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# Single Amino Acids as Additives Modulating $\text{CaCO}_3$ Mineralization

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## 1. Introduction

Biom mineralization has become an important theme in the fields of biology, chemistry, and materials science. It is an ability of organisms to produce organic-inorganic composite structures, which mainly serve a function for storage, protection or skeletal support. What makes biomineralization such an interesting subject of research is the simplicity organisms are able to modify their surroundings into complex materials. If you simply compare biogenic and geologically produced minerals with the same chemical compositions, you will notice a substantial difference in ultrastructure and subsequently, difference in properties.

There are many different examples where biomineralization can be witnessed, like in sea coral, teeth or eggshells. The diversity of structures, minerals and macromolecules that build up these tissues is impressive (1, 2, 3). Diatoms are able to precipitate silica from the environment to create their ornate skeletons. Another example of biomineralization is the mineral magnetite with its ferromagnetic properties. Not only it is found in many microorganisms as a structural component, but it can furthermore be used for navigation along the Earth's magnetic field. In all these diverse examples of biomineralization processes, a common theme can be found in each case—mineralization occurs in the presence of an organic matrix that appears to direct mineral and ultrastructural morphology (4-6) (7) (8) (9-11) (12). A closer look at the mineralized tissues of certain invertebrates shows that  $\text{CaCO}_3$  is the predominant mineral composition utilized, but have completely different structural features from its geological counterparts. It is this same geological mineral, which is usually brittle and very unstable under shear forces, that provides strength and structure to invertebrates. The combination of organic and inorganic materials and the control of growth make these biologically mineralized structures mechanically tough and highly resilient materials (13).

In Nature,  $\text{CaCO}_3$  can be found in three different forms of mineral-modifications (14). The most frequent and thermodynamically stable form is calcite. It consists of trigonal crystals and appears mostly transparent or milky in solution. Also well-known are vaterite and aragonite. They both have the same chemical stoichiometry but form a crystalline lattice that is different from calcite due to the differences in packing of the unit cells in each respective polymorph. Aragonite is metastable and slowly transforms to calcite after some time.

Aragonitic crystals have a prismatic structure. Vaterite is also metastable and is rare, but when it does form, it occurs in orthorhombic unit cells (Figure 1).

A significant form of  $\text{CaCO}_3$  and until recently, found to be as ubiquitous as its crystalline forms, emerges in the first stage of pre-nucleation, is amorphous calcium carbonate (ACC). Addadi and coworkers found that an amorphous phase, actually composed of several independent phases, was involved in the formation of certain biomineralized structures (15) (16). Gilbert et al. reports the thermodynamics of ACC I and ACC II types during transformation to crystalline products (17). Gebauer and coworkers demonstrated that these two different species of ACC control the subsequent formation of calcite or vaterite. Furthermore, they showed that a part of  $\text{Ca}^{2+}$  ions forms neutral equilibrium clusters before nucleation of the supersaturated solution arises (18). Several groups have also investigated the influence of different additives on the nucleation of  $\text{CaCO}_3$  (19-22). Amongst others, polycarboxylates which usually serve as inhibitors of the formation of scale in laundry detergents or dishwaters were used to investigation inhibition of nucleation (23). The experiments revealed that additives and acidic polymers have very different effects on nucleation such as adsorption of calcium ions or an influence on soluble-cluster formation (18). These previous studies show that nucleation of calcium carbonate is a complex, multistep process whereby additives often are a major determinant driving crystallization of  $\text{CaCO}_3$  (18) (24).

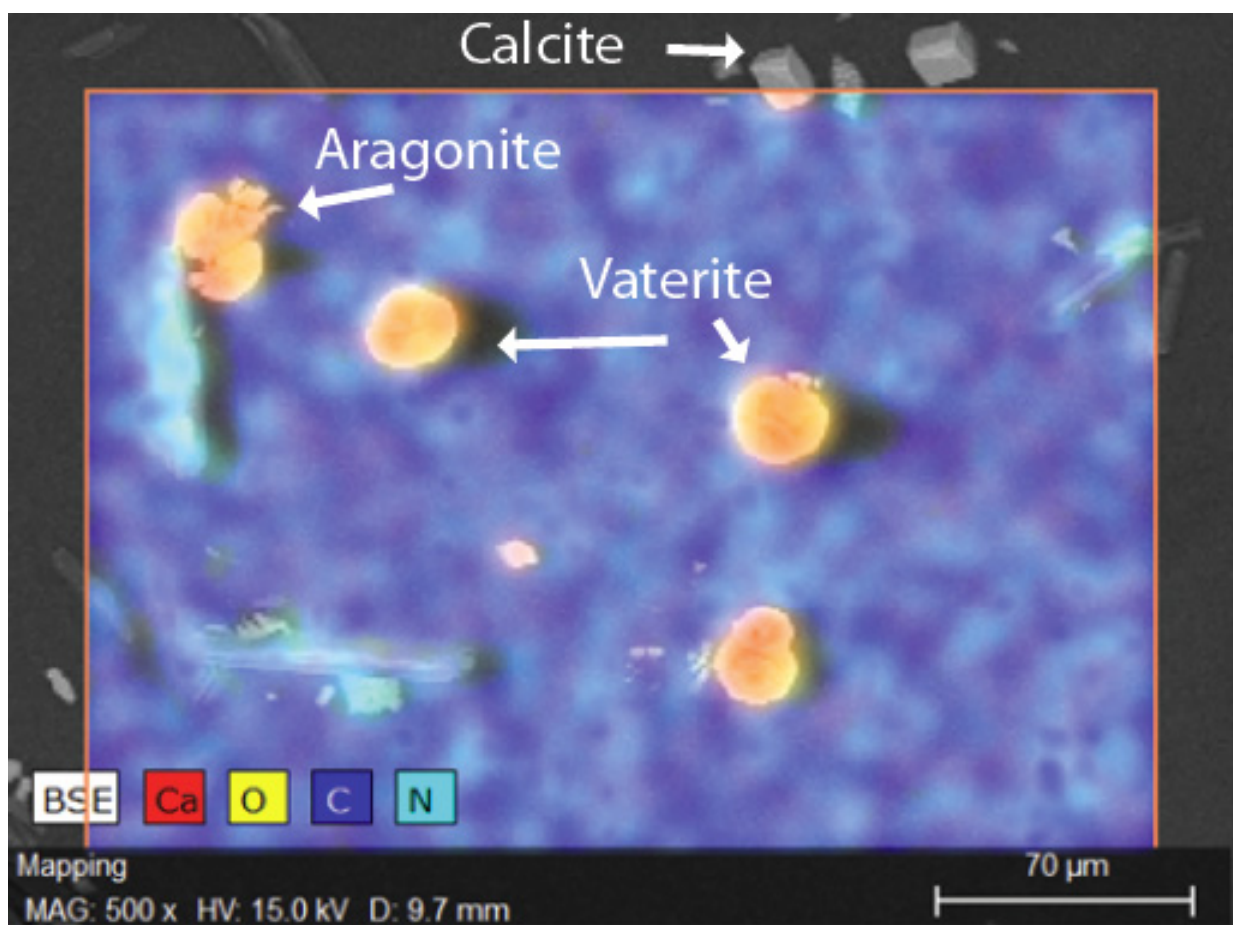


Fig. 1. SEM-EDX image of the three various polymorphs (calcite, aragonite, and vaterite) of  $\text{CaCO}_3$  mineral

