Highly-Defective Nanocrystals of ZnS Formed via Dissimilatory Bacterial Sulfate Reduction: a Comparative Study with their Abiogenic Analogues

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Abstract

The physicochemical properties of a (nano)mineral are strongly affected by its formation processes, and thus, may indicate the (nano)mineral's formation environment and mechanism. This correlation, although relevant to a myriad of geological, environmental, and material-science processes, has not yet been fully appreciated and systematically explored. Here, using the Zn-S system, we demonstrate that biological and abiotic processes at similar experimental conditions can produce distinctive particle size, morphology, and crystal structure in the formed ZnS. Specifically, bacterial sulfate reduction led to the formation of highly-defective nanocrystals of mixed sphalerite and wurtzite in a range of ~ 4 - 12 nm. By comparison, the abiotic procedures of titration- or diffusion-controlled precipitation resulted in the formation of polycrystalline aggregates that contained randomly-oriented, ultrafine crystals below ~2-3 nm. The poor crystallinity in the abiogenic samples, regardless of the sulfide addition rates, reveals

an overall nucleation-dominated, crystal growth-restricted pathway for the formation of ZnS from low-temperature aqueous solutions. The difficulty in the ZnS crystallization likely stems from the intrinsic surface instability of the ZnS growth units (i.e., in the form of nanoclusters) resulting from the dipole-dipole interactions of the unit with surrounding water molecules. In the biogenic samples, the ZnS crystallinity was significantly improved, indicating that the presence of bacterial metabolites somehow promoted the crystallization process. With evidence for the enlarged {111} planes in the biogenic nanocrystals, we attribute this enhancement mainly to the selective interaction of the bacterial metabolites with polar faces of the ZnS growth units, which might have effectively screened the dipole moments in the growth units and enabled their crystallographic assembly. By revealing the intrinsic difficulty and specific pathways for the ZnS crystallization, we also present viable explanations for the exclusive occurrence of structural defects in {111} planes of the biogenic nanocrystals. The findings of our study provide insight into the origin and fate of metal sulfide nanoparticles in the environment, and have implications for biomineralization, bioremediation of metal-contaminated sites, and bacterial production of functional nanomaterials.

1. Introduction

Metal sulfides (e.g., ZnS), especially those in the nanoscale regime, are of intense interest in the geological, environmental, and material-science sectors for a multitude of processes and applications, including the biogeochemical cycling of elements (Pirrie et al., 2003; Smieja-Krol et al., 2010; Scott et al., 2012; Yoon et al., 2012), environmental remediation (Radhika et al., 2006; Castillo et al., 2012; Pala and Brock, 2012), resource recovery (Bawden et al., 2003; Kucha et al., 2010; Saunders 2012), ecotoxicity evaluation (Santos et al., 2010; Leigh et al.,

2012), biomineralization (Zbinden et al. 2001; Moreau et al., 2004; Posfai and Dunin-Borkowski, 2006), the origin and evolution of life (Mulkeydjian 2009), and the development of functional semiconductors (reviewed in Fang et al., 2011). The popularity of research involving nano-sized metal sulfides partly originates from their widespread distribution in natural and anthropogenic systems. As is being recognized, nano-clusters, nano-crystals, and nano-aggregates of Fe-, Zn- and Cu-sulfides constitute a major fraction of the metal load in anoxic oceanic, sedimentary, freshwater, and deep-sea vent environments (e.g., Labrenz et al., 2000; Orberger et al., 2003; Hochella et al., 2005; Luther and Rickard, 2005; Yucel et al., 2011; Hofacker et al., 2013).

Counter-intuitively, evidence shows that nano-metal sulfides are not confined to anoxic environments as colloids of ZnS, CdS, and CuS can persist in oxic waters for months, transporting metals between different environments and ecological niches via metal-sulfide associations (Theberge et al., 1997; Rozan et al., 2003; Sukola et al., 2005; Priadi et al., 2012). In addition, recent environmental studies confirmed the presence of ZnS nanoparticles in domestic and industrial sewage and sludge (Lombi et al., 2012; Kim et al., 2014; Ma et al., 2014), and suggested that sulfidation is a dominant process for the dissolved metals in these engineered systems.

