]. This is because studying the pathogenesis of RP is difficult due to the complexity of the renal and urinary environment where it is formed. The hypotheses regarding the roles of the various macromolecules that are present in urine range from suggesting that they could either promote or inhibit stone pathogenesis, depending on their state or under differing conditions [5, 17, 26, 36–47].

Out of the many different types of stone formers, RP deposits seem to be consistent among one particular group termed idiopathic calcium oxalate stone formers, or ICSFs. This group forms calcium oxalate renal stones without any systemic disorder and make up about 80% of all stone-formers [8, 16, 24, 31, 33, 39, 41, 42, 48–51]. The lack of a known cause (such as various urinary imbalances) for the majority of patients who develop stones also contributes to the difficulty of understanding and treating this painful and recurring disease. In addition, there is currently an absence of animal models of RP formation. Neither hyperoxaluria nor hypercalciuria produce calcium deposits similar to RP [12, 14, 27, 29, 52]. While the calcium oxalate stones induced on the renal papilla of rats may be similar to those formed in humans with primary hyperoxaluria, the animals are fed ethylene glycol or injected with sodium oxalate to induce hyperoxaluria, which is not representative of the physiological mechanism of human calcium oxalate nephrolithiasis [12, 27, 52–54]. Experimentally inducing hypercalciuria in the rat models leads to calcium phosphate crystal deposition in the tubular lumens and pelvic space, not at sites of RP formation in humans [27]. Disadvantages of an animal model also include the high costs associated with breeding, care, and execution of gene knockout experiments and variable control. Thus, we are developing an *in vitro* model system, which is complimentary to *in vivo* studies because it provides the ability to examine how each variable, separately or in controlled combinations, influences the mineral formation. This is particularly valuable when considering the urinary environment, which has variable conditions (such as pH and ionic species) as well as macromolecular species. Therefore, our goal is to develop an *in vitro* model system of the two stages of stone formation, where in the first stage we can examine the mineralization of renal tissue with calcium phosphate, and in the second stage, examine how this “biomimetic Randall’s plaque” then influences the overgrowth of calcium oxalate into a “biomimetic stone”, while controlling the various macromolecular and urinary species that might influence these two distinctly different processes [14, 26, 38, 55–57].

From a materials science perspective, the morphology of RP and stones can provide signatures to suggest how the crystals may have been formed. Specifically, the RP morphologies resemble those of calcium-based mineral products formed by an amorphous precursor [26, 28, 38, 48, 55], and particularly those seen by our group when using the polymer-induced liquid-precursor (PILP) process (Figure 3). In this PILP process, negatively-charged polymers (acidic polypeptides or proteins) sequester positively charged calcium ions, which along with the respective counter-ions (such as carbonate, phosphate, oxalate) causes the solution to undergo liquid liquid phase separation, producing hydrated,

ion-enriched nano-droplets [28, 55, 58, 59]. These so-called PILP droplets are so highly hydrated that this amorphous precursor has a fluidic character [55, 60]. This allows the droplets to coalesce into smooth coatings and films before crystallizing, producing interesting morphologies and features in the mineral products unlike crystals produced by the classical crystallization mechanism [26, 28, 55, 59, 60]. The PILP process was first identified with calcium carbonate (CaCO_3) [59], however the minerals found in kidney calcifications, calcium phosphate (CaP) and calcium oxalate (CaOx), seem to behave similarly in our model systems [61].

