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Overview of biopolymer-induced mineralization: What goes on in biofilms?

Alan W. Decho*

Department of Environmental Health Sciences, The Arnold School of Public Health, University of South Carolina, Columbia, SC 29208, USA

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ABSTRACT

Bacteria are associated with mineralization and dissolution processes, some of which may enhance or compromise the physical stability of engineered structures. Examples include stabilization of sediment dikes, bioplugging, biogrouting, and self-healing of concrete and limestone structures. In contrast to 'biologically controlled' precipitation (*e.g.* shells) of eukaryote organisms, microbial precipitation primarily results from two major processes: (1) 'biologically induced' precipitation, where microbial activities generate biogeochemical conditions that facilitate precipitation; and (2) 'biologically influenced' precipitation, where passive interactions of extracellular biopolymers and the geochemical environment drive precipitation. A common location for such biopolymers is the microbial 'biofilm' (*i.e.* cells surrounded within a matrix of extracellular polymeric substances (EPS)). EPS biofilms occur commonly in both natural environments and many engineered surfaces. Emerging evidence now suggests that EPS inhibit, alter or enhance precipitation of calcium carbonate. Functional groups on EPS serve as initial nucleation sites, while other moieties function to control extent and types (*e.g.* crystals vs. amorphous organominerals) of precipitation. Understanding how to control, or even manipulate, precipitation/dissolution processes within the confines of EPS matrices will influence long-term structural integrities of materials. The present overview explores properties of EPS, and their potentially destructive (dissolution) and constructive (precipitation) effects on precipitation. Initial insight is offered for understanding how biopolymers might be controlled for applied purposes.

1. Introduction

Precipitation of calcium carbonate ($CaCO_3$) is a widely occurring natural process and sequesters a vast reservoir of carbon. $CaCO_3$ precipitation is involved in the formation of animal and protozoan shells, tropical sands, coral reefs, etc. Precipitation by macro-organisms is often used for specific purposes (*e.g.* formation of shells or teeth) (Addadi and Weiner, 1992; Bäuerlein, 2004), and its macro-products of $CaCO_3$ are clearly visible. Their formation is 'biologically controlled' and occurs at atomic/molecular-scales, where nucleation and growth of the biomineral is a carefully controlled, cell-mediated process (Barabesi et al., 2007). However, precipitation can also be 'biologically induced' or 'biologically influenced' (Dupraz et al., in press). Here, microorganisms are most often involved. While both types of precipitation typically occur outside of cells, a distinction between the two processes are important. 'Biologically induced' precipitation is a direct result of microbial activities, which generate the biochemical conditions necessary to facilitate precipitation. 'Biologically influenced' precipitation is a passive process, and a result of interactions between extracellular biopolymers and the geochemical environment.

Recently, in civil and environmental engineering research, microbially mediated effects on $CaCO_3$ precipitation process have gained increasing interest for both their positive and negative roles in: (1) biogrouting; (2) reducing performance due to scaling and fouling of reverse osmosis filters and anaerobic membrane bioreactors; (3) the 'self-healing' of cracked statues, building and bridges; (4) the use of dried sewage floc as building material; (5) the stabilization of sediments; and (6) reducing the leaching of contaminates in soils. In contrast, uncontrolled dissolution of $CaCO_3$ has negative effects, compromising the physical stability (and aesthetic appeal) of bridges, monuments, historical buildings, monuments and statues, etc. (Hoke and Turcotte, 2004; McNamara and Mitchell, 2005). Many of these destructive processes are, in part, assisted by microorganisms and, more specifically, attached forms of bacteria, called biofilms.

Understanding the molecular-scale events of these processes will be necessary for controlling both precipitation and dissolution for applied purposes. Therefore, it is now becoming increasingly relevant, from a structural and environmental engineering standpoint, to understand how to carefully control biomanipulations of bacteria for precipitation and dissolution purposes. The present overview focuses on bacterially mediated precipitation; specifi-

* Tel.: +1 803 777 6584; fax: +1 803 777 3391.
E-mail address: awdecho@mailbox.sc.edu.

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cally how the extracellular polymeric substances associated with bacterial biofilms affect the process of $CaCO_3$ precipitation from molecular- to macro-scales. A background, basic fundamentals and state of knowledge are presented in the context of understanding the biopolymer-mediated precipitation process.