Both biological and abiotic processes can lead to the formation of nano metal sulfides in the environment. Dissimilatory sulfate-reducing bacteria (dSRB), which utilize SO_4^{2-} as a terminal electron acceptor and discharge reduced sulfur species to the environment, are perhaps the most important facilitators of metal sulfide precipitation in low-temperature geological settings. Field evidence revealed that spherical aggregates of ZnS nanocrystals were formed in the biofilms composed of sulfate-reducing bacteria in a flooded Pb-Zn mine (Labrenz et al.,

2000; Moreau et al., 2004). Interestingly, similar aggregates of ZnS nanoparticles were found in the mineralized layers of the Pompeii worms near a deep-sea vent, which was interpreted as the organism's detoxifying mechanism against high-concentration metals via symbiosis with sulfate-reducers (Zbinden et al. 2001). The role of dSRB in producing ZnS nanoparticles was also demonstrated under simulated environmental conditions (Peltier et al., 2011; Castillo et al., 2012). For examples, Castillo et al. showed that micron-sized aggregates of sphalerite and wurtzite nanoparticles were produced in the presence of mixed SRB communities at room temperature (~21°C). Hydrothermal emissions also contribute to the natural inventory of nano metal sulfides by releasing excessive sulfide and metals to the ocean environment (Hochella 2012). Specifically, precipitates of Zn- and Fe-sulfide nanoparticles were detected in the low-temperature diffuse flows (Hsu-Kim et al., 2008) and also in the high-temperature plumes (Yucel et al., 2011; Gartman et al., 2014) of black smokers at a spreading center in the Pacific Ocean. Being exceedingly small in size, nano-metal sulfides tend to acquire high mobility and/or reactivity under aqueous conditions, and thus, it remains a challenge to understand the origin, transport routes, and fate of the sulfide nanoparticles identified from various geological and environmental scenarios.

Metal sulfides (e.g., ZnS) are also intrinsic semiconductors, of which the nano-forms are used in a variety of applications like solid-state lighting, high-resolution flat screen displays, and targeted cancer imaging. Among the efforts to synthesize metal sulfide nanostructures of specific physicochemical properties, the biological pathways via microbial sulfate-reduction are gaining noticeable traction being less energy-intensive and eco-friendly. To date, a variety of microorganisms including bacteria, algae, yeasts, and fungi have been, or are being, tested for the ability to synthesize metal sulfide nanoparticles (Bai et al., 2006; Mandal et al., 2006; Zhou

et al., 2010; da Costa et al., 2012; Duran and Seabra, 2012; Hazra et al., 2012; Edwards et al., 2013; Miradeh et al., 2013; Su et al., 2013; Villa-Gomez et al., 2013; Mala and Rose, 2014; Moon et al., 2014).

Yet, across the different domains of research, it is striking that a basic understanding for the difference between biogenic and abiogenic nano-metal sulfides in terms of size, morphology, crystal structure, and surface reactivity is largely unavailable. Further, the mechanisms involved during the biological formation of ZnS are poorly understood. This lack of information provided motivation for our current study.

Here, using a Zn-S system, we have investigated whether the biogenic sulfide minerals formed via bacterial sulfate reduction may be distinguishable from their abiogenic analogues using the mineral's morphology, crystal structure, and/or reactivity. We chose Zn-S as the model system mainly because (1) the nano-forms of ZnS are highly relevant for biogeochemical cycles and industrial applications (reviewed above), and (2) ZnS phases are exclusively monosulfides (i.e., sphalerite and wurtzite) with one oxidation state for Zn and S, thereby making it possible to focus the comparison on the sulfides' morphology, crystal structure, and defect attributes. The results of our study showed that bacterial sulfate-reduction can lead to the formation of highly defective nanocrystals with distinctive particle-size, morphology, crystal-structure, and reactivity (in terms of non-oxidative dissolution) in comparison to their abiogenic analogues, indicating that the formation of these two types of ZnS involved different mechanisms. In this study, we discuss in depth about the formation mechanisms for biogenic versus abiogenic ZnS nanoparticles. Our findings have relevance for the biogeochemical cycling of metals and sulfur, and for the environmental and industrial processes that involve ZnS formation from aqueous solutions.

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117 2. Materials and Methods

118 2.1 Chemicals and Solutions

119 The chemicals and reagents used in the experiments were of ACS- or higher grade
120 (mostly purchased from Sigma-Aldrich®), and ultrapure water (18 MΩ•cm, Millipore®) was
121 used for the preparation of all solutions. Unless indicated otherwise, sample preparation and
122 other experimental procedures were conducted under anoxic conditions.