The PILP process can emulate many of these mineralogical signatures seen in RP and stones (Figure 2) [55]. For example, the smaller mineral deposits in the basement membrane of RP appear as electron dense, spherical objects, around 50 nm in diameter, while the larger deposits show alternating rings of mineral (apatite) and organic matrix (Figure 2c & d) [4, 6, 8, 9, 14, 20, 23, 26, 62]. We hypothesize that the spherules seen in the basement membrane may be due to phase separation of PILP droplets, which could accumulate and fuse into larger structures due to their fluidic character. We propose that the banded appearance could be due to a diffusion-limited exclusion of the proteins or other impurities as the precursor phase solidifies and crystallizes. During crystallization, impurities are often excluded, but in this case, can only be excluded a short distance because the crystallization is taking place in a densifying amorphous phase; thus one might expect it to lead to entrapped layers of organics [55]. Such a diffusion-limited mechanism has been demonstrated using both polarized light microscopy and a fluorescently-tagged polymer during the crystallization of calcium carbonate from the PILP process (Figure 4c & d) [26, 55]; however, the type of calcium-based mineral and the size of the spherules differs substantially from RP, where the CaCO_3 spherulites were 10s of microns in size, while the spherules in RP are on the order of 10s of nanometers in size. Therefore, this is an interesting feature that deserves further study to determine if it is relevant to the calcium phosphate spherules observed in RP.

Although it has been theorized that mineral first forms as spherules in the basement membrane which then spreads into the interstitium [14, 28, 55, 60], some evidence suggests the mineral starts can also start by heterogeneous nucleation of crystals by the lipids of matrix vesicles present in the basement membrane (Figure 5a), which then grow into the interstitial collagen (Figure 5b) [14, 63]. Collagen is generally a poor nucleator of hydroxyapatite, and requires relatively high supersaturations, and even then, crystals nucleate predominantly on the surface of the fibrils and grow into a large crust coating the surface. However, using acidic polymer additives, we have been able to promote intrafibrillar mineralization of the collagen via the PILP process. Our hypothesis is that the fluidic properties of the amorphous precursor droplets enable them to be drawn into the gaps and grooves of the collagen fibrils by capillary forces. Others have suggested that solid amorphous precursor nanoparticles diffuse into the gap zones of the collagen fibrils [64]. In either case, the collagen fibrils become infiltrated with the amorphous mineral precursor, which then crystallizes into nano-sized HA crystals embedded within the interstices of the collagen fibril (Figure 4a & b). Notably, and similar morphologies of banded collagen (suggestive of intrafibrillar mineral) are seen in the interstitial plaque (Figure 5b) [14, 28, 55, 58, 60, 65]. Thus it is possible that the collagen is helping to stimulate the mineral formation, as we have observed in our model system, where the acidic polymer is highly

inhibitory to crystallization in solution, but when a collagen matrix is added, the mineral nanodroplets are sequestered directly into the fibrils. Interestingly, intrafibrillar mineral often occurs before any mineral is found on the surface of the fibrils, which is the opposite of the conventional crystallization (without polymer), where the mineral spherulites forms predominantly on the surface of the fibrils. It has been shown that just underneath or at the surface of the papillary surface epithelium where the plaque has broken across (Figure 6a-b) spherulites similar with fine, radial crystallites, sometimes appears embedded underneath the mineralized fibrous collagen layer (Figure 6c-d); however, these deposits are typically much more dense than the porous spherulites seen in conventional crystallization [14, 30]. This crystal morphology may have occurred as the mineral is not confined by the collagen at the papillary tip.

While supersaturated conditions are necessary for mineral formation in kidney stones, the thermodynamics and kinetics of the processes are also heavily influenced by molecules that can act as promoters, inhibitors, or in some cases both (for instance, proteins may act differently depending on the degree of phosphorylation, etc.) [5, 17, 26, 28, 36–46, 55]. The hypothesis that RP may initiate via a PILP-like process is suggested because the renal and urinary environment contains many different acidic macromolecular species, and such polyelectrolytes could conceivably induce or promote the non-classical crystallization pathway under the right conditions [61]. In fact, RP as well as the stones themselves contain various amounts of proteins and polysaccharides, including albumin, globulin, Tamm-Horsfall glycoprotein (THP), prothrombin fragment 1, inter- α -trypsin inhibitor (bikunin), osteopontin (OPN), and calprotectin, all of which could conceivably contribute to forming an amorphous phase, or even a PILP-like phase, as demonstrated for various acidic polymers in our group [66–68]. These components are termed crystal matrix proteins, and constitute 1–5% of RP and urinary stones, implying that they may have some influence in the stone-formation process [10, 31, 34, 36, 38, 39, 41, 50, 51, 55, 69–71].