## 2. Amino acids

Several groups have examined the effect of biological additives, proteins in their native conformations, on the effects of calcium carbonate mineralization. From secondary to tertiary conformations, several aspects of structure modulate the effect--including the magnitude of effect specific proteins have on crystallization. To simplify the understanding of these protein interactions, we attempt to describe the influence of their constituent amino acid groups. In this manner, structure is removed in order to determine the specific effects of amino acids on crystallization. There are twenty so called natural amino acids, which differ in their side chain functional groups (25). Eight of them are considered to be essential that means that they cannot be produced from other compounds by the human body (25). Some of these relevant amino acids are found to modulate calcium carbonate mineralization. Their characteristics are briefly described in the following sections.

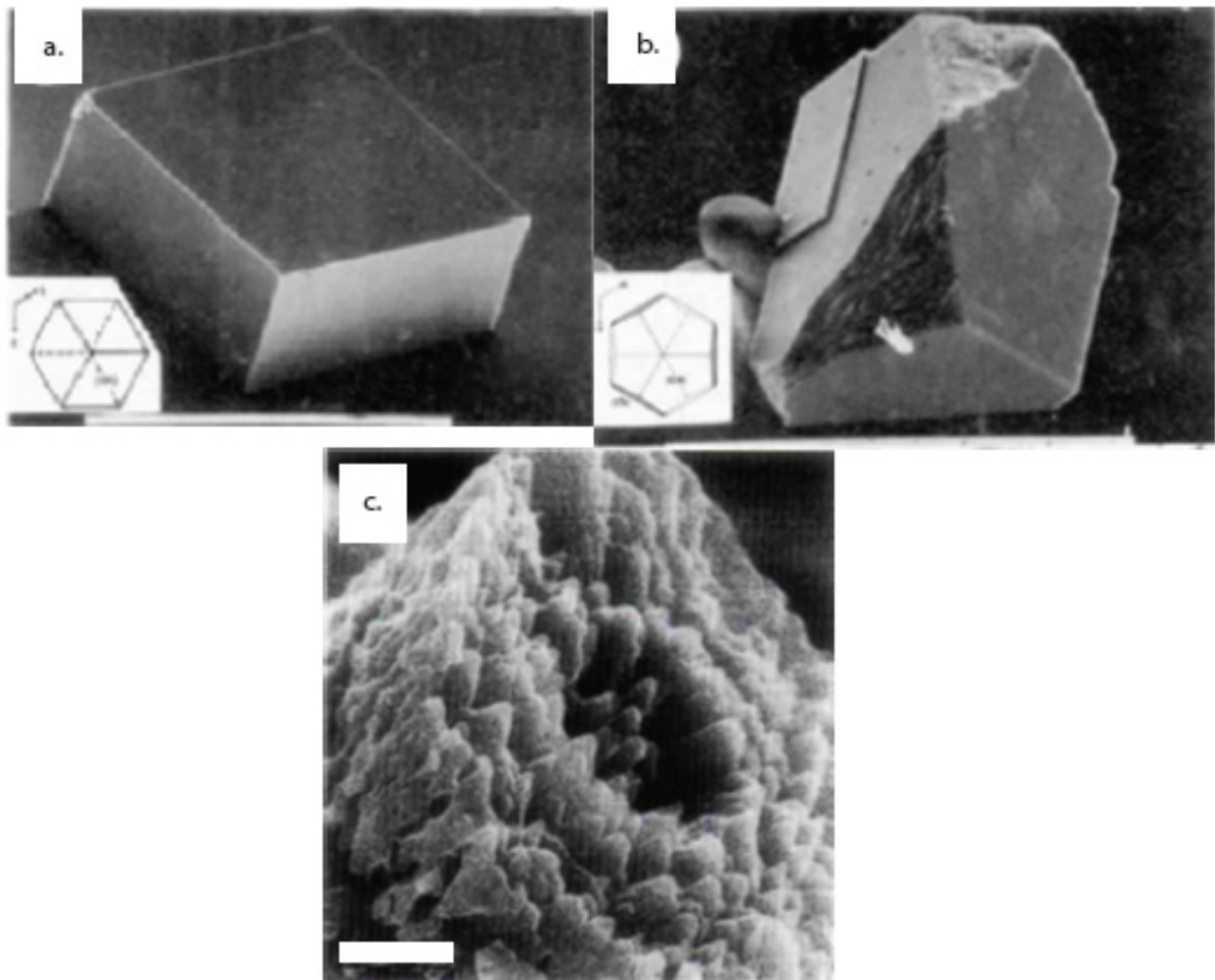


Fig. 2. Various morphologies of  $\text{CaCO}_3$  single crystalline precipitates (a.) geological calcite (b.) calcite grown in the presence of sea urchin spicule matrix proteins [scale bar = 100  $\mu\text{m}$ ] (c.) fracture surface of a broken sea urchin spicule [scale bar = 500 nm]

Arginine is a basic amino acid with a  $pK_a$  of 12.48. The side chain contains a complex guanidinium group which is positively charged under neutral, acidic and even most basic conditions. This explains the alkaline characteristics. Asparagine is a polar amino acid with a carboxamide group in its side chain. Asparagine as well as arginine are nonessential amino acids. Glutamic acid belongs to the acidic amino acids with a  $pK_a$  of 4.1. Its side chain contains a carboxylic acid at the end. Furthermore, glutamic acid does not belong to the essential amino acids. In contrast, methionine is one of the few essential amino acids. Together with cysteine, these amino acids are the only two whose side chain consists of a sulphur group. Proline has a unique structure among the twenty amino acids because of its secondary  $\alpha$ -amino group. The cyclic structure of proline shows nonpolar behavior. Serine is a polar amino acid due to the hydroxyl group in the side chain. Valine is an essential amino acid. Together with leucine and isoleucine, it belongs to the branched-chain amino acids. Due to its alkyl side chain, it shows a nonpolar character (25).