123 2.2 SRB Growth and Biogenic ZnS

124 The cells of *Desulfovibrio desulfuricans* (purchased from DSMZ, a.k.a., ATCC 29577)
125 were maintained in a modified Postgate's medium (#63) at 30°C before being inoculated at 1%
126 (v/v) into a modified metal toxicity medium (mMTM). The compositions and pH of #63 and
127 mMTM are listed Table 1. The cells were allowed to grow for ~ 72 h to reach mid-exponential
128 phase in the metal-free mMTM (the time profiles for optical density at 450 nm, acetate and
129 dissolved sulfide concentrations, and TOC are shown in Appendix Fig. S1), and then used to
130 inoculate the Zn-amended mMTM at 1% (v/v). The final concentration of Zn was set at 1 mM in
131 the amended medium. Whitish precipitates of ZnS were gradually formed in the medium as the
132 bacterial cells grew, and were separated by centrifugation at 8000 g for 5 min. The solid pellet
133 was then dispersed in 20 mL of 0.1 % (m/m) Triton-X solution containing 100 µg of lysozyme
134 and 100 µg of proteinase K for 1 h at 45 °C to lyse and remove the associated bacterial cells,
135 before separated again by centrifugation (8000 g, 5 min). Postgate's medium was only used for
136 the initial recovery and maintenance of the bacterial cells and sequential inoculation at 1% (v/v)
137 through mMTM medium was considered an effective measure to ensure that residual metabolites

from previous culture media were sufficiently diluted as to not impact the formation of nano-ZnS.

2.3 Abiogenic ZnS

The first type of abiogenic ZnS (labeled as abio-ZnS1 hereafter) was produced by titrating aqueous sulfide into a ZnCl₂ solution at room temperature (i.e., ~ 25 °C). Specifically, HEPES-buffered solutions of 15-mM Na₂S (solution 1) and 2-mM ZnCl₂ (solution 2), were prepared immediately prior to use, and 50 mL of solution 1 was titrated into 50 mL of solution 2 at a controlled speed of ~ 30 mL/h using a syringe pump. The formed precipitates were then separated from the solution by centrifugation (8000 g, 5 min). To reduce the addition rate of sulfide, we also adopted a diffusion-controlled pathway to synthesize the second type of abiogenic ZnS. In this case, 50 mL of HEPES-buffered solution containing a total concentration of 15 mM sulfide was added into double-layered dialysis tubing (500D), suspended in 50 mL of solution 2 at a room temperature of ~ 25°C (labeled as abio-ZnS2 hereafter). Precipitates resultant from the diffusion of sulfide and Zn were then separated from the solution by centrifugation. Both abio-ZnS1 and abio-ZnS2 samples were incubated in 20 mL of 0.1 % (m/m) Triton-X solution containing 100 µg of lysozyme and 100 µg of proteinase K following the same procedure as that for the biogenic samples before being analyzed. This specific step was mainly to remove the effects of different preparation procedures on the biogenic versus abiogenic samples.

2.4 Characterization

Powder X-Ray Diffraction The primary phases of the biogenic and abiogenic solids were analyzed using a Miniflex II XRD (Rigaku) equipped with a Cu K α source, with individual scans from 10° to 80° 2 θ (0.02 step) and a scan speed of 0.1.

High-Resolution Transmission Electron Microscopy The specimens for TEM analyses were prepared by loading dilute samples on the ultrathin carbon films of gold grids (from Ted Pella, Inc.). The dried specimen were then examined using a JEOL-2100 TEM, which was equipped with a Silicon drift-type energy dispersive X-ray spectroscopy (EDS) detector and two slow-scan CCD cameras, GATAN Orius SC200D CCD and Gatan Ultrascan, respectively, operating at 200 kV. The primary particle size and aggregation state of the abio-ZnS samples were further analyzed using a FEI Titan TEM with a field emission source operated at 300 kV. Annular dark field imaging of the Abio-ZnS samples was performed on the FEI Titan operated in scanning transmission electron microscopy (STEM) mode.

Qualitative Dissolution Experiments To characterize the dissolution behavior of the biogenic and abiogenic ZnS nanoparticles, we submerged the TEM specimen of abio-ZnS1 and bio-ZnS in 250 mL of hydrochloric acid solution (pH 3). The solution was constantly stirred at ~100 rpm in exposure to the atmosphere at room temperature (~22°C). The reacted samples were then analyzed at 12-48 h intervals using the JEOL-2100 TEM.

3. Results

3.1 Abiogenic ZnS: Aggregates of Ultrafine Nanoparticles

The ZnS formed via the titration procedure (abio-ZnS1) was identified as sphalerite by XRD (Fig. 1a), and the average crystal size was estimated to be 2 nm, 1 nm, and 2 nm, respectively, in the (111), (220), and (311) directions using the Scherrer equation. This low crystallinity in the abio-ZnS1 samples was confirmed by the HRTEM analysis (Fig. 2a-b), which revealed that they were composed of nanoparticle aggregates with a size range of ~15-40 nm. Closer examination of these abio-ZnS1 aggregates showed that they contained randomly-

oriented fine particles (mostly below $\sim 2\text{-}3\text{ nm}$) of sphalerite. The measured d-spacing for the displayed lattice fringes was $\sim 3.12\text{ \AA}$ (highlighted in Fig. 2a), indicating that the dominant faces for the primary particles were $\{111\}$ planes. The ZnS formed via the diffusion procedure (abio-ZnS2) exhibited similar particle-size and morphology, shown in Fig. 1b and Fig. 2b.