A protein of great interest is osteopontin (OPN or uropontin, the urinary form of OPN), which has been shown to be important in the pathogenesis of renal stones and, as mentioned, has been identified as one of the proteins in the matrix layer [4, 7–9, 14, 27, 31, 36, 39, 41–43, 46]. It also has been shown to be present in the concentric laminations found in RP (where it was immunostained)[9]. OPN is usually believed to be inhibitory to nucleation [55, 72–74], but with high supersaturations (or other conditions) it can induce the PILP process, and has been shown to do so *in vitro* (Figure 4b) [66]. In addition, OPN undergoes significant post-translational modification; the degree of phosphorylation, glycosylation, or both could change it from an inhibitor to a promoter of crystallization [17, 34, 40, 45, 72, 75]. In fact, studies have even shown that there are differences in the properties of OPN in urine from stone formers compared to non-stone formers [40]. Therefore, OPN was a protein of keen interest to initiate our studies. We have in fact done prior studies with OPN (extracted from milk) to see if it would behave similarly to polyaspartate since it is an intrinsically disordered and acidic protein. We found that, even though this protein is inhibitory to crystal formation via the classical nucleation and growth pathway, it can induce the PILP process and form an amorphous precursor, and that this pathway can actually promote the mineralization of collagen fibrils [66].

The aim of this study is to develop a biomimetic Randall's plaque model system so that the role of various species in the first stage of idiopathic stone formation can be studied *in vitro*. Our hypothesis is that several of the macromolecules present in urine and renal tissues may induce the PILP process, and that this process may be responsible for some of the features observed in the calcium phosphate RP in the basement membrane and interstitium. We will then examine how different variables, separately or in combinations, influence such processes, with the ultimate goal of developing strategies to avoid stones, or treatments and therapeutics to ameliorate RP and thus stone formation [38, 45, 55].

Materials and Methods

As mentioned, the main theory of Randall's plaque formation is that calcium phosphate deposits initially form in the basement membrane of the thin loops of Henle, and these mineral deposits then spread into the interstitial tissue [4]. Thus, a material consisting of both the basement membrane and interstitial tissue components was ideal for this study. MatriStem® materials (kindly provided by ACell, Inc.) are comprised of a natural decellularized porcine urinary bladder matrix (UBM) and have both an intact epithelial basement membrane surface and tunica propria surface [76].

For mineralization experimentation, MatriStem® sheets (7 cm × 10 cm, with a thickness of ~0.0550mm [77]) were cut into ~2 cm × 2 cm sections and were mineralized using a 4.5 mM calcium and 2.1 mM phosphate solution in tris buffer at a pH of 7.4, which is based on a recipe that we have used in our prior PILP experiments [58, 60]. For inducing the polymer-induced-liquid precursor (PILP) mineralization process, 50 µg/ml of poly-L-aspartic-acid (pAsp•sodium salt, MW = 27 kDa, polydispersity index of 1.00–1.20, Alamanda Polymers) or OPN (~30 kDa, which was extracted from bovine milk [78, 79]) was added to the solution, while the control contained no polymer [28]. MatriStem® samples were removed after the course of 4 days for the OPN conditions and 7 days for the pAsp conditions, rinsed thoroughly in dI-water, and lyophilized. The different mineralization times were due to the observation that bulk precipitation in the solution occurred for OPN after 4 days, which is usually an indicator that full intrafibrillar mineralization has already occurred, after which mineral will then start to build up on the surface of the scaffold. These scaffolds, along with a non-mineralized MatriStem specimen, were then mounted on scanning electron microscopy (SEM) stubs and coated with carbon for analysis using a JEOL 6400 SEM. For further analysis using transmission electron microscopy (TEM) and diffraction, the samples mineralized with pAsp were removed from the solution, rinsed, and immediately immersed in 4% paraformaldehyde in phosphate buffer (pH 7.4) and fixed for a minimum of 3 days. After fixing, the samples were rinsed in phosphate buffer, dehydrated via graded ethanol solutions, and embedded in LR white hard resin (Electron Microscopy Sciences). Thin sections (approximately 0.05–0.08µm) were cut and analyzed with a Hitachi 7600 TEM or a JEOL 200CX TEM. These were compared to TEM images of native Randall's plaque that was conducted previously in our lab. All other chemicals were purchased from Fisher unless otherwise noted.

