

# *In situ* electron microscopy studies of calcium carbonate precipitation from aqueous solution with and without organic additives



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## ARTICLE INFO

### Article history:

Available online 4 June 2013

### Keywords:

Biom mineralization  
*In situ* electron microscopy  
Crystallization  
Calcium carbonate  
Electron microscopy

## ABSTRACT

For the understanding of mineral formation processes from solution it is important to obtain a deeper insight into the dynamics of crystal growth. In this study we applied for this purpose a novel atmospheric scanning electron microscope that allows the investigation of CaCO<sub>3</sub> particle formation in solution under atmospheric conditions with a resolution of approximately 10 nm. Furthermore it permits the *in situ* observation of the dynamics of crystal evolution. With this tool the precipitation of CaCO<sub>3</sub> was studied in the absence and presence of additives, namely poly(acrylic acid) and poly(styrene sulfonate-co-maleic acid) which are known to influence the crystal growth rate and morphology. We determined particle growth rates and investigated the formation and dissolution dynamics of an observed transient phase, believed to be amorphous calcium carbonate. This technique also enabled us to study the depletion zones, areas of lower intensity due to reduced ion concentrations. Ion flux rates were obtained from the depletion zone width, which amounted to several μm assuming the formation and dissolution dynamics of amorphous calcium carbonate being the rate determining process. This assumption was confirmed since the obtained fluxes were found to be in good agreement with fluxes derived from the experimentally observed crystal growth rates.

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## 1. Introduction

The role of a transient amorphous precursor phase for crystal formation has been identified as crucial for the precipitation of a mineral phase in many biological systems (Terminé and Posner, 1967; Raz et al., 2002; Addadi et al., 2003; Mahamid et al., 2010). Therefore, understanding the growth and dissolution dynamics of this phase and its properties is of paramount importance for unraveling the mechanisms behind the formation of highly complex skeleton- and shell structures formed by vertebrates and invertebrates. Biological systems exert a tight control over composition, shape, ultrastructure and polymorph selection of the mineralized phase from the nano- to the mesoscale. One important pathway of control is the introduction of polymers with different functionalities, in particular proteins and polysaccharides produced by the organism, which lead to variations of nucleation, growth rates, polymorphs and shape of the mineral phase (Belcher et al., 1996; Chasteen and Harrison, 1999; Arias and Fernández, 2008; Kwak et al., 2009). The interaction of these additives with the amorphous

precursor phase has attracted great attention since it is believed that its understanding helps to explain the promotion and inhibition of mineralization as well as the shape control by organisms (Gower and Odom, 2000; Chen et al., 2009; Verch et al., 2011).

The mechanism behind precipitation remains an area of intensive research. As one result of this research it becomes increasingly clear that the formation of precursor phases and agglomerates follows hierarchical patterns from the atomic level to the mesoscale and by that determining the functionality of the resulting organic/mineral composite (Aizenberg et al., 2005). In recent years various *in situ* techniques have been applied in order to study the involved steps and the dynamics of calcium carbonate crystallization in solution. *In situ* X-ray diffraction for instance permits the study of the evolution of the crystallinity (Wolf et al., 2008) or particle sizes (Bots et al., 2012). However, it is not possible to observe the development of an individual crystal/particle as the data obtained are usually averaged over all particles present in the pathway of the X-ray beam. Atomic force microscopy (AFM) and electron microscopy facilitate imaging with desirable, high spatial resolutions. *In situ* AFM as a surface sensitive method allows for example the visualization of the step growth of crystals (Wasylenki et al., 2005), but traditionally suffers from relatively low scan speeds, though high-speed atomic force microscopes have been developed in recent years (Toshio, 2012). Traditional electron microscopy, on the other hand, requires a high vacuum to avoid

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electron scattering. This boundary condition makes it impossible to study biological or similar materials in their native – often aqueous – environment. Cryo-electron microscopy offers a way to maintain the vacuum and the fluid environment – albeit in a frozen state – and providing so called quasi time-resolved information about the crystallization process of calcium carbonate (Pouget et al., 2009). However, the samples are often altered during the sample preparation procedure such that it is frequently impossible to distinguish between genuine features and artifacts introduced during the sample preparation for imaging (Hurbain and Sachse, 2011).

Novel approaches to this problem make use of specially designed microscopes or holders that allow for electron imaging through thin membranes keeping the investigated sample in a liquid environment while still making use of the high spatial resolution provided by the electron microscope (de Jonge et al., 2010; Nishiyama et al., 2010).

In this work we employed a novel technique to study precipitation from a supersaturated solution of calcium and carbonate ions: an atmospheric scanning electron microscopy (ASEM) (Nishiyama et al., 2010). This technique allows imaging of crystal formation within the liquid phase under atmospheric conditions and facilitates the study of growth dynamics of  $\text{CaCO}_3$ . In addition to experiments in the absence of additives, we investigated the  $\text{CaCO}_3$  growth in the presence of macromolecules that are known to affect the crystal formation. We utilised poly(styrene sulfonate-co-maleic acid) (PSS-MA) and poly(acrylic acid) (PAA) for this purpose. The first polymer is an amorphous  $\text{CaCO}_3$  (ACC) stabilizing mesocrystal mediator (Song et al., 2008) while the latter polymer is known to stabilize polymer induced liquid precursor (PILP) phases and ACC (Gower and Odom, 2000). In industrial applications it is frequently used as an anti-scaling and dispersing agent (Gill, 1999).