3.2 Bacteriogenic ZnS: Crystal Structure and Defects

By comparison, the biogenic samples were composed of much larger crystals showing little aggregation. Specifically, the bio-ZnS was primarily identified as sphalerite with three distinctive peaks at 2θ s of 28.2° , 47.5° , and 56.4° , respectively, by XRD (Fig. 1c), and the average crystal size was determined to be 6 nm in the (111) , 4 nm in the (220) , and 4 nm in the (311) directions using the Scherrer equation. Based on the HRTEM analysis, the bio-ZnS samples consisted of mono-dispersed nanocrystals with a primary size of $\sim 5\text{-}12\text{ nm}$. Although most of these nanocrystals were sphalerite (e.g., Fig. 3a and c-f), the presence of wurtzite was also confirmed based on the calculated interplanar spacing and angles using the corresponding FFT (e.g., see Fig. 3b). With the evidence of an entire nanoparticle displaying the wurtzite structure in the biogenic sample, we predict that the total volume fraction of nanoparticles having the wurtzite structure would be no more than a few % based on the Rietveld analysis using the corresponding peaks in the XRD data. The bio-ZnS nanocrystals exhibited a variety of morphologies, among which triangular 2D morphologies with larger crystal sizes were frequently observed (Fig. 4). We calculated the d-spacing for the exposed triangular faces of relevant nanocrystals and identified such faces as sphalerite $\{111\}$ planes. It is noteworthy that a positive relationship was found between the crystal-size of the bio-ZnS nanocrystals and the enlargement of their $\{111\}$ faces.

More interestingly, we found that a majority (i.e., > 80%) of the biogenic nanocrystals contained structural defects, such as twins and stacking faults (see examples in Figs. 3c-f). And in association with these defective regions, irregularities commonly existed in the lattice fringes (e.g., Fig. 5). To fully understand the change in the atomic structure of sphalerite caused by the introduction of simple twins and stacking faults, we analyzed two specific examples in our study (Fig. 6). The defects mostly extended along the {111} close-packed layers of the sphalerite structure (as illustrated in Fig 6 and the references cited within), and the original cubic stacking order (fcc) in the defect-free nanocrystals was modified into the hexagonal packing order (hcp) representing the wurtzite phase wherever a twin boundary or stacking fault was introduced. Thus, the occurrence of wurtzite structure “locally” in association with structural defects is well supported by this structural analysis. Other than the simple twins or stacking faults, we also confirmed the presence of multiple twins and/or stacking faults in the bio-ZnS samples (e.g., Fig. 5b and c), which was more important in generating irregularities in the lattice fringes. Further, the presence of structural defects can cause different surface relief in the observed nanoparticles (e.g., Fig. 5a).

3.3 Dissolution Behavior: Bacteriogenic versus Abiogenic ZnS

In the TEM-based, qualitative dissolution experiments, we observed different dissolution behaviors for the bio-ZnS versus abio-ZnS1 samples. For most bio-ZnS nanocrystals, the dissolution followed a certain retreating direction (e.g., arrows in Fig. 7a-b) rather than an equidimensional pattern of shrinkage. And HRTEM images suggest that the dissolution (of bio-ZnS nanocrystals) started where the structural defects (i.e., twins and stacking faults) intersected the nanocrystals’ surface (e.g. Fig. 7 c-d). In a few cases, fragmentation-type processes also seemed to be under way (e.g., lower right of the encircled area in Fig. 7e). For the abio-ZnS1 samples,

we were unable to recognize apparent dissolution directions or patterns however, mainly due to the samples' high aggregation state and small primary particle sizes, coupled with the randomness in the particle orientations. Judging from the average size and morphology of the reacted abiogenic samples, we suggested an equi-dimensional shrinkage pattern for their dissolution behavior.

4. Discussion

4.1 *Formation of ZnS from Simple Aqueous Solutions: A Nanocluster-Based Process*

The formation of ZnS from low-temperature aqueous solutions involves the formation and assembly of nanostructures (i.e., clusters). As has been demonstrated by previous studies (Kortan et al., 1990; Helz et al., 1993; Daskalakis and Helz, 1993; Luther et al., 1999; Hamad et al., 2002), the nucleation of ZnS occurs with the formation of sub-nanoscale rings [i.e., $\text{Zn}_3\text{S}_3(\text{H}_2\text{O})_6$ with a size of $\sim 7 \text{ \AA}$] via aggregation of soluble complexes. And cross-linking of these rings results in nanoscale clusters [i.e., $\text{Zn}_4\text{S}_6(\text{H}_2\text{O})_4^{4-}$ or $\text{Zn}_6\text{S}_4(\text{H}_2\text{O})_4^{4-}$ with a size of $\sim 9.7 \text{ \AA}$] that are structurally analogous to sphalerite. The sphalerite-like clusters are considered the basic building blocks for the final precipitates of ZnS.