However, it is not yet known which amino acids are responsible for the different tasks concerning crystal nucleation and formation, especially their functional side groups. From this these questions about function and structural characteristics, it is particularly interesting to investigate the effects of crystallization of  $CaCO_3$  with the addition of different amino acids as additives. Crystal morphology as well and crystallization kinetics in  $CaCO_3$  formation have been specifically examined in the presence of specific amino acid sequences (Figure 2).

All twenty amino acids have been surveyed and the amino acids that had dramatic morphology effects on the  $CaCO_3$  precipitates in comparison to their geological analogs were further characterized with other methods.

### 3. Vapor-diffusion crystallization

The precipitation of calcium carbonate offers a model system for the investigation of nucleation and subsequent crystal growth (26). A simple, but still effective method to monitor various precipitations is the “vapor diffusion technique.” In the case of  $CaCO_3$ , it is based on the decomposition of  $(NH_4)_2CO_3$  or  $NH_4HCO_3$  into  $CO_2$  and  $NH_3$  (Figure 3). The precipitation of a solid phase is described by the following equations:



whereby Equation (1) describes the formation of bicarbonate ions from  $CO_2$  and water as an initial step to formation of calcium carbonate in Equation (2). The reaction is performed in a desiccator which provides an isolated environmental chamber. In our case, the “vapor-diffusion crystallization” technique, is a variation of “vapor diffusion,” has been used. For this purpose, a dish of  $NH_4HCO_3$  solution was located at the bottom of the chamber. Drops of  $CaCl_2$  solution were placed on cover slides, which were located on a shelf above the solution (Figure 3). Despite its easy handling, this method has also disadvantages. Disadvantages include low reproducibility of the precipitation process and difficulties in monitoring (26). Another disadvantage is the dependency of the precipitation on the volume of solution and desiccator (26).



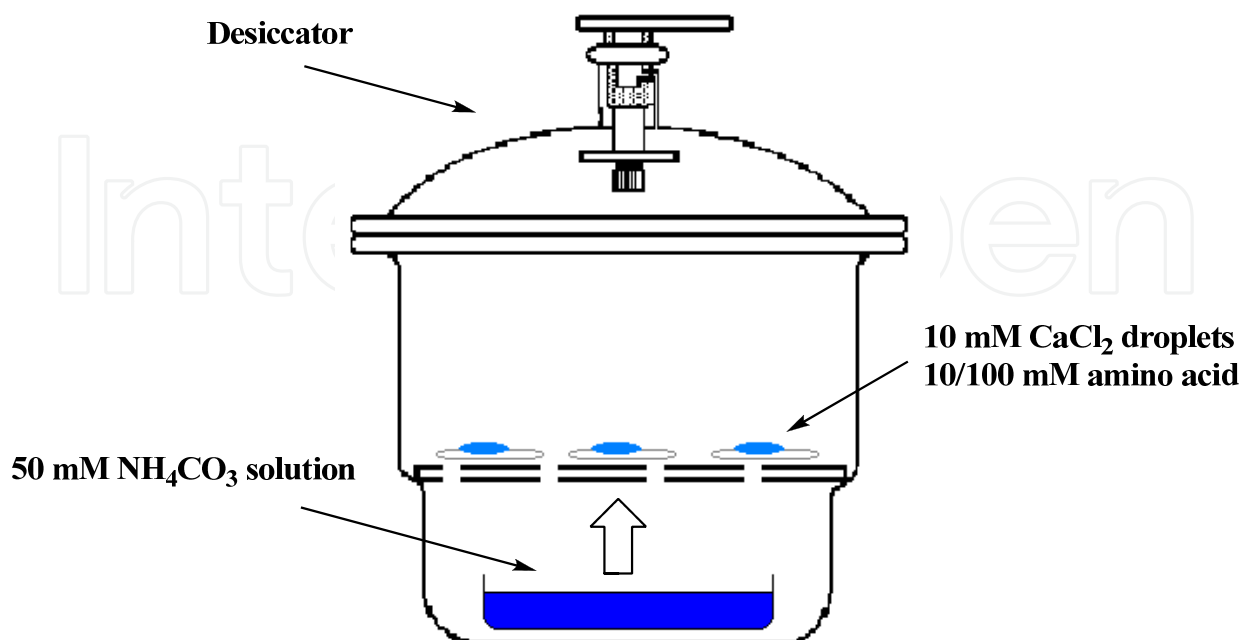


Fig. 3. Diagram of the vapor diffusion sitting drop crystallization experiment. At the bottom of the desiccator is a dish with 50 mM NH<sub>4</sub>CO<sub>3</sub> solution. On a shelf above are placed cover slides with 10 mM CaCl<sub>2</sub> solution.

#### 4. *In situ* potentiometric titration

To observe the kinetics of CaCO<sub>3</sub> crystallization, from ion clusters to post-nucleation aggregates, *in situ* potentiometric titration has been utilized. Specifically, the precipitation of CaCO<sub>3</sub> mineral products in the presence of amino acids in solution can be observed and compared to reference situations.

The basis of this measurement is based on the potential differences across a membrane and deriving from this a quantity that can be correlated to ion concentrations. By utilizing the Nernst equation, a potential to determine the amount of free Ca<sup>2+</sup> ions and bound Ca<sup>2+</sup> ions in solution can be determined as follows:

$$E = E_0 + \frac{RT}{nF} \ln \left( \frac{[\text{free Ca}^{2+} \text{ ions}]}{[\text{bound Ca}^{2+} \text{ ions}]} \right)$$

where  $E_0$  is the starting chemical potential,  $R$  is the gas constant,  $F$  is Faraday's constant, and  $T$  is temperature.

The other quantities of the equation are measured by a commercial, computer controlled titration system equipped with pH and Ca<sup>2+</sup> selective electrodes to measure the respective quantities in solution as CaCl<sub>2</sub> is titrated into the reaction solution (Figure 4). The quantity can then be correlated to the concentration of Ca<sup>2+</sup> ions in solution and as well concentration bound Ca<sup>2+</sup> ions.