1.1. Carbonate biomineral precipitation: the basics

Carbonate precipitation is essentially a function of carbonate alkalinity and the availability of free calcium ions (Dupraz et al., in press). The two are combined as a saturation index (SI) (Stumm and Morgan, 1996). Generation of carbonate ions occurs through a series of processes referred to as the 'alkalinity engine' (see Dupraz et al., in press). Concentrations of both free carbonate and Ca^{2+} ions must exceed saturation in order for precipitation to occur (Mullin, 1992), relative to the solubility product of calcite. When the SI is greater than 0.8, $CaCO_3$ precipitation occurs (Kempe and Kazmierczak, 1994), while further study has posited a 10-fold supersaturation as being necessary for precipitation (Arp et al., 2001).

In the absence of organic molecules and under conditions of relatively low supersaturation, the formation of $CaCO_3$ follows a classical model of crystallization involving homogeneous crystallization of calcium carbonate (Mullin, 1992), where crystals grow until they are of mesoscopic size (*i.e.* micrometers) (Cölfen and Mann, 2003). Once precipitated, the partial pressure of CO_2 plays a major role in determining the type of charge at equilibrium. A calcite surface, for example, is negatively charged in CO_2-free water, and becomes progressively positive-charged with increasing CO_2 concentrations (Eriksson et al., 2008). Continuous dissolution and re-precipitation of ions take place at the calcite surface.

The saturation conditions also affect the structural progression of precipitate formation. Under conditions of high supersaturation (*e.g.* 10–100-fold), there was a step-wise process to the crystallization/precipitation that occurs over very short (*i.e.* millisecond) time scales (Rieger et al., 2007). First, the initial mixing $CaCl_2$ and Na_2CO_3 solutions form an emulsion-like structure. Then, the structure decomposed to $CaCO_3$ nanoparticles, which then aggregated to form vaterite spheres several micrometers in diameter. Finally, the spheres are transformed via dissolution and recrystallization to form distinct calcite rhombohedra. The study showed that the nanoparticle stage is a critical stage. If sufficient organic polymers (*e.g.* polycarboxylic acid) are added, the nanoparticles are stabilized (against compaction and recrystallization), and then form amorphous precipitates.

In biomineralization, organic molecules are used to initiate precipitation, influence the continued growth of the precipitate, or selectively inhibit precipitation. Although the focus here is on microbial processes, shells produced by animals offer a useful starting point for understanding how ordered interactions of organic molecules affect precipitation. The shell of an abalone, for example, consists of calcite in the outer prismatic layer, and aragonite in the inner nacreous layer (Mann et al., 2007). Shells and other forms of biominerals typically contain relatively small amounts (*e.g.* <10%) of organic molecules (*e.g.* proteins, glycoproteins, proteoglycans and polysaccharides) that control crystal nucleation, orientation, size and phase (Lowenstam and Weiner, 1989; Simkiss and Wilbur, 1989; Wheeler et al., 1981; Addadi and Weiner, 1985; Pipich et al., 2008). The precipitate is formed over an insoluble organic matrix, which acts as a structural mold and contains nucleation sites. The organic matrix, or more specifically their proteins, is intimately associated with the resulting precipitate. The two shell layers of the abalone contain different sets of proteins that have been shown in the laboratory to induce crystallization and modify the growth of crystals (Belcher et al., 1996). Earlier studies suggested that most nucleation proteins were highly acidic (*i.e.* p*I* <4; where p*I* is the pH at which molecule has no net charge) (Addadi and Weiner, 1985; Mann, 2002; Wheeler et al., 1981; Fu et al., 2005; Gotliv et al., 2005). More recently, less-acidic proteins, having a p*I* near 7.8, have also been isolated (Eriksson et al., 2008). Of greater importance than acidity, however, is the proper spacing of positively charged and negatively charged residues on amino acids (*e.g.* histidine, arginine, and aspartic acid) using neutral amino acids (*e.g.* cysteine). Further, the sorption of certain proteins to a calcite surface is used to inhibit subsequent dissolution and conversion to aragonite. Under these conditions, the crystal surfaces become covered with holes due to specific protein binding, and subsequent inhibition of crystal growth (Mann et al., 2007), a property that contributes strength and stability to the abalone shell.

1.2. The microbial biofilm environment

Bacteria and other microorganisms are major agents that induce or influence the precipitation (and dissolution) of $CaCO_3$ through their activities and/or products (Barabesi et al., 2007). In general, activities of microorganisms have two net effects: (1) organic carbon production, mainly through photosynthetic CO_2 fixation; and/or (2) organic carbon mineralization/decomposition. Several major functional groups (or clades) of microorganisms participate in these activities: (1) photolithoautotrophs (*i.e.* primarily the cyanobacteria that carry out oxygenic photosynthesis); (2) aerobic heterotrophs (that carry out CO_2 respiration), (3) fermenters, (4) anaerobic heterotrophs (primarily sulfate-reducing bacteria), (5) sulfide oxidizers, (6) anoxyphototrophs (*i.e.* purple and green sulfur bacteria), and (7) methanogens. Certain microbial metabolic activities create carbonate alkalinity and therefore promote precipitation, while other activities increase dissolved inorganic carbon (DIC) and/or produce organic acids that could lead to pH decrease and net carbonate dissolution (Dupraz et al., in press).