Results

Figure 7 shows an SEM micrograph of the Matristem® scaffold before mineralization. The interstitium (abluminal) side shows a fibrous, irregular, porous structure (Figure 7a & b). The basement membrane (luminal) side, however, has a relatively smooth, tightly bounded morphology (Figure 7c & d). The abluminal side is consistent with the typical fibrous nature of the tunica propria in the interstitial tissue, being based on type I collagen fibrils, while the luminal side is consistent with basement membrane composition [76], which is composed primarily of laminin, type IV collagen and heparan sulfate proteoglycan.

Figure 8 shows a representative SEM micrograph of the Matristem® specimen mineralized via the conventional crystallization process (no polymer additive). Hydroxyapatite (HA) spherulitic clusters are found to have nucleated on both the membrane of the luminal side and on the surface of the collagen fibers in the abluminal side. Large, individual spherulites range in size from approximately 0.5–5µm, and are porous due to the separate nature of the platy polycrystals, which is unlike the tiny dense spherules that are commonly found in Randall's plaque. This crust-like appearance on the abluminal side is similar to our findings in prior studies for the conventional mineralization control reaction [28, 60]. The PILP mineralized scaffolds using pAsp additive on the other hand show an entirely different morphology (Figure 9). The collagen fibrils in the abluminal side appear thicker (Figure 9a), which is consistent with intrafibrillar mineral (because the fibrils do not collapse when placed under vacuum when mineral has displaced water). And instead of the HA spherulites nucleating on the surface, the collagen fibrils appear relatively smooth, presumably due to the majority of the mineral being intrafibrillar, and any extrafibrillar mineral tends to be smooth due to the coalescence of the fluidic calcium phosphate precursor from the PILP process [28, 55, 59, 60]. Because identifiable crystal features are not seen on the collagen, energy dispersive spectroscopy (EDS) was used to confirm the presence of mineral inside the fibers. The corresponding EDS spectrum in Figure 9c shows a large calcium and phosphorous peak in the specimen, thus indicating the presence of a large amount of calcium phosphate mineral; given that very little mineral is observed on the surface, the majority of this CaP must therefore be inside the fibrils. This is similar to our previous studies in which dense collagen sponges and demineralized bone were mineralized with the PILP process [28, 60, 66, 80], and similar EDS peak counts could be correlated with 60 to 70 wt% mineral, as determined by thermogravimetric analysis. On the luminal side, faint, tiny bright spots can be seen, which appear to be embedded within the membrane (Figure 9b, arrows). We believe these bright specks are mineral phase because calcium phosphate would be expected to give a higher z-contrast in SEM. These small specks are spherical and similar in size to the ~50 nm spherules in the initial plaque deposits found in the basement membrane seen in Randall's plaques, but further studies at high magnification with TEM were needed to determine if the spherules have the signatory concentric laminations found in RP [4, 7, 8, 10, 14, 20, 21, 23, 26, 62]. The surface also contained a few external HA crystals, but the amount was significantly less than that of the conventional crystallization.

Figure 10 shows the Matristem® scaffolds mineralized with OPN additive. The results are similar to the pAsp mineralization, with intrafibrillar mineralization of collagen on the abluminal side (Figure 10a), and the appearance of spherules on the surface or embedded

below the membrane on the luminal side (Figure 10b). However the amount of mineral appears to be much higher for the OPN sample even though the mineralization time was less (but this needs to be confirmed using thermogravimetric analysis).