In our study, the crystallinity described for the abiogenic samples suggests that the ZnS formation from simple aqueous solutions is a nucleation-dominated, crystal growth-restricted process, regardless of the sulfide addition rates. Viewed from the standpoint of the references just above, the ZnS nucleation and growth can be described as formation and crystallographic assembly of sphalerite-like clusters.

We attributed the high nucleation tendency in the abiotic solutions to the combined thermodynamic and kinetic drive for the nucleation process. In detail, the thermodynamic threshold for the ZnS nucleation, determined using the solubility of sphalerite (i.e., $K_{sp} = 2.5 \times 10^{-22}$ at 25°C) as a guideline, falls below ppb levels in terms of Zn^{2+} and/or sulfide concentrations, indicating that the energy barrier for the nucleation is negligible in our experimental systems. Meanwhile, the reaction kinetics for the formation of sub-nanoscale rings and sphalerite-like clusters was formerly evaluated, and shown to be extremely fast (Luther et al., 1999), which corroborated the observed dominance of ZnS nanoparticles ≤ 2 nm.

The restriction in ZnS crystal growth, on the other hand, reveals an intrinsic difficulty for the condensation and crystallographic assembly of sphalerite-like nanoclusters. Such difficulty likely stems from the structural instability of the nanoclusters, mainly resulting from the permanent dipole moments existent in the structure. Although controversy remains regarding the definition of “nanoclusters”, we use the word here *sensu lato* to include both basic cluster structures [i.e., $\text{Zn}_6\text{S}_4(\text{H}_2\text{O})_4^{4-}$] and larger nanostructures (up to 5 nm) that are constructed of the basic ones. Evidenced by both experimental and computational studies (Wright et al., 1998; Leung and Whaley, 1999; Rabani 2001; Spano et al., 2003; Zhang et al., 2003; Burnin et al., 2005), ZnS and other metal-chalcogenide (i.e., CdS) nanoclusters undergo significant relaxation and reconstruction of their (surface) structure under aqueous conditions due to the dipole-dipole interactions of the structure and surrounding water molecules, and as a result, these nanoclusters cannot maintain their sphalerite-like configuration. This water-induced instability for the ZnS nanoclusters fully justifies the difficulty in their crystallographic assembly, which led to the observed ultrafine crystal size and high aggregation in the abiogenic samples.

4.2 Effects of Biological Components on ZnS Crystallization

In comparison, the biogenic samples exhibited significantly enhanced crystallinity, indicating that the presence of bacterial cells and metabolites promoted the crystal growth of ZnS. Although a few previous studies have demonstrated the close association between extracellular organic matter and biogenic nanoparticles [e.g., Moreau et al. (2007) confirmed the incorporation of proteins in the spheroidal aggregates of biogenic ZnS nanocrystals produced via bacterial sulfate reduction], the mechanisms involved in the metabolite-ZnS growth interactions are generally poorly understood. Here we attribute the enhancement of the biogenic ZnS crystallization observed in this study mainly to the complexation and stabilization effects of the bacterial metabolites.

A number of biomolecules and organic ligands that are present in the cell culture medium of sulfate-reducing bacteria (e.g., acetate, amino acids and polysaccharides) can form relatively stable complexes with Zn^{2+} under aqueous conditions. For example, acetate is known to mediate the concentrations of free Zn^{2+} by forming Zn-acetate complexes under relatively rapid reaction kinetics (Giordano and Drummond, 1991; Gyoryova and Balek, 1993; Shock and Koretsky, 1993; Delolme et al., 2003; Borg and Liu, 2010). In the biological system of our study, acetate was produced along with sulfide at a ratio of 2:1, and thus, the availability of free Zn^{2+} might be effectively limited, resulting in a slow-down of the ZnS nucleation. This retarded nucleation rate could promote crystal growth indirectly by giving more flexibility to the assembly of the formed nuclei (i.e., nanoclusters). Besides acetate, other (poly)anionic species such as phosphate, amino acids, and polysaccharides, existent in the biological system, are also capable of complexing Zn^{2+} (Bruland, 1989; Deonaraine et al., 2011). Such complexation effects of bacterial metabolites is not equivalent to simply modifying the nucleation rate of ZnS, however, because no enhancement was observed in the crystal size of the abiogenic ZnS samples when the sulfide

addition rate was dramatically decreased in the abiotic system (i.e., abio-ZnS1 vs. abio-ZnS2). This result indicated that the bacterial metabolites are also involved in the crystallization of ZnS via other mechanisms.