Microbially mediated precipitation in nature occurs in a wide range of environments, such as the formation of microbialites (Burne and Moore, 1987; Mann and Nelson, 1989; Riding, 1991; Arp et al., 1999), stromatolites (Freytet and Verecchia, 1998; Reid et al., 2000), ooid sand grains (Reitner et al., 1997; Brehm et al., 2006), ocean whiting events (Thompson et al., 1997), desert crust soils (Garcia-Pichel, 2002), soil precipitates (Lian et al., 2007), thrombolites (Kennard and James, 1986), hypersaline mats (Ludwig et al., 2005), and beachrock (Neumeier, 1999).

Many examples of microbially mediated calcification listed above are coupled to photosynthetic production (Soetaert et al., 2007). This is because oxygenic photosynthesis drives the pH away from an unstable neutral point and shifts the carbonate equilibrium to promote precipitation in carbonate-saturated water (Soetaert et al., 2007), especially in freshwater environments. This occurs because photosynthesis removes CO_2 from the environment, often more rapidly than it can be replaced via diffusion, generates O_2 and raises pH. Therefore, bicarbonate dissociates into CO_2 and OH^- creating the alkaline conditions that favor precipitation (Arp et al., 2001; Soetaert et al., 2007). In contrast, aerobic respiration processes, which oxidize organic matter, generally result in $CaCO_3$ dissolution (Jørgensen and Cohen, 1977). Organic carbon derived from photosynthesis can also be oxidized anaerobically by sulfate-reducing microorganisms, and result in precipitation (Visscher et al., 1998).

However, the microbial communities of natural environments lack the carefully controlled precipitation environments of their eukaryote counterparts that precipitate $CaCO_3$. The types of precipitates and the conditions under which the precipitates are formed, are often more variable, and less predictable (Boquet et al., 1973; Perry et al., 2007). Microorganisms, however, can accomplish a

level of environmental control within the confines of a 'microbial biofilm'.

A biofilm is a group of microbial cells that are enclosed within a matrix of extracellular polymeric substances or EPS (Neu, 1994; Decho, 2000a). Biofilms occur ubiquitously in a wide range of environments (Stoodley et al., 2002). It is assumed that the biofilm environment allows cells to conduct extracellular activities, such as enzymatic degradations of organic matter, chemical communication, coordination of gene activities, and nutrient exchange, with greater efficiency than their free-living counterpart cells suspended in water. The biofilm also is a microenvironment where precipitation can be facilitated or inhibited over microspatial scales (*i.e.* μm to mm) and exhibit some spatial organization. The biofilm and its associated EPS matrix, therefore, serve as a useful starting point for investigating how organic molecules influence the precipitation process.

1.3. The EPS matrix

The EPS are produced and secreted by bacteria and other microorganisms to facilitate attachment and buffer their immediate extracellular environment (Decho, 1990). A wide range of organic molecules is found in association with the EPS matrix; some of which are structural components, while others are sorbed or localized molecules. The distinction between the two, however, is not often clear. While many EPS are highly hydrated, most of the water is not directly bound to the EPS (Schmidt and Flemming, 1999). Instead, the matrix has been described as 'immobilized water', since the majority of the water is simply localized there (Sutherland, 2001a). The highly hydrated nature of many EPS represents an environment where ions and/or molecules can accumulate to reach higher concentrations (compared to the overlying bulk phase), especially when they are produced or released more rapidly than diffusion or other forms of mass transfer can remove them (Sutherland, 2001b). Such localized accumulations of ions, such as O_2, Ca^{2+} and H^+, have been measured within the EPS matrices of microbial mats (Visscher et al., 1998; Braissant et al., 2009; Decho et al., 2009).

A great deal of literature exists on the various monomeric compositions of EPS polysaccharides of bacteria under different growth conditions, and will not be explored in-depth here (for reviews, see Decho, 1990; Sutherland, 2001b; Wotton, 2004). The EPS matrix is now known to contain several different major categories of molecules whose roles and involvement in precipitation are still under study. In addition to polysaccharides, proteins and glycolipids have been found, sometimes abundantly, as part of the EPS matrix. A growing body of research studies now acknowledges the presence and role(s) of nucleic acids (*e.g.* DNA) and other classically perceived information molecules, as perhaps an important structural component of the EPS matrix (Whitchurch et al., 2002).