TEM of native plaque found in the interstitium revealed that the crystal products are fine textured, suggesting that they are probably intrafibrillar and surrounded by collagen (Figure 11) (i.e., not as large as the crystals in spherulitic clusters, as shown in the control reaction). Some crystals appeared elongated and needled-like in morphology, while others were more non-descript, which is typical of platy crystals when viewed perpendicular to the very thin platelets, while edge-on views of platelets will show dark streaks in TEM. Therefore, it is difficult to discern in TEM if the crystals are platelets or needles, as has been discussed extensively by the bone community. In addition, some crystals are organized in a radial texture, which, as mentioned, is likely mineral that was not constrained by the collagen such that spherulites structures form. This morphology is more typical of synthetic hydroxyapatite and such textures have been shown to be a signature of the calcium phosphate plaque found in idiopathic calcium oxalate stone formers [14]. Electron diffraction analysis (Figure 11b) indicates that the deposits were composed of mostly crystalline or semi-crystalline hydroxyapatite.

TEM analysis was conducted for identifying the two specific characteristics of RP; the basement membrane spherules and the mineralized collagen in the interstitium. To note, the different contrast between the native plaque and the “mimetic” plaque is due to the different voltages used during electron microscopy analysis (200keV vs. 80keV respectively). Figure 12 shows TEM images of microtome sections (~80 nm thickness) of the “biomimetic plaque” prepared using pAsp. These sections were cut as to identify the basement membrane. While regions of aligned crystals are likely collagen associated mineral as seen in Figure 11 (Figure 12a-b), some sections show small isolated spherules (Figure 12c-e). Some spherules that were less 10nm were not able to be magnified further (Figure 12c & d). However, at high magnification (Figure 12f), the larger spherules (Figure 12d) do not exhibit concentric laminations which may be due to the fact that they were not stained. It is also not certain if these regions are basement membrane or just an earlier stage of mineralization of the interstitium, where these initial mineral nucleation sites may actually be collagen associated, and thus further studies are needed to discern if laminated spherules are formed in our system. Figure 13 shows the interstitium of the PILP mineralized scaffolds, which had a very similar appearance to the native plaque when examined with TEM. Both needle-like (Figure 13b) and plate-like (Figure 13c) nano-sized HA crystals were found, as well as regions with radially-oriented crystals producing circular features. Given that we know from the SEM images that the biomimetic plaque consisted predominantly of collagen associated crystals (in the interstitial side), this suggests that the small crystal size is due to the constraints of the surrounding collagen fibrils. The circular features suggest this region may contain a bundle of collagen fibrils in cross-section, which seem to be surrounded by more prominent crystals with a needle-like texture. Selected area electron diffraction (SAED) showed the same semi-crystalline or crystalline hydroxyapatite identity (Figure 13d), which as further confirmed using X-ray diffraction (data not shown).

Discussion

Based on its morphological features, we propose that Randall's plaque may involve non-classical crystallization pathways induced by the large variety of species in the urinary environment. Thus mineralization of the native tissue scaffolds is a useful starting place to develop an *in vitro* model system for examining the crystallochemical interactions that might occur during the initial stage of idiopathic calcium oxalate stone formation, the Randall's plaque.

We have shown that scaffolds consisting of both basement membrane and interstitium were able to be mineralized through the PILP process, using both OPN and an acidic polypeptide as a protein mimic. The two polymer additives behaved similarly, which might seem surprising from a biochemistry perspective. But we have argued that this is because the PILP process is non-specific, meaning that it does not require specific protein-crystal binding interactions; in fact, the mineral phase is initiated prior to the formation of any crystal faces, and simply requires interaction of the polyelectrolyte with hydrated ion clusters (or condensed phase) [81].