In our studies, the titrations were carried out in a 10 mM carbonate buffer. The carbonate buffer contained a mixture of  $\text{Na}_2\text{CO}_3$  and  $\text{NaHCO}_3$ . The pH-value was set to 9.75. For precipitation, a 10 mM  $\text{CaCl}_2$  solution was used. The following amino acids were used as additives: asparagine, arginine, glutamic acid, methionine, proline, serine and valine. Their concentrations were 10 mM and 100 mM, respectively. All titrations were performed at room temperature. The experiment was carried out in a 100 ml beaker filled with 10 ml of 10 mM carbonate buffer accomplished with 10 mM/100 mM amino acid. Before every titration, constant pH values were assured using 100 mM sodium hydroxide and 10 mM hydrochloride acid. The application rate was 5  $\mu\text{l}$  for NaOH/ HCl and 10  $\mu\text{l}$  for  $\text{CaCl}_2$  solution. For the titration of 100 mM asparagine, glutamic acid and arginine required more alkaline buffer solution was required. For the titration with glutamic acid, a  $\text{NaCO}_3$ / NaOH buffer was used. Its pH-value was set to 10.4. In case of asparagine and arginine, the pH-value of  $\text{NaCO}_3$  buffer was set by manually adding 1 M NaOH to get a pH of 9.75. All titrations were carried until reaching mineral precipitation.

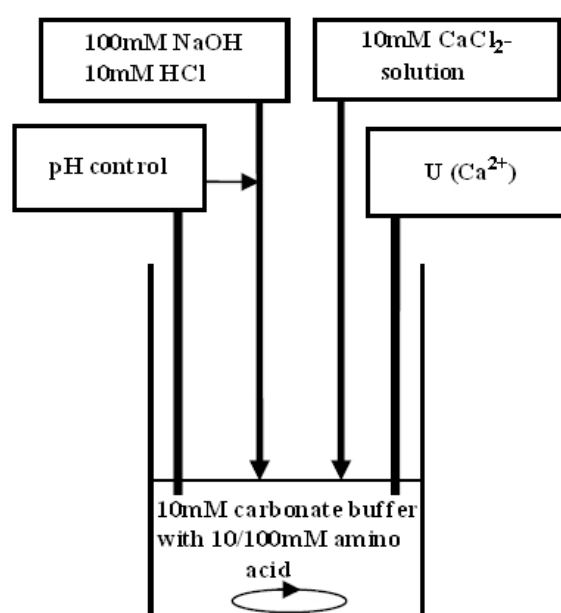


Fig. 4. Scheme of the titration experiment. 10mM  $\text{CaCl}_2$ -solution was titrated into a beaker containing 10mM carbonate buffer with 10mM/100 mM concentrations of amino acid. For constant pH, 100 mM NaOH/ 10 mM HCl were added, respectively. The titration was monitored with a  $\text{Ca}^{2+}$  ion electrode.

## 5. Discussion

The effects of specific amino acids on the crystallization of  $\text{CaCO}_3$  were investigated. For this purpose, “vapor-diffusion crystallization” measurements were used to initially survey the amino acids having an effect on crystal morphologies. All twenty natural amino acids were surveyed and the resulting crystals were characterized by various techniques such as light microscopy and scanning electron microscopy (Table 1,2). In the most interesting cases of an amino acid, further investigations were used to characterize the specific amino acids. Subsequently, *in situ* potentiometric measurements were conducted to determine the differential kinetics involved in each of the amino acid cases.

	Amino acid	Properties	Average size of crystals
1.	Reference ( CaCl <sub>2</sub> only)	Accumulation of undefined crystals; few calcite structures	13 μm (only calcite structure)
2.	Alanine	Some calcite crystals; few round undefined structures; widely scattered	4 μm
3.	Aspartic acid	Accumulation of calcite-like crystals; smooth borders	10 μm
4.	Cysteine	LM: Accumulation of vaterite-like clusters SEM: only calcite-like-clusters (very small amount)	9 μm
5.	Glutamine	Calcite crystals	14 μm
6.	Glycine	Accumulation of vaterite-like forms; a few calcite clusters	17 μm
7.	Histidine	Vaterite and calcite structures	23 μm
8.	Isoleucine	Calcite structure; widely scattered	17 μm
9.	Leucine	Calcite structures; few vaterite-crystals; widely scattered	16 μm
10.	Lysine	Small crystals; calcite structures; chains of crystals	6 μm
11.	Phenylalanine	Vaterite/calcite structures; widely scattered	16 μm
12.	Threonine	Accumulation of calcite crystals; sharp borders	11 μm
13.	Tryptophan	Vaterite/calcite structures; accumulation of clusters	12 μm
14.	Tyrosine	Vaterite/calcite structures	17 μm

Table 1. Description of crystalline precipitates mineralized in the presence of amino acids (10 mM) in 10 mM CaCl<sub>2</sub>-solution as observed in polarized light microscopy(V: 15 μl), duration: 72 hours

Two series with a 10 mM and the other with 100 mM amino acid concentration were carried out as an approximate “low” and “high” concentration, respectively (Table 1, 2). It could be observed that, in general, in both cases the calcium carbonate crystals possess the similar size dimensions (between 10 and 50 μm) and show calcite-like forms (Figure 5, 6). Nevertheless, several differences can be recognized, particularly for the seven selected samples. Here, more vaterite-like structures than calcite in the samples with a lower amino acid concentration can be observed. Moreover, the size of the formed CaCO<sub>3</sub> crystals were rather smaller (between 5 -30 μm). It can be observed that amino acids have a distinct effect on the crystallization of CaCO<sub>3</sub> (Figure 5,6). In contrast, the reference situations (without additives), only small trigonal crystals with sharp edges were found. The CaCO<sub>3</sub> samples with amino acid show usually rounded crystals with smooth edges and partially entirely new structures. The amino acids arginine, asparagine, glutamic acid, proline, methionine, serine, valine were subsequently determined to produce dramatic effects in crystallization and chosen for further characterization (27).



