

INFLUENCE OF ADDITIVES ON STABILIZATION AND MINERALIZATION PATHWAY
OF AMORPHOUS CALCIUM CARBONATE NANOPARTICLES

BY

QI MA

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Environmental Engineering in Civil Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

Adviser:

Associate Professor Rosa M. Espinosa-Marzal

ABSTRACT

Amorphous calcium carbonate (ACC) is of high interest because of its critical role in biomineralization. ACC can be a precursor of crystalline calcium carbonate phases that are more energetically stable. ACC is not only stored in organisms for long time for the subsequent mineralization of new tissues, but it can also be the final carbonate phase in various organisms. The presence of organic additives has been shown to affect the mineralization pathway in solution by either inhibiting the aggregation of ACC or stabilizing it.