It should be noted that the osteopontin that was used here was extracted from bovine milk, which is generally more phosphorylated than OPN from other sources found in bone, so it could differ in its inhibitory or promotory activity, as well as ability to induce the PILP process. Indeed, studies have shown that higher phosphorylation can correspond to a higher degree of mineralization, as well as to a high inhibitory activity [72, 75, 82, 83], but these studies did not use the PILP system. Therefore, it would be interesting to test other forms of OPN for their influence on the PILP mineralization, and their interaction with tissue matrices.

While the control reaction, without polymer additive, led to similar crystal morphologies on the luminal and abluminal side (as an external crust of spherulites), the PILP reaction led to dramatically different mineral textures on the two surfaces, and these textures more closely resemble those found in native RP. The collagen in the interstitium is closely associated with the mineral deposits, as seen in TEM micrographs of RP. In addition, TEM and diffraction analysis of the "mimetic" plaque showed an impressive resemblance to native plaque containing small thin nano-crystals, which in the case of the biomimetic plaque, we know to be associated with the organic collagen matrix. While the tiny specks on the luminal side in the SEM micrographs may be related to the early stage spherules found in the basement membrane of RP, we had difficulty locating these regions in the TEM sections and verifying their identity. This may be due to the fact that basement membrane is very thin (approximately 50–100 nm [84]) compared to the interstitium, and thus sectioning only that portion poses challenges.

For our next set of experiments, we were fortunate to be able to obtain decellularized kidney papilla here at the University of Florida (from the labs of Dr. Edward Ross and his colleagues at the College of Medicine, Division of Nephrology, and Dr. Christopher Batich in the Department of Materials Science & Engineering), who have developed a protocol for decellularization of kidneys, so we will be able to test tissues that are even a closer mimic to

the native structures found in the kidney. Also, there is a greater chance for the detection of early stage spherules as there is a much greater area of basement membrane in the numerous kidney tubules that will be exposed during sectioning. With these kidney tissues, we will also examine the scaffolds at earlier time points to try and catch the earliest stages of mineral formation, to determine which matrices are more likely to promote the mineral first, the basement membrane or collagen fibrils in the interstitium. Of course, one must keep in mind that when these tissues are decellularized, the ion transport into the tissue could be substantially different without the cells. Nevertheless, this model system can still help identify how the mineral precursors interact with different tissue matrices, as well as species present in the urinary environment.

Conclusion

Our preliminary studies with the Matristem® decellularized tissues seem very promising in that they are leading to mineralogical features that mimic those seen in RP, and they are showing striking differences when the mineralization pathway follows the non-classical PILP process, supporting our hypothesis that some of the acidic urinary macromolecules could play an influential role in inducing a PILP-type process. Of course one would not expect the body to secrete proteins that would deliberately lead to stones, but we believe that they are secreted due to their inhibitory activity (or as an inflammatory response), which may be successful at preventing crystal nucleation in most cases, but if the protein becomes overwhelmed, such as at higher supersaturations (or due to other factors), they could then lead to a crashing out of mineral via this non-classical pathway.

Eventually, experiments involving the overgrowth on calcium oxalate on this “biomimetic” RP, both *in vitro* using a flow system, and *in vivo* using rat models, will be implemented. If this biomimetic RP indeed serves as a suitable nidus for mineral overgrowth under physiological urinary conditions, and if the “biomimetic” stones have similar morphological features as those found in native idiopathic CaOx-CaP composite stones, we believe this will provide a viable model system for studying the pathogenesis of this complex disease.

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Fig. 1. Randall's plaque theory of stone formation in idiopathic calcium oxalate stones, adapted. Crystalline deposits of calcium phosphate first form in the basement membrane of the thin loops of Henle and spread into the interstitial tissue. This plaque eventually breaks through the urothelial lining of the renal papilla, where it becomes exposed to the urinary space, allowing for calcium oxalate overgrowth into a stone to occur. (Reprinted from ref [4], Copyright 2010, with permission from *Springer Berlin Heidelberg*)

Fig. 2.