	Amino acid	Properties	Average size of crystals
1.	Reference (CaCl <sub>2</sub> only)	Sharp borders; widely scattered; only calcite structures	31 μm
2.	Alanine	Sharp borders; widely scattered; only calcite structures	16 μm
3.	Aspartic acid	Branched undefined structures	Not measurable
4.	Cysteine	Accumulations of round vaterite-like clusters; some calcite crystals	Not measurable
5.	Glutamine	Calcite structures; accumulation of clusters	15 μm
6.	Glycine	Smooth borders; widely scattered; calcite form	11 μm
7.	Histidine	Smooth borders; widely scattered; calcite form	Not measurable
8.	Isoleucine	Calcite structures	11 μm
9.	Leucine	Some calcite crystals; few undefined structures	27 μm
10.	Lysine	Small accumulations of crystals; calcite-like forms; smooth borders	Not measurable
11.	Phenylalanine	Small crystals; calcite-like structures	9 μm
12.	Threonine	Calcite-like structures; widely scattered between amino acid	50 μm
13.	Tryptophan	Calcite structures; widely scattered between amino acid	Different sizes
14.	Tyrosine	A few calcite structures; crystals covered by amino acid	Not measureable

Table 2. Description of crystalline precipitates mineralized in the presence of amino acids (100 mM) in 10 mM CaCl<sub>2</sub>-solution. (V: 15 μl), duration: 18 hours observed in polarized light microscopy

In the “*in situ* titration” experiment, the exact phases (saturation, supersaturation, nucleation and growth) of calcium carbonate crystallization in the presence of amino acids can be monitored (Figure 7). In this experiment the titration was also performed with 10 mM and 100 mM amino acid concentration. Thereby, a 10 mM CaCl<sub>2</sub> solution was titrated in a beaker with 10 mM carbonate buffer with an amino acid. The titration was monitored with a Ca<sup>2+</sup>- and pH-electrode. During titration the pH-value was kept constant to exclude pH effects on the calcium carbonate crystallization. There were problems with the samples 100 mM arginine, asparagine and glutamic acid. In these cases, the amino acids were too acidic, so that the standard buffer did not perform to keep the pH of the solution constant. For glutamic acid, a new buffer consisting of 10 mM sodium carbonate and 1 M sodium hydroxide, was used. For the amino acids asparagine and arginine, the buffer solution was set up manually before titration. In this 1 M NaOH was added to the carbonate buffer until pH achieved 10. It should be noted that in these cases, the volume of the carbonate buffer was not 10 ml anymore. It is hence difficult to compare these titrations with the other samples due to different volumes.

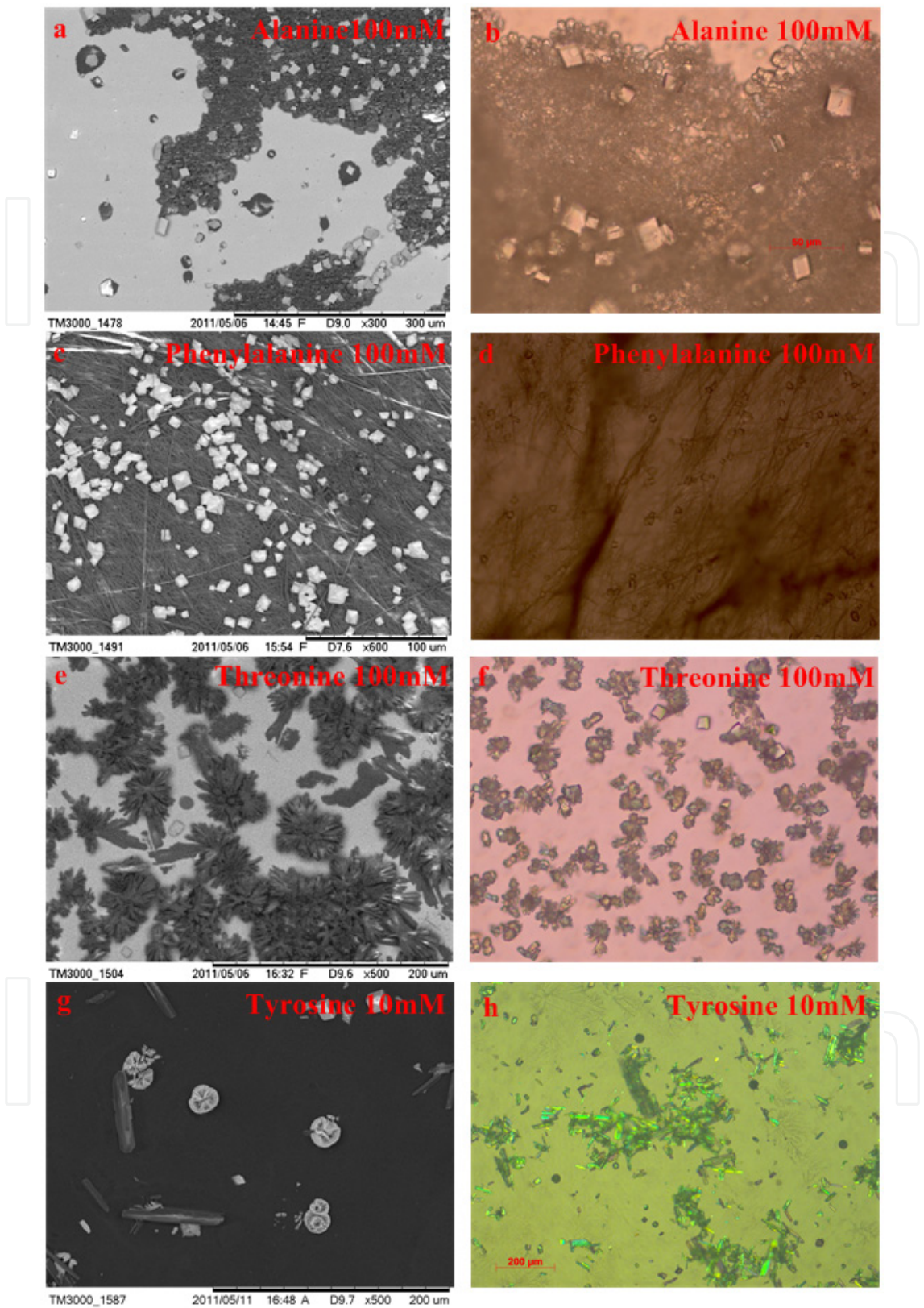


Fig. 5. Light microscopy and SEM pictures of crystalline precipitates after CaCO<sub>3</sub> vapor diffusion crystallization. The left column shows SEM recordings. The right one light microscope images, accordingly.