EPS consist of both charged (*i.e.* positive or negative) and uncharged (*i.e.* neutral) moieties. Determining the molar ratios of different sugar monomers or amino acids in EPS are helpful in understanding the net charges of such polymers. However, it should be realized that once EPS enters natural systems, it is likely to be rapidly modified. For example, bacteria possess the capabilities to enzymatically modify EPS compositions, both *pre*- and *post*-secretion (Sutherland, 1995). Under natural conditions, geochemical (and photochemical) transformations occur, further modifying the composition. Thus, natural EPS likely exists as a 'continuum' of molecular-sizes, and compositional and degradation states. From a precipitation standpoint, it is important to determine which specific molecules will be important in contributing to precipitation facilitation or inhibition. The functional relevance of EPS compositions will relate to how specific monomers, or more specifically their functional groups, interact, and bind ions that participate in precipitation.

Typical carbohydrate monomers found in EPS include both charged and uncharged forms: D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, L-guluronic acid, D-mannuronic acid, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine (Sutherland, 2001b). Functional groups that are present on charged EPS monomers are especially important in guiding molecule–molecule interactions, and influencing the macro-properties of EPS and their ability to conduct organo-mineral precipitation. Functional groups include carboxyls, hydroxyls, phosphates, amines, and sulfates. The types of interactions between/among EPS and their functional groups may vary (Flemming et al., 2007). Weak interactions, such as London (dispersion) forces, electrostatic interactions, and hydrogen bonds can theoretically occur. Carboxyl groups, for example, can display different electrostatic interactions. They may repel each other, or attract each other when divalent cations are present (Mayer et al., 1999). Acetylation of polysaccharides may contribute to the formation of hydrophobic pockets (*i.e.* microdomains, see below) through London dispersion forces, while deacetylation increases cooperativity of EPS capsular strands, as they undergo a transitional change from random coils to ordered helices in the capsular EPS of bacteria (Cui et al., 1999). Hydrogen bonds provide a major interaction in the overall binding forces between EPS molecules.

These same molecule/molecule interactions contribute to the binding of polymer molecules to a crystal surface. Recently, certain monomers, associated with specific polysaccharides involved in coccolith shell formation, have been shown to sorb to existing crystal facies and inhibit the continued growth of calcite precipitates at those locations (Yang et al., 2008). This occurred through the interaction of functional groups on the calcite crystal and specific monomers (*e.g.* α-D-forms of xylose, rhamnose, galactose and mannose). H-bonds linked the monomers to the calcite surface. Adsorption energies between the sugars and the crystal surface ligands were measured (and modeled). Under the laboratory conditions used by the authors, OH groups on the saccharides demonstrated stronger relative adsorption strengths with the CO_3^{2-} terminations of the crystal, when compared with Ca^{2+} terminations (Yang et al., 2008). Complexation, therefore, occurred mainly through linkages of OH groups on saccharide monomers and CO_3^{2-} (carbonate) groups on the crystal surface. This further implied that sugar monomers on EPS exhibit strong affinities for carbonate ions.

Certain types of EPS monomers efficiently sequester Ca^{2+} and Mg^{2+} ions (Flemming, 1995). It follows that the presence of acidic functional groups on EPS will inhibit precipitation through the binding of Ca^{2+} ions. For example, the Ca^{2+} cation has two charged (+) sites available for complexation to negatively charged functional groups, such as a carboxylic acid, sulfate, or phosphate groups. Ca^{2+} ions often form bidentate bridges between two adjacent EPS molecules having negatively charged groups (Geesey and Jang, 1989). The formation of bindentate complexes lowers the concentration of free Ca^{2+} ions (*i.e.* that are available for precipitation), and could inhibit precipitation (Kawaguchi and Decho, 2002a). The calcium binding capacities of different types of EPS extracted from bacteria have been shown to range from 50 mg Ca/g EPS to 180 mg Ca/g EPS (summarized in Dupraz et al., in press). This has supported the general idea that one function of many EPS is to inhibit precipitation through Ca chelation.

Since certain monomers on EPS are highly labile to bacteria, they could be selectively removed by bacterial degradation of EPS.

Bacteria in microbial mats were found to rapidly utilize uronic acids and other monomers (Decho et al., 2005). Since uronic acids are charged monomers, their removal will result in a net loss of carboxylic functional groups on EPS, and consequently, a reduced ability of EPS to complex Ca^{2+} cations. However, the localized 'concentration' of ions (*e.g.* Ca^{2+}) cannot occur unless there is relatively rapid release coupled with mass transfer resistance (by the EPS). Mass transfer resistance, in part, will be a function of the physical state of the EPS, which are influenced by the types and relative frequencies of bonding between EPS molecules. Removal of specific moieties is known to change the polymeric solubility, flexibility, and steric conformations of EPS polysaccharide components (Sutherland, 2001a). Doing so potentially exposes nucleation sites (on EPS) for unidentate binding of Ca^{2+} ions to commence $CaCO_3$ precipitation.