Histology and TEM images of the earliest stages of Randall's plaque. (a) Light micrographs of stained sections shows that spherules first appear in the basement membrane of the thin loops of Henle (arrows). (b) Further accumulation of mineral was then seen throughout the interstitium. (c) Closer examination using TEM shows early stage calcium phosphate spherules with alternating light (mineral) and electron dense (stained organic) rings. (d) Individual spherules are ~50 nm in diameter. There is no evidence of crystallites at this early stage, and their round form (without radial polycrystals) suggests they may form via an amorphous precursor. Magnifications are $\times 25,000$ for (c) and $\times 70,000$ for (d). (Reprinted from ref [9] Copyright 2005, with permission from *Nature Publishing Group*)

Polymer-Induced Liquid-Precursor (PILP) Process

Fig. 3.

The PILP process was first identified with CaCO_3 . (a) Liquid-liquid phase separation occurs as the negatively-charged polymer sequesters calcium and phosphate ions (or clusters, or condensed phases), along with entrapped water, to form ion-enriched nano-droplets. (b) These droplets are so highly hydrated that the amorphous precursor has fluidic character, which allows coalescence into a continuous film. (c) The film solidifies and undergoes an amorphous to crystalline transformation, (d) creating a continuous crystalline film of CaCO_3 . (Reprinted from ref [59], Copyright 2000, with permission from *Elsevier Inc.*)

Fig. 4.

Mineralization via the polymer-induced liquid-precursor (PILP) process. (a) Proposed mechanism of intrafibrillar mineralization of collagen via the PILP process; (i) PILP nanodroplets adsorb to and infiltrate into the gap zones of a collagen fibril. (ii) Precursor solidifies into amorphous calcium phosphate and crystallizes, (iii) resulting in the fibrils being embedded with nano-crystals of hydroxyapatite. (b) SEM of a type-I collagen sponge mineralized by the PILP process using OPN as the PILP-inducing acidic macromolecule. Inset shows an EDS spectrum of the same region, demonstrating that the collagen fibrils contain calcium phosphate, even though it is not apparent on the surface. (c) Polarized light microscopy of a film of CaCO_3 which was deposited by the PILP process using polyaspartate additive. Concentric laminations are seen during the amorphous-to-crystalline transformation. (d) Fluorescently-labeled polyaspartate demonstrates that the 'transition bars' are created by diffusion-limited exclusion of the polymer since it is an impurity to the crystallization. ((a) Reprinted from ref [28], Copyright 2007, with permission from *Elsevier Inc*; (b) Reprinted with permission from ref [66], Copyright 2014, with permission from *Elsevier Inc*.; (c & d) Reprinted from ref [59], Copyright 2000, with permission from *Elsevier Inc*.)

Fig. 5.

TEM micrographs of tissue sectioned from the interstitium of the early stages of RP from the same specimens as the samples shown by SEM in Fig. 5. (a) Spherical entities, which are thought to be matrix vesicles, also seem to promote mineralization. A banded but non-mineralized collagen fibril can be seen directly below the larger mineralized vesicle. (b) TEM micrograph of calcifying collagen fibrils in RP displays a distinct banding pattern, as noted by the arrows, which matches the d-spacing of native collagen. The same banding pattern can be deciphered on a neighboring fibril that has not yet mineralized, marked as **C**. (Bar = 1µm) (Reprinted from ref [14] Copyright 2012, with permission from *Elsevier Inc.*)

Fig. 6.

SEM micrographs of the renal papillae from biopsies of a patient with idiopathic stones undergoing percutaneous nephrolithotomy. (a) RP appears as a protrusion on the papilla. Papillary surface epithelium (E) is sloughing, exposing subepithelial surface (SE). Dotted scale bar indicates 100 µm. (b) Higher magnification demonstrates surface epithelium, subepithelial surface and area of exposed fibrous layer (F) beneath. Dotted scale bar indicates 33.3 µm. (c) Higher magnification of the fibrous layer which is from exposed collagen-based interstitial tissue. Dotted scale bar indicates 20 µm. (d) Spherulites of various diameters under fibrous layer. At this mature stage, the spherulites appear to contain radial crystallites, but the texture of the polycrystals is very fine. Dotted scale bar indicates 15 µm. (Reprinted from ref [14] Copyright 2012, with permission from *Elsevier Inc.*)