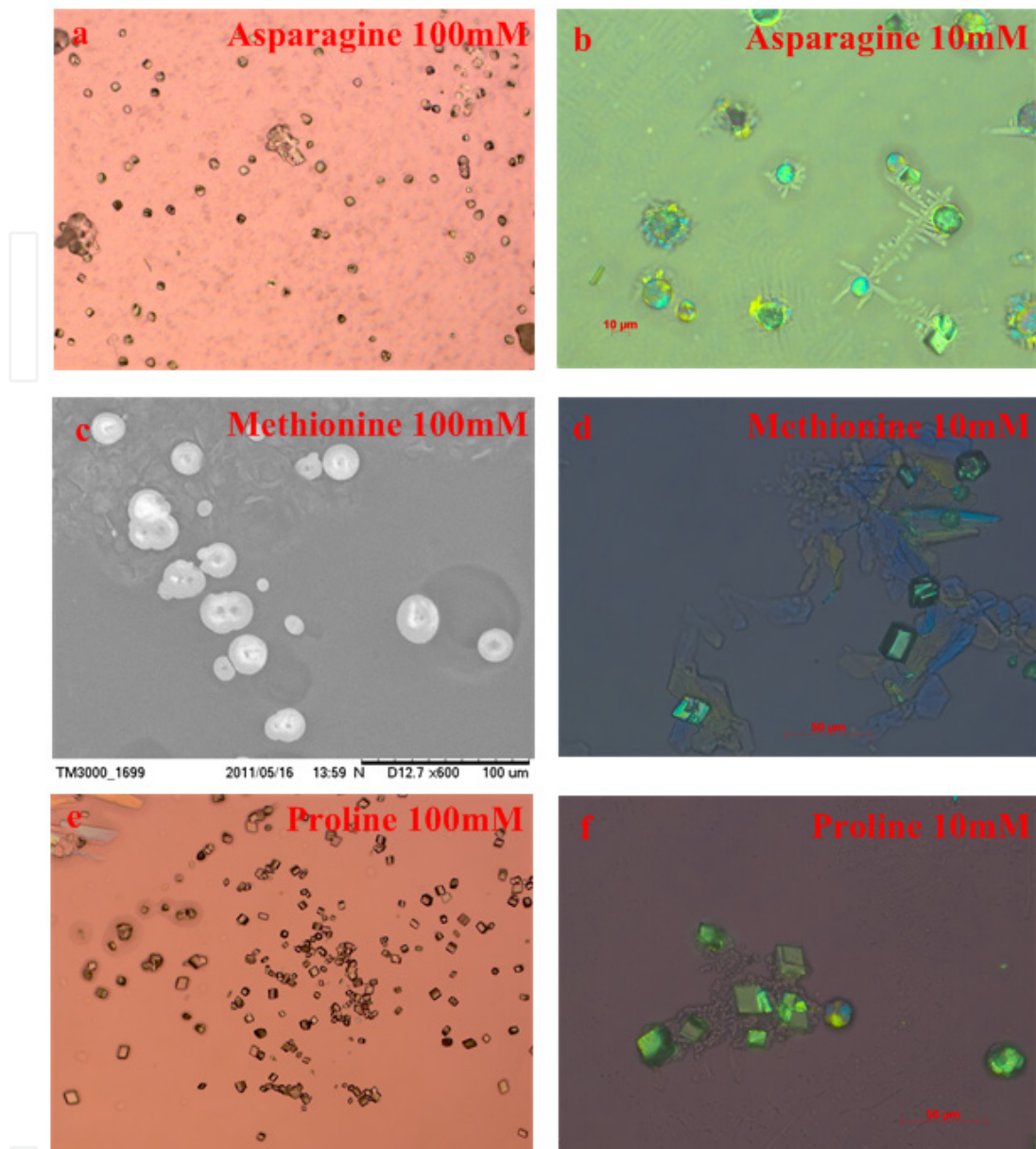


Fig. 6. Light microscopy and SEM images of  $\text{CaCO}_3$  crystals after vapor-diffusion crystallization in the presence of asparagine, methionine or proline.

All titration curves show the typical features of a Lamer diagram. The greatest effect on the nucleation in the 10 mM experiment was found to be glutamic acid (Figure 7). In comparison to the reference, the free calcium concentration was four times larger before nucleation started. It is surprising that the samples proline and arginine have even a negative effect because the nucleation  $\text{CaCO}_3$  started earlier than in the reference. It can be possible that the interaction of  $\text{Ca}^{2+}$  ions with the amino acid leads to a lower activation barrier and thus to an earlier nucleation stage. In the second titration assay with 100 mM, valine showed the biggest effect on the nucleation of calcium carbonate. In this case, an obviously higher concentration of free  $\text{Ca}^{2+}$  ions as in the reference was measured. Nevertheless, the comparison of both graphs (10 mM / 100 mM) shows some interesting observations. By increasing the amino acid concentration ten times higher, a doubling of the



critical concentration of calcium ion can be observed and the nucleation also took twice as long. Consequently, it can be said that the interaction of amino acid molecules and  $\text{Ca}^{2+}$  ions lead to a delay of nucleation and further to a change in crystallization conditions. Since in this these titration measurements (10 mM/100 mM) at two concentrations, these results have to be dealt critically. Subsequent measurements should provide certainty on the exact interactions regulating the crystallization processes.

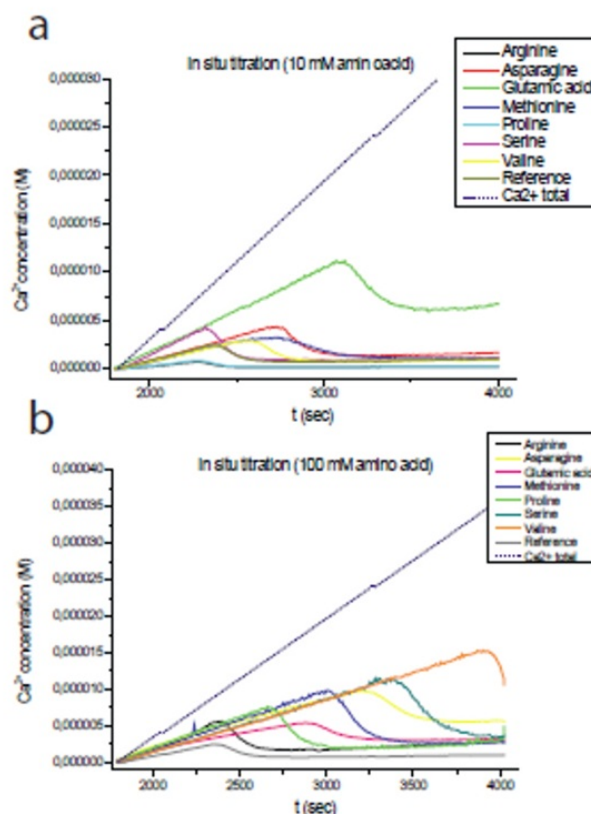


Fig. 7. Titration curves of calcium carbonate precipitation in the presence of a) 10 mM and b) 100 mM concentration amino acids