Steric conformational changes of an EPS molecule will affect which functional groups are unavailable (*i.e.* hidden) for ion sorption. The steric conformation of a molecule, in part, reflects which portions (*i.e.* moieties) of a molecule are exposed and available for complexation. Neu (1996) first suggested that certain amphiphilic (EPS) polymers may anchor such that their hydrophilic regions are exposed. These steric modifications have dramatic effects on their physical properties. Small changes in pH, hydration, and partial degradation will change the steric configuration of a molecule. This has the potential to open up new functional groups for cation (or anion) binding, and potential nucleation sites (Kawaguchi and Decho, 2002a). Braissant (Braissant et al., 2009), using laboratory titration experiments, showed that free Ca^{2+} ions are complexed by several different functional groups on EPS from microbial mats. However, it is clear that *in situ* microspatial heterogeneity within EPS over spatial scales of nanometers (nm) to micrometers (μm) will be important in understanding the inhibition or facilitation of precipitation, and second, that bulk analyses of EPS compositions and properties will limit this critical microspatial information.

1.3.1. Microdomains

The EPS gel matrix, by its intrinsic nature, is thought to consist of small, localized areas or 'microdomains' where certain chemical properties are substantially enhanced or enriched. Microdomains, a term first coined by polysaccharide chemists, have the potential to impart specific chemical properties to the EPS over very small spatial scales (*e.g.* nm to μm) (Kawaguchi and Decho, 2002b). The EPS matrix is essentially a hydrogel that consists of a 3-dimensional polymeric network imbedded in water and having microenvironments that are in thermodynamic equilibrium with the surrounding medium (Verdugo et al., 2004). Studies of gels have shown that the polymeric molecules can be either covalently linked together, which provides a relatively stable, non-dispersing network, or can exist as a tangled, hydrophobic, or ionic linkages that can readily disperse or contract (Tanaka, 1992). Hence, certain portions of gels (*e.g.* tangled networks) can change their size and density rapidly in response to pH, ionic concentration, or temperature, while other portions (*i.e.* covalently linked networks) can remain relatively stable (Viney et al., 1993). The mobility of polymeric molecules within a gel is constrained by local diffusion within the free path length between cross-links (Verdugo et al., 2004). The physical chemistry of such networks will be important in understanding the relevance of nanometer-sized patchiness that is thought to exist in natural polymeric environments (Azam, 1998).

Microdomains could include the presence of highly ordered H-bonding between adjacent EPS molecules, excluding water, and result in net hydrophobic microzones. Acetylation of EPS can promote the formation of such hydrophobic domains (Mayer et al., 1999). The net result of such microdomains, from a functional standpoint, is the presence of localized areas within EPS where precipitation is either strongly inhibited or facilitated.

1.3.2. Spatially organized precipitation within microbial mats

Natural microbial mat systems provide an informative platform from which to observe and understand precipitation, or its inhibition, and the broader sediment stabilization process. In photosynthetically driven systems, such as microbial mats (De Brouwer et al., 2002; Stal, 2003) autotrophic production by cyanobacteria provides large inputs on a daily basis of labile EPS, and low molecular-weight (LMW) organic carbon that fuels heterotrophic degradation and further EPS modifications. Decho et al. (2005) followed the uptake and conversion of C-14 bicarbonate into cells, EPS, LMW exudates and CO_2. Daily degradation by heterotrophic bacteria converted much of the recently secreted cyanobacterial EPS and LMW components to CO_2 within a 24 h period, leaving behind a more refractory remnant EPS (Decho et al., 2005).