Fig. 7. MatriStem® urinary bladder matrix prior to mineralization. The abluminal surface (a, b) shows the fibrous nature of the tunica propria while the luminal surface (c, d) has a smooth, tightly bounded morphology consistent with basement membrane structure

Fig. 8. MatriStem® mineralized without polymer additive. Both the luminal (a) and abluminal (b) surfaces are shown after 7 days of mineralization. Both surfaces have a high degree of extrafibrillar precipitates of HA spherulites. (c) A higher magnification image shows the typical platey morphology of hydroxyapatite crystals produced without polymer additive. (d) EDS shows a presence of both calcium and phosphorous at very high levels

Fig. 9. MatriStem® mineralized using the PILP process (with pAsp additive) after 7 days, and corresponding EDS spectra. (a) The abluminal surface contains a few small HA crystals, but most of the scaffold contains intrafibrillarly mineralized collagen fibrils, as verified with EDS (c). (b) The luminal surface appears to have no obvious HA crystals, yet upon closer examination, one can see small bright spots (arrows), which are thought to be mineral since they show higher Z contrast. (d) A small amount of calcium can be detected in the EDS spectrum as well

Fig. 10. Matristem® mineralized with OPN additive for 4 days and their corresponding EDS spectra. The results are similar to the pAsp mineralization, with intrafibrillar mineralization on the abluminal side (a) and possible spherules on the luminal side (b). However the amount of mineral appears much higher for the OPN sample even though the mineralization time was less than that used for pAsp. The relative counts for the Ca and P peaks seems very high for the luminal side (d), but considering them in relation to the carbon peak, it can be seen that there is much lower mineral content than on the abluminal side (c)

Fig. 11.

- (a) Transmission electron micrograph of a thin section of native Randall's plaque. Note both the needle-like and plate-like crystal morphologies, and areas of radially arranged crystals.
- (b) Corresponding selected area electron diffraction pattern indicates that the deposits are crystalline or semi-crystalline hydroxyapatite

Fig. 12.

Transmission electron micrographs of microtome sections (~80 nm thickness) of “biomimetic plaque” prepared using pAsp additive in Matristem® mineralization. (a & b) Regions of aligned crystals (which are more readily identified when viewed edge-on) are collagen associated mineral. (c) This section shows a heavily mineralized region neighboring a region with very small isolated spherules. (e) While the spherules in c-d are too small to identify further, other non-collagenous areas show larger, spherules. At high magnification (f), the spherules in (d) do not exhibit concentric laminations; however, these samples were not stained, so organic rings will not be obvious. It is also not certain if the regions in (c-f) is basement membrane or just an earlier stage of mineralization of the interstitium, where these initial mineral nucleation sites may actually be collagen associated, as suggestive of the neighboring fibrillar structures seen in (d). The less mineralized region

seems too thick to be basement membrane (d,e), except for the region shown in (c), so further studies are needed to discern if laminated spherules are formed in our system. Scale bars are all 100 nm, except for panels (b) & (e), which are 500 nm

Fig. 13.

Transmission electron micrographs of microtome sections (~50 nm thickness) of “biomimetic plaque” prepared using pAsp additive in Matristem® mineralization. (a) Low magnification showing a thin section of partially mineralized tissue on top of the holey carbon film, where the regions with dark contrast are dark due to mineral (not staining). (b) Circular features suggest this region may contain a bundle of collagen fibrils in cross-section, which seem to be surrounded by more prominent crystals with a needle-like texture. (c) At high magnification, both plate-like and needle-like crystals are seen, which may be due to crystal orientation effects, similar to the plaque. Crystals appear to be nano-sized. (d) Electron diffraction of the region shown in (c) verifies that the deposits are crystalline or semi-crystalline hydroxyapatite