Light microscope measurements showed that the formed crystals were much smaller in the titration experiment than the “vapor-diffusion crystallization” method. Since it was difficult to observe crystals or different structures at all with light microscopy due to the size of the crystals, further SEM and TEM images were taken. With SEM, only scattered agglomerations of  $\text{CaCO}_3$  were found, but there was a significant difference among the samples. All samples of 100 mM amino acid concentrations showed round forms in contrast to the dilution experiment. Here, only clusters of calcite-like structures were found, which have rounded edges and smooth corners. Perhaps higher concentrations of amino acids can promote thermodynamically unfavored amorphous phases of calcium carbonate. In diluted samples, further TEM measurements show only round structures which developed into big agglomerations (Figure 8, 9). Exceptions were found in the samples arginine and asparagine. In these samples, also calcite-like forms could be found. Comparison of SEM and TEM data (titration experiment 10 mM amino acid concentration), show no definitive conclusions between the two measurements. At a first

glance, the preparation of the samples for SEM and TEM are different and thus provide different results. The more important point is that each measurement type operates at different lengthscale. The SEM pictures are in a size range of few micrometers, whereas the TEM in contrast operates at the nanometer range and additionally the samples have to be very thin (few nanometers) to see visualize. The very small round crystals and clusters that are seen on the TEM images are not detectable with SEM or LM. The much bigger crystals, which later usually develop into calcite-like structures, can be observed with light microscopy or SEM.

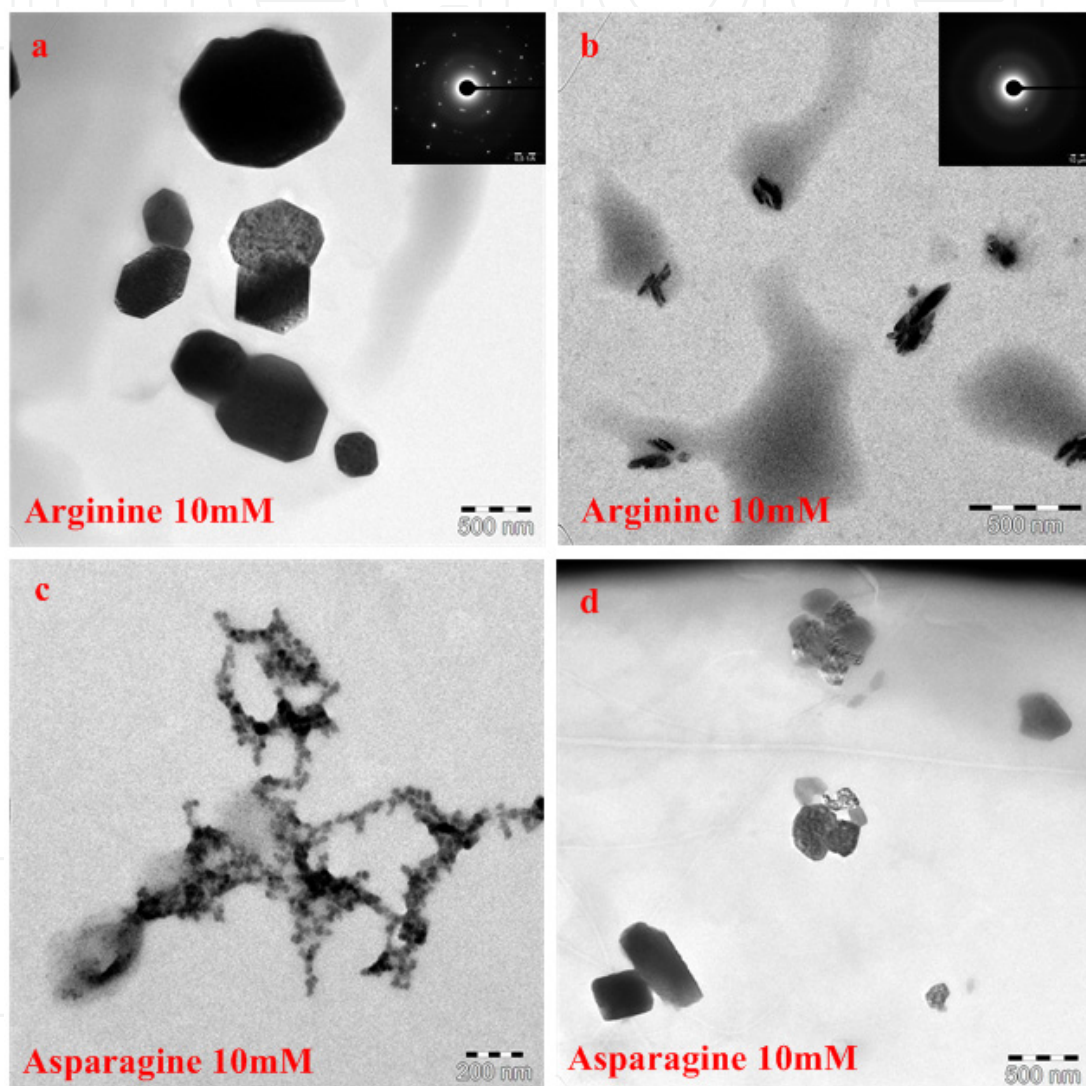


Fig. 8. TEM from the *in situ* potentiometric titration measurements with 10 mM amino acid concentration a & b) arginine, c & d) asparagine.

It is assumed that the different stages of crystal growth of calcium carbonate are measured in each of the different measurements under LM, SEM, and TEM. After nucleation, there are the two types of ACC I or ACC II. This could be the small round structures, which can be seen on the TEM pictures. After some time, more and more round crystals agglomerate to the bigger cluster as seen in Figure 9d and 9g, for instance.