One mechanism of ZnS growth under the influence of microbes is likely related to the stabilization effects of bacterial metabolites on the polar faces of the ZnS nanoclusters/nanoparticles (Barnard et al., 2010). In detail, the biogenic nanocrystals of sphalerite were found to be dominated by particularly enlarged {111} faces, indicating that these polar faces were preferentially inhibited from growth in the presence of bacterial cells and metabolites. Such selective stabilization of the {111} faces likely provided an important mechanism for the sphalerite-like nanoclusters/nanoparticles to overcome their structural instability induced by water. Specifically, the {111} faces, being the only polar planes in sphalerite structure (as illuminated in Fig. 8 a-c), are most susceptible to interactions with polar molecules (like water) in their vicinity, which may cause the surface reconstruction and disorder previously reported in ZnS nanoclusters (Zhang et al., 2002; Spano et al., 2003; Gilbert et al., 2004). Therefore, minimizing the polarity of the {111} faces (e.g., via adsorption of biomolecules available) in the biological system can effectively stabilize the sphalerite-like structure in the formed nanoclusters/nanoparticles, thereby furthering their crystallographic assembly. Significantly, we also noticed that, the enlargement of {111} faces in the biogenic samples became more apparent in larger nanocrystals, for example, nanocrystals composed of {111} faces and showing triangular 2D morphologies were frequently identified in the nanocrystal >10 nm (Fig. 4). These results provided even stronger evidence for the correlation between the enhanced ZnS crystallization in the biological system with the stabilization of polar faces in the ZnS growth units.

In terms of the stabilization effect, a range of biomolecules available in the bacterial metabolites were likely involved, and among them, the amphiphiles (e.g., phospholipids and amino acids) that contain polar head-groups and nonpolar hydrocarbon tails were likely most effective in screening the dipole moments of the sphalerite nanostructures. Previous studies on the synthesis of ZnS nanoparticles (Meulenberg and Strouse, 2001; Moore and Wang et al., 2006; Liu et al., 2011) showed that using amines and ethylene glycols as solvents could effectively prevent the surface relaxation of metal-chalcogenide nanoclusters and nanoparticles, largely in line with our experimental results and understanding. Moreover, recent studies (Jain et al., 2015 and references cited therein) on biogenic nanoparticles reveal that proteins and carbohydrates in extracellular polymeric substances in anaerobic reactors have strong interactions with the formed nanoparticles, governing their colloidal stability via modifying surface charge.

4.3 Structural Defects Associated with ZnS Crystallization

The occurrence of structural defects in the biogenic nanocrystals is likely a direct consequence of the instability of ZnS (i.e., sphalerite) nanostructures in aqueous environments, and meanwhile, reflects the metabolite-mediated, nanocluster-based crystallization pathway. Specifically, the dipole-dipole interactions between ZnS nanoclusters and water molecules can cause significant structural strains. Although previous computational studies (Spano et al., 2003; Hamad et al., 2005) suggested that such strains could modify original sphalerite-like structures to bubble-like polyhedral structures, the latter ones have not been reported in experimental work yet (to the best of our knowledge). Instead, stacking disorders marking phase transitions were frequently observed in nanocrystals of metal chalcogenides (Murray et al., 1993; Tolbert and Alivisatos, 1994). Therefore, the water-induced structural strains in the ZnS nanostructures might

be more likely released via variations in the stacking orders of the {111} faces (e.g., stacking faults) rather than relaxing the overall nanocluster structure. Further, the crystallization of ZnS in the biological system in this study was revealed to involve the assembly of individual sphalerite-like nanoclusters, particularly stabilized at and enlarged in their {111} faces by the bacterial metabolites. Thus, the common occurrence of structural defects (i.e., twins and stacking faults) also manifests a relatively high probability of imperfect lattice matching of two {111} surfaces coming together in the presence of the surface-stabilizing biomolecules.

We closely examined a large fraction of the twins and stacking faults identified in the biogenic nanocrystals (e.g., Fig. 9), and found that these defects (i.e., both stacking faults and twins) exclusively occurred at {111} planes. This finding strongly corroborates the roles proposed for the {111} faces in stabilizing ZnS nanostructures and in epitaxially merging different ZnS nanoclusters.

We included several examples (in Fig. 9) to demonstrate the different sizes and morphologies of the nanocrystals that accommodated the observed structural defects. In Fig. 9a-b, the associated nanocrystals were relatively small (e.g., 3-4nm) and the defects seemed to be an integral part of the nanocrystals. By contrast, the defects in Fig. 9c-d apparently resulted from the epitaxial merging (a.k.a., oriented attachment) of relatively large individual nanoparticles. Here we note that these two types of twins (Fig. 9a-b vs. Fig. 9c-d), in essence, were formed via similar pathways, both through the crystallographic assembly of sphalerite-like nanostructures, but for the former, the unit size was much smaller (i.e., nanoclusters below or ~ 1 nm) than those for the latter (i.e., nanocrystals of ~ 5 nm). As the nanocrystals grew bigger in size, their epitaxial merging also resulted in higher crystallographic anisotropy in the newly formed crystals (e.g., Fig. 9c-d).