## 2. Experimental details

### 2.1. Atmospheric scanning electron microscopy

The experiments were performed using a JEOL JASM-6200 scanning electron microscope (ClairScope™). A schematic drawing of the experimental setup is shown in Fig. 1. The instrument consists of two components. The lower component is kept under vacuum and comprises an inverted atmospheric SEM and a backscattered electron (BSE) detector, while the upper component remains under atmospheric pressure with an optical microscope allowing also for fluorescence imaging not used in our studies. Both components are separated by a  $\sim 100$  nm thin silicon nitride membrane ( $\text{SiN}_x$ ,  $250 \times 250 \mu\text{m}$  lateral length) in the centre of a disposable 35 mm diameter plastic culture dish, through which the electrons can penetrate and interact with the sample that is maintained under

atmospheric pressure. Electrons backscattered by the sample and atoms in the solution are used for the image formation. This design allows for *in situ* studies of crystallization processes through the  $\text{SiN}_x$ -membrane. However, the mean free path of electrons through liquids is very limited and hence the information depth using an aqueous solution under the imaging conditions applied in our experiments is in the range of several  $\mu\text{m}$  (Morrison et al., 2012).

The instrument was initially used at 30 kV for best resolution and penetration depth, but most experiments were performed at 20 kV to reduce electron beam damage to the sample.

### 2.2. Sample preparation

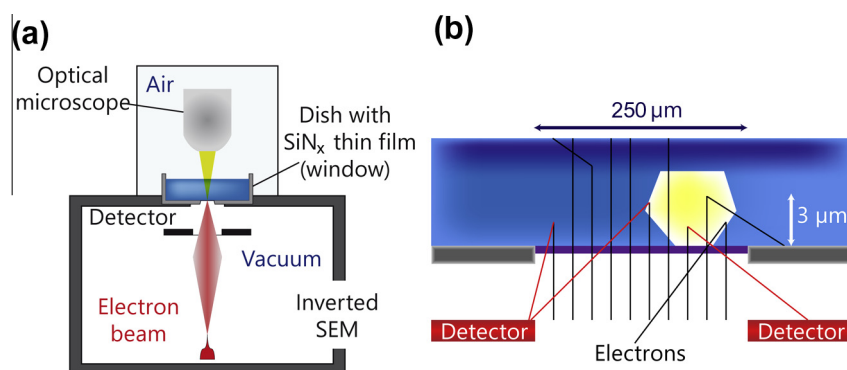
Aqueous solutions of  $\text{Na}_2\text{CO}_3$  (10 mM, Sigma–Aldrich, Cat.No. 223530) and  $\text{CaCl}_2$  (10 mM, Sigma–Aldrich, Cat.No. 223506) were freshly prepared using deionized water (18.2 M $\Omega$  cm). This concentration was chosen since it provides nucleation times below approx. 30 min at room temperature, which is approximately the lifetime of the  $\text{SiN}$  membranes under electron irradiation, allowing a direct observation with ASEM. PSS-MA (total final concentration: 350 and 85 mg/L) and PAA (5 mg/L), when applied, were added to the  $\text{CaCl}_2$  solution. The sample dishes for the ASEM studies (JEOL Welwyn Garden City U.K.) were treated with an air plasma for 40 s directly before the experiment in order to hydrophilize the membrane surface. The solutions were filtered through a  $0.22 \mu\text{m}$  filter before use. The calcium ion solution was added to the dish and the reaction started about 5 min later by adding the same volume of  $\text{Na}_2\text{CO}_3$  solution. After the ASEM observations, the supernatant reaction solution was removed from the dish. After drying under air the crystals were examined in a conventional SEM (FEI Sirion S-FEG). Poly(4-styrenesulfonic acid-co-maleic acid sodium salt) (Cat.No. 434566) and poly(acrylic acid) (Cat.No. 416029) were purchased from Sigma–Aldrich and used without further purification. All experiments were conducted at least twice: 4 times in the absence of polymer additives, twice in the presence of PSS-MA and 3 times in the presence of PAA.

The crystal growth rates were evaluated by applying a threshold to the image pixels and by determining the crystal area using the freeware ImageJ software (Schneider et al., 2012). The depletion zone widths were derived from 20 pixels wide line scans across the crystal area.

## 3. Results and discussion

### 3.1. Precipitation without additives

To study the precipitation we investigated the formation of  $\text{CaCO}_3$  crystals growing from a supersaturated aqueous solution



**Fig. 1.** Schematic for the experimental setup of the atmospheric SEM (ASEM) used in this work. (a) Arrangement of inverted SEM at the bottom and petri dish at the top. (b) Imaging is performed through a  $\text{SiN}_x$  membrane at the bottom of the petri dish containing the supersaturated  $\text{CaCO}_3$  solutions.

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