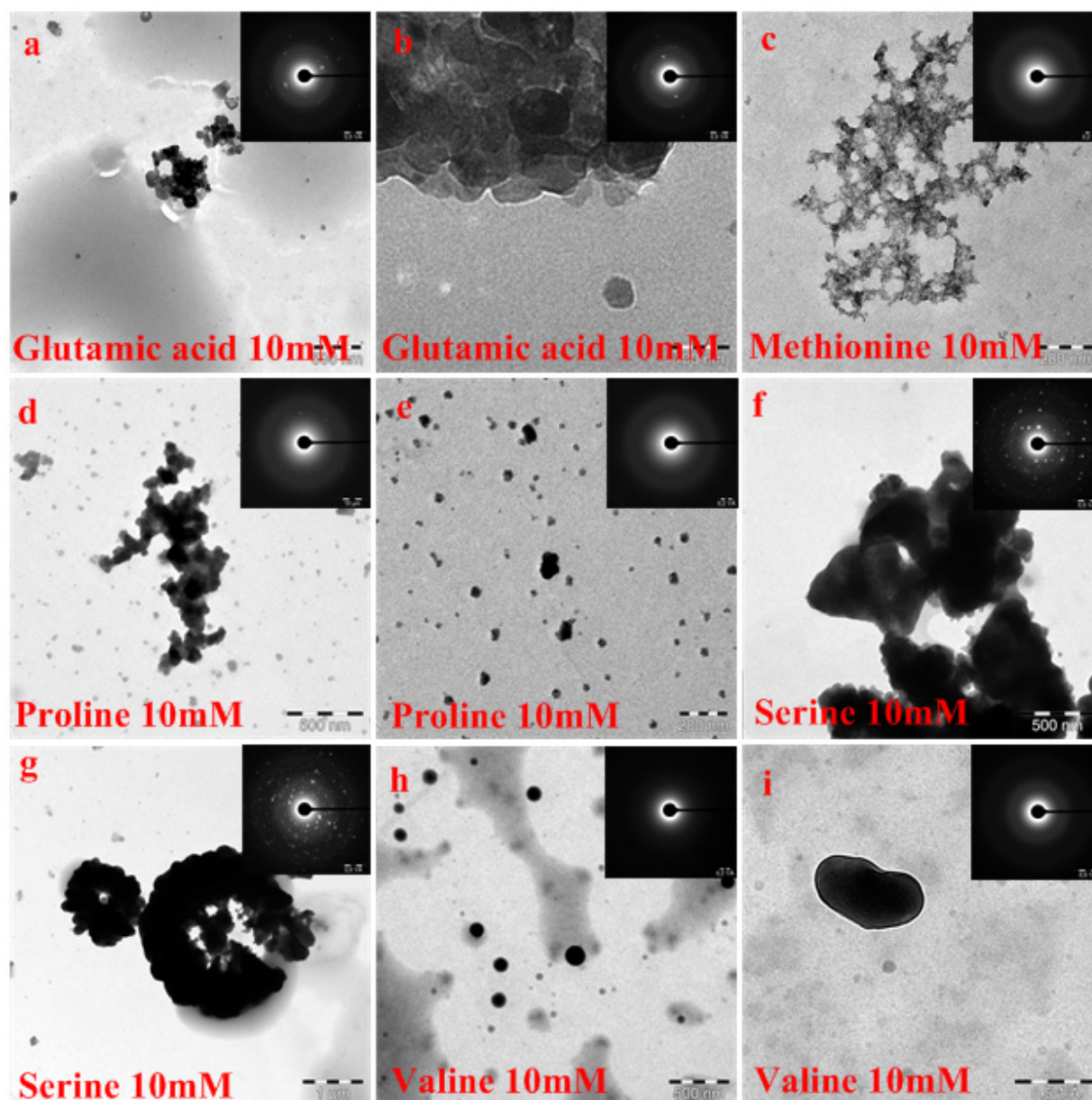


Fig. 9. TEM from *in situ* potentiometric titration measurements with 10 mM amino acid concentration a & b) glutamic acid, c) methionine, d & e) proline, f & g) serine and h & i) valine.

Finally, the instable clusters convert to a thermodynamically stable crystal structure, like calcite or vaterite, which can be seen on the TEM pictures (Figure 10). The amino acids can serve as nucleation- and growth- promoting molecules for calcium carbonate, reducing the activation energy of nucleation and facilitating the crystal growth. Interactions at these early stages of nucleation can prefer or inhibit specific crystal shapes and hence, control structures during calcification. In the “in-situ-titration,” much smaller crystals can be produced. The “vapor-diffusion crystallization” experiment provides  $\text{CaCO}_3$  more points for heterogeneous nucleation than “in-situ-titration.” This lowers the activation barrier and thus, results in faster growth and finally larger crystals. In conclusion, it was shown that specific amino acids have a distinct effect on calcification. Depending on the type of amino

acid and concentration, calcium carbonate can form different structures. Round structures, like vaterite are often favored instead of the thermodynamically stable calcite as intermediate structures. Furthermore, the type of crystallization also plays a decisive role as shown here in the aforementioned two different crystallization situations.

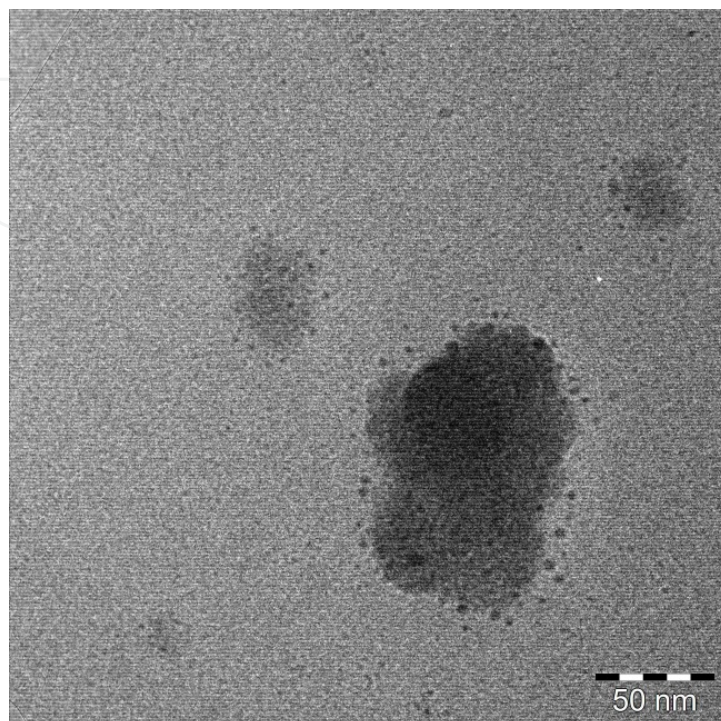


Fig. 10. TEM of a titration experiment with 10 mM of proline. A CaCO<sub>3</sub> crystal surrounded of many small crystals is shown.

## 6. Outlook

Use of *in situ* solution based techniques to study mineralization in the presence of additives is a powerful tool to determine kinetic as well as thermodynamic parameters involved in driving mineralization processes. Specifically, with the ability to determine the binding kinetics of pre-nucleation clusters, amorphous aggregates, or inhibition kinetics, Nature's use of additives in regulating mineral growth and development can be closely followed as it occurs. With the addition of other *in situ* physical measurement methods (i.e. *in situ* AFM), the ability to study the influence of additives, both small molecules as well as supramolecular complexes, can be accomplished with better observations of key events like nucleation or ripening stages in mineralization. By fully elucidating these processes in biomineralization, a better understanding on composing stronger tougher materials is achieved such that synthetic materials can be tailored with similar materials and mechanical properties found in Nature.

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