Interestingly, the atomic structure for the twins identified in the biogenic nanocrystals (Fig. 6) was similar to that previously illustrated for bulk crystals (Minceva-Stefanova, 1981; Pósfai and Buseck, 1997; Srot et al., 2003), suggesting that the instability of ZnS crystals and the formation of structural defects are intrinsic to the ZnS crystallization process under different P-T conditions. Further, similar twins and stacking faults were also reported in synthetic ZnS nanoparticles produced with the assistance of organic capping agents at elevated temperatures, e.g., 170°C (Murray et al., 1993; Tolbert and Alivisatos, 1994; Nanda et al., 2000; Huang et al., 2003).

4.4 Implication for ZnS Nanoparticles in the Environment

The distinctive size, morphology, and crystal structure exhibited by the biogenic nanocrystals in our study may provide new restraints for the origin of ZnS nanoparticles that are detected in various natural and anthropogenic settings. Both crystalline and amorphous ZnS nanoparticles have been previously identified in nature. For example, spherical aggregates of defective nanocrystals of ZnS were found in association with bacterial biofilms in a Pb-Zn mine (Labrenz et al., 2000; Moreau et al., 2004). HRTEM analysis revealed that the primary particle size for these biogenic aggregates was in a range of 3-5 nm, consistent with our results. Similar aggregates of ZnS were also recognized in sludges from a wastewater treatment plant, and the primary crystals showed a size range of 4-7 nm (Kim et al., 2014). In comparison, irregular-shaped aggregates of ZnS were identified in the anoxic sediments of the Clark Fork River (Hochella et al., 2005) and in the oxic water columns of the Seine River (Priadi et al., 2012). Further analyses revealed that the ZnS in the samples from these two scenarios was completely amorphous. Besides the information preserved in the nanoparticles (e.g., crystal size and

structure), the biogenic nanoparticles of ZnS were also more physically confined in their mobility due to the presence of bacterial cells and extracellular polymeric substances that facilitated the formation of biofilms (exemplified in Moreau et al., 2007). This limitation effect of the cells and extracellular polymers could promote a more uniform morphology in the secondary aggregates of the biogenic ZnS (Fig. S3), also shown in Moreau et al., 2004.

Reversely, the variation in size, aggregation-state, and crystallinity of the ZnS nanoparticles formed via different pathways also has an impact on the nanoparticles' reactivity (e.g., dissolution and the capacity to adsorb contaminants) and fate in the environment. In our study, although we could not obtain quantitative data regarding the dissolution rates of biogenic versus abiogenic nanocrystals at this time, we qualitatively estimated the percentage for the diminution of biogenic versus abiogenic nanocrystals/aggregates at pH 3 for a duration of 24 h. While most of the abiogenic aggregates had disappeared by the end of the dissolution experiment, significant fractions of the biogenic nanocrystals remained (as shown in Fig. 7). If we assume that initial loadings of biogenic versus abiogenic ZnS nanoparticles (by weight) were comparable on the TEM holders, our TEM analysis-based estimation strongly suggests that the larger nanocrystals of biological origin are more resistant to dissolution under acidic conditions than the abiogenic aggregates composed of much finer nanoparticles. This is consistent with previous understanding that dissolution rates increase with decreasing particle sizes (Liu et al., 2008, 2009). Additionally, the aggregates of poorly crystalline ZnS may also exhibit high adsorption capacity for toxic metals, for example, the samples of amorphous ZnS from the riverbeds of the Clark Fork River incorporated relatively high concentrations of arsenic and lead at the ZnS nanoparticles' surfaces (Hochella et al., 2005).

The relationship between the ZnS nanoparticles' physicochemical properties and formation mechanisms identified in our study may provide important insight regarding the origin and fate of ZnS nanoparticles in natural and engineered environments. It may be premature, however, to conclude on what criteria may be used to distinguish the biogenic versus abiogenic ZnS in the field. The major reason for this is that our current results are based on relatively nascent nanoparticles formed in the biological and abiotic systems. As the ZnS nanoparticles collected from the field may have different ages, it becomes equally important to understand how these nascent biogenic and abiogenic nanoparticles are modified in their physicochemical properties by aging. A follow-up study on the in-situ transformation of biogenic versus abiogenic ZnS nanoparticles is under investigation.

In summary, our data showed that biological and abiotic processes under the same temperature and pressure conditions can lead to distinctive particle sizes, morphology, and crystal structure in the formed nanoparticles of ZnS. Overall, the formation of ZnS from low-temperature aqueous solutions is a nucleation-dominated, crystallization-restricted process. And the difficulty in the ZnS crystallization likely stems from the surface instability of the sphalerite-like nanostructures, induced by the dipole-dipole interactions between the structure and surrounding water molecules. The presence of the bacterial cells and metabolites in the biological system plays an enhancing role in the crystallinity of the formed ZnS nanoparticles, likely via preferentially stabilizing the polar faces (i.e., {111}) of the growth units. This metabolite-mediated, nanocluster-based crystallization pathway also results in the common occurrence of structural defects such as twins and stacking faults in the biogenic nanocrystals of ZnS.

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647

Appendix Table 1. Composition of cell culture media (in g·L⁻¹) used for the biological experiments

	Modified Postdate's (#63)	Modified Metal Toxicity Medium	Zn-Amended Modified Metal Toxicity Medium
K ₂ HPO ₄	0.5	-	-
NH ₄ Cl	1.0	-	-
(NH ₄) ₂ SO ₄	-	1.23	1.23
Na ₂ SO ₄	1.0	0.91	0.91
CaCl ₂ ·2H ₂ O	0.1	0.06	0.06
MgSO ₄ ·7H ₂ O	2.0	1.0	1.0
Na-lactate	2.0	4.6	4.6
Yeast extract	1.0	0.05	0.05
Tryptone	-	0.5	0.5
Ascorbic acid	0.1	-	-
Na-thioglycolate	0.1	-	-
FeSO ₄ ·7H ₂ O	0.5	-	-
ZnCl ₂	-	-	0.11
HEPES	-	5.5	5.5
pH	7.8	7.2	7.2

Figure

Figure 1. X-ray diffraction analyses for the abiogenic (a-b) and biogenic samples (c) show that the obtained ZnS nanoparticles were primarily sphalerite. The reference peaks and corresponding faces as well as d-spacing for sphalerite (blue tick marks) and wurtzite (red tick marks) are also indicated.

Figure 2. Representative transmission electron micrographs for the abiogenic ZnS. (a-b) High-resolution bright-field micrographs of AbioZnS1 and AbioZnS2, respectively (the regions where the nanoparticles did not overlap with each other were also highlighted); (c) STEM-annular dark field (ADF) micrograph for AbioZnS2.

Figure 3. Transmission electron micrographs of the bio-ZnS nanocrystals showing various sizes, morphologies, and structural attributes. Examples of structural defects in the bio-ZnS samples are highlighted. The zone axis for each micrograph was indexed using their FFT patterns (refer to supplementary Fig. S2 for more information). The nanocrystal displayed in (b) has been identified as wurtzite whereas the rest as sphalerite.

Figure 4. Transmission electron micrographs of the bio-ZnS nanocrystals showing triangular 2D morphology. The d-spacing for the revealed faces of these nanocrystals is exclusively ~ 3.13 Å, consistent with that of sphalerite {111} planes.

Figure 5. Transmission electron micrographs for the bio-ZnS nanocrystals showing anomalies (highlighted) in the lattice fringes. Such anomalies are locally associated with structural defects. For example, multiple twins (white lines and arrows) are present in (c). The zone axes for the nanocrystals in these images were all identified as sphalerite [110].

a. Perfect

b. Twins

c. Single Stacking Fault

Figure 6. Analysis for the atomic structure of (a) defect-free and (b-c) defect-containing bio-ZnS nanocrystals. The transmission electron micrographs are obtained in this study, and the corresponding atomic models are modified from previous studies (Pósfai et al., 1988; Srot et al., 2003). (The atomic models only show one possible orientation for the arrangement of Zn and S.) Both the identified twin (highlighted by yellow dash line) and stacking fault (highlighted by green dash line) extend along the sphalerite {111} planes, modifying the original cubic stacking-order into hexagonal ones (i.e., {001} in wurtzite) at the twin boundary or stacking faults.

Figure 7. Transmission electron micrographs for the bio-ZnS nanocrystals after the qualitative dissolution experiments for ~ 24 h. The original and dissolution morphologies for the nanocrystals as well as the dissolution direction are indicated using dotted lines and arrows.

Figure 8. Projected atomic structures on the major planes of sphalerite (a-c) and of wurtzite (d), obtained using CrystalMaker™. (a) Zn/S-terminated $\{111\}$, (b) $\{110\}$ and (c) $\{001\}$ of sphalerite. (d) $\{001\}$ of wurtzite.

Figure 9. Transmission electron micrographs for the bio-ZnS nanocrystals showing structural defects. (a-b) Twins that appear intrinsic to the nanocrystals; and (c-d) twins and stacking faults that are likely from epitaxial merging of individual nanocrystals.