In the lithifying microbial mats of marine stromatolites different bacterial functional groups form distinct horizontal layers. The cyanobacteria-rich layer produces abundant EPS on a daily basis (Decho et al., 2005) that efficiently binds free Ca^{2+} ions (Kawaguchi and Decho, 2002b). This binding largely inhibits precipitation despite saturation concentrations of both Ca^{2+} and carbonate ions in the overlying water. Immediately above this is a layer containing abundant sulfate-reducing microorganisms (SRM) (Decho et al., 2009), which live and metabolize under oxic conditions (Jørgensen, 1994; Canfield and Des Marais, 1991; Visscher et al., 1992). The SRM produced compositionally different forms of EPS, which contain abundant functional groups that bind Ca^{2+} ions (Braissant et al., 2007). However, a relatively continuous layer of both crystalline and amorphous microprecipitates forms in proximity to this EPS. It is currently thought that if only one binding site is complexed with a Ca^{2+} ion, the other charge on the calcium is available for complexation with a carbonate ion to initiate (*i.e.* nucleate) $CaCO_3$ precipitation. The activities of the two functional groups, together, result in a concerted effort to limit precipitation to specific locations in the mats, called micritic lamina, which posits adaptive roles for the greater community. Thus, the organized, repeating layering of $CaCO_3$ precipitation in microbially produced stromatolite structures may be viewed as an evolutionarily primitive analog for organized precipitation, when compared with those of eukaryote tissues.

2. Microbial precipitation of $CaCO_3$

Microbial precipitation of $CaCO_3$ occurs by two general mechanisms (Trichet and Defarge, 1995). First, precipitation can occur as a direct result of bacterial activities. This is referred to as 'microbially induced' precipitation and requires the activities of living cells. Urea-hydrolysis, sulfate reduction, and denitrification are three examples of this type of precipitation process (Visscher et al., 1998, 2000).

Second, microbial precipitation can occur by an indirect mechanism, called 'microbially influenced' precipitation (Dupraz et al., in press). This type of precipitation occurs, not in association with bacterial activities, but instead passively through the interactions of the extracellular organics (of bacteria) and the geochemical environment. The geochemical environment near cells is a result of microbial processes and mass transfer resistance, and since they are not mutually exclusive, it is difficult to separate these effects *in situ*. In practice, however, both processes typically occur in spatial and temporal proximity (Weiner and Dove, 2003). Here, the EPS matrix becomes tightly intertwined with precipitation processes.

2.1. Crystalline vs. amorphous precipitates

Once nucleation has commenced, the calcium carbonate precipitate occurs in one of several forms: (1) as a growing crystal (*i.e.* a purely mineral form) or (2) as an amorphous precipitate (*i.e.* a mineral plus organic complex), or (3) as a mixture of the two. Within the confines of the EPS matrix (and biofilms) the precipitate forms exist along a continuum, ranging from individual highly ordered crystals to disordered clusters of organic-rich amorphous precipitates. Classifying precipitates, however, becomes less tangible. Upon examination with scanning electron microscopy (SEM) or X-ray diffraction (XRD) precipitate clusters within EPS contain both amorphous and crystalline forms, which merge together (Dupraz et al., in press). Much work has been conducted using organic hydrogels as analogs of precipitation processes that occur in shells, corals, teeth, and otoliths (summarized in Estroff et al., 2004). It can be speculated that specific moieties (*i.e.* anionic functional groups), such as carboxyls, phosphates, amines, and sulfates, act as nucleation sites for carbonate precipitation. This would require the unidentate binding of a divalent Ca^{2+} cation to EPS, and its subsequent complexation with a carbonate (CO_3^{2-}) anion would initiate the formation of a precipitate. The orogeny of such complexes is not well-understood.

2.1.1. Crystalline minerals

Calcium carbonate occurs in five crystalline forms (Mullin, 1992; Addadi et al., 2003). These are calcite, aragonite, (*i.e.* the two most common biological forms), vaterite, monohydrocalcite and ikaite. Crystalline forms are highly ordered and consist of the mineral only (*e.g.* calcium and carbonate), and have a defined physical and chemical structure that is discernible with XRD, Fourier transform infrared spectroscopy (FT-IR) and other spectroscopic/chemical analyses. Aragonite typically exhibits an orthorhombic structure (*i.e.* three unequal axes at right angles) with acicular crystals, while calcite has a trigonal rhombohedral structure (*i.e.* six-sided) (Verrecchia and Verrecchia, 1994). Mineral crystals often contain 'substituent elements'. For example, the ion Mg^{2+} is often substituted for Ca^{2+} in $CaCO_3$ precipitates under marine conditions owing to the high relative abundances of Mg^{2+} ions in seawater. This results in high-magnesium (*i.e.* 'high-mag') calcite. However, substitutions are not simple replacements of one ion for another. A recent study by Chen et al. (2005) showed that substitution of Ca^{2+} by Mg^{2+} was inhibitive towards carbonate precipitation. However, when polysaccharides are present, stable growth of Mg-rich precipitates occurs (Raz et al., 2000). This implies the importance of an organic matrix in the formation and stabilization of high-mag calcite. Other trace elements, such as strontium, uranium, thorium and gold can also replace Ca^{2+} to be incorporated into crystals and other $CaCO_3$ precipitates. Further information regarding the crystal structures of calcium carbonate minerals can be found in an excellent review (Addadi et al., 2003).

Substituent anions also influence the type of mineral formed and its relative stability. For example, carbonate anions can be substituted with PO_4^{3-} or SO_4^{2-} ions, and even contribute to resistance to dissolution (Spanos et al., 2006). Common examples include the formation of gypsum ($CaSO_4$). This 'evaporite' mineral often precipitates under hypersaline (*i.e.* high salinity) during approaching anhydrophilic (*i.e.* evaporation) conditions (Warren, 2006), and during the surface degradation of carbonate statues and buildings (McNamara and Mitchell, 2005). Interestingly, gypsum precipitates under the high ionic conditions (>200 parts per thousand ppt salinity) of hypersaline ponds and in the presence of abundant organic matter (EPS), and still forms well-ordered crystals (*i.e.* not amorphous precipitate). It is not known if this results from the greater affinity of the sulfate anion for the calcium cation, when compared with $CaCO_3$.

2.1.2. Amorphous precipitates

Under natural conditions, it is likely that few crystals will be entirely inorganic in composition. Different types of organics, ranging from decaying organic matter to EPS to specific proteins in EPS, have been implicated in the formation of amorphous biominerals (Sagemann et al., 1999; Braissant et al., 2003; Bosak and Newman, 2005). Amorphous calcium carbonate precipitates are generally less stable, and more soluble than their crystalline counterparts (Addadi et al., 2003). Their formation involves the incorporation of organic molecules with the mineral matrix (Benzerara et al., 2006; Rodriguez-Navarro et al., 2007) with the former likely acting as precipitation nuclei or precipitation inhibitors. The amorphous precipitate matrix, therefore, exhibits considerably less predictable mineral structure, than its crystalline counterpart, when analyzed using XRD analyses (Decho, unpublished).

A fundamental question is: how does a crystal or amorphous precipitate begin and grow? Organomineral formation typically begins with an organic substrate (*i.e.* molecule), which acts as a precipitation nucleus. Results of a laboratory study, using EPS isolated from precipitated layers of marine stromatolites, showed that specific portions of EPS served to initiate precipitation under superstaturated conditions (Kawaguchi and Decho, 2002a). This only occurred, however, if the EPS was anchored to beads. Further analyses revealed that the protein fraction was connected with precipitation. The authors postulated that acidic tails on proteins may have served as precipitation nuclei. Further additions of unbound EPS under similar conditions resulted in the generation of amorphous precipitates (Kawaguchi and Decho, 2002b). Dupraz et al. (in press) recently outlined several steps for precipitation within the EPS matrix of microbial mats: (1) local increases in alkalinity and pockets of supersaturation; (2) formation of amorphous calcite gels; (3) production of nanospheres from a mixture of amorphous calcite and acidic EPS molecules; and (4) nanospheres acting as seeds for further carbonate precipitation. EPS also influences the shapes of precipitate structures. A wide range of calcium carbonate precipitate shapes have been observed using scanning electron microscopy produced under from both natural and laboratory conditions (Kawaguchi and Decho, 2002a,b; Braissant et al., 2003; Bosak and Newman, 2005; Bontognali et al., 2008). These include: smooth rhombohedra, needles, dumbbells, spherulites, and nanometer spheroids, having size ranges from microns to nanometers (Dupraz et al., in press).

2.1.3. Dissolution and inhibition of $CaCO_3$ precipitation

Dissolution of $CaCO_3$ is central to the damaging effects on $CaCO_3$ structures, but also to its reorganization. Carbonate dissolution results in extensive damage to marble, limestone and concrete associated with statues, buildings and bridges (Kanellopoulou and Koutsoukos, 2003; Hoke and Turcotte, 2004), and occurs under acidic conditions (*e.g.* <pH 7.0) (Morse and Arvidson, 2002). When organic molecules such as EPS, amino acids and dextrans sorb to carbonates (Hazem et al., 2001; Hardikar and Matijevic, 2001; Braissant et al., 2003; Perry et al., 2005), continued precipitation slows and dissolution is inhibited. Additions of diphosphates have been shown to reduce dissolution of marble (Spanos et al., 2006). Carbonate anions can be substituted with PO_4^{3-} or SO_4^{2-} ions, and contribute to resistance to dissolution (Spanos et al., 2006). Sorption of sugar monomers and amino acids also have been shown to slow the dissolution of calcite under laboratory conditions (Yang et al., 2008; Yoshino and Kagi, 2008). However, it is not yet known how additions and incorporation of trace elements (mentioned above)

into the $CaCO_3$ mineral matrix influences (*i.e.* reduces) its susceptibility to dissolution.

Dissolution is also critical to the transformations between different forms of $CaCO_3$. The transformation of natural aragonite crystals to calcite was shown to occur via an interface-coupled dissolution–precipitation mechanism (Perdikouri et al., 2008). Also, sulfate-reducing bacteria (SRB) have even been utilized as an alternative cleaning technology for stone monuments to convert (via dissolution and re-precipitation) the black gypsum fouling crust to calcite (Atlas et al., 1988; Cappitelli et al., 2006) without the undesirable products (*e.g.* sodium sulfate) that result from traditional chemical treatments (Cappitelli et al., 2007). Sorption of molecules to precipitate surfaces can also inhibit further precipitation. A recent study by Chen et al. (2005) showed that substitution of Ca^{2+} by Mg^{2+} was inhibitive towards carbonate precipitation.

3. Enzymatic hydrolyses within EPS

The EPS matrix has been referred to as an immobilized enzyme system (Sutherland, 2001a,b). Enzymes are an active part of natural biofilms and are localized within the EPS matrix in proximity to cells. These enzymes are called extracellular enzymes, and are not physically connected to cells. Other enzymes, called ectoenzymes, are bound to cell-surfaces. Localization of extracellular enzymes within the EPS matrix allows organic molecules that are bound to or trapped by EPS to be hydrolyzed, with the hydrolysis products remaining in proximity to cells, where they can be taken up efficiently via diffusion by cells (Sutherland, 1995). It has been assumed that cells benefit from localization of extracellular enzymes within the EPS matrix.

The macromolecular composition of the EPS matrix changes over time, in part, due to the activities of extracellular enzymes (Sutherland, 2001a). A wide array of extracellular enzymes, which hydrolyse polysaccharides, proteins, and many other forms of molecules, have been isolated from laboratory cultures of EPS-producing bacteria (Sutherland, 1995). It is possible that partial degradation of EPS by these enzymes can result in newly available functional groups, which then have different calcium chelation or nucleation properties. Also, enzymatic hydrolyses of EPS can result in 'hydrate pockets', within which precipitation of needle-like crystals can occur. Such pockets and needles have been observed in lithifying marine microbial mats (Decho, 2000b; Dupraz and Visscher, 2005). It has not yet been determined, however, how extracellular enzymes are localized within the EPS matrix. The enzymes may be trapped within the hydrated pockets, or alternatively, are bound to EPS (Flemming et al., 2007). This bonding would provide to the enzyme an enhanced level of stability against denaturation. However, empirical evidence to support such ideas has been limited.

3.1. What lies ahead?

A major focus of future research resides in determining how organic matter be isolated or synthesized that will produce predictable precipitate matrices, and which can be manipulated? Certain types of EPS are well-suited to inhibiting $CaCO_3$ precipitation. For example, freshly secreted EPS from the cyanobacterium *Schizothrix* sp. (involved in stromatolite formation), inhibits $CaCO_3$ precipitation (Kawaguchi and Decho, 2002b). Such types of EPS can be used to develop approaches to localize precipitation and its inhibition over varying spatial scales and conditions.

Research on biopolymer-mediated precipitation is in its academic infancy, but is poised to rapidly gain attention. Special attention must be afforded to understanding the atomic/molecular-scale controls on biomineralization, and crystal formation in the presence of organics. Two foci are immediately relevant: first, the use of extracellular enzymes becomes immediately relevant to manipulating precipitation processes. The commonly used urease enzyme is cell-associated, hence requires the presence and viable activities of living cells. One biotechnological possibility, is to localize (and stabilize) urease enzymes within microscopic protective EPS gel beads, rather than living bacteria. Then, the microscopic beads, in lieu of bacterial cells, can be injected and used to conduct controlled precipitation.

Second, the study of natural precipitation systems will likely provide novel approaches to control and manipulate precipitation. For example, the EPS within the lithifying mats of marine stromatolites may represent, what can be described as, a primitively developed regulation of precipitation (*e.g.* a primitive attempt at a microbial shell?). Regulation appears to be accomplished by the separation of different forms of EPS where precipitation inhibition, nucleation, and enhancement occur over small microspatial scales (*i.e.* microns to millimeters). A detailed understanding of these systems presents an opportunity to mimic such EPS conditions experimentally, and manipulate the controlled precipitation of $CaCO_3$ in an organized manner. Some stromatolites (*e.g.* Lee Stocking Island, Bahamas) are towering monolithic structures extending meters above the sediment, and have been built by microorganisms and existed relatively intact in the presence of strong daily currents for (estimated) thousands of years. Through an understanding of such macro-scale structures, controlled manipulations for applied purposes may be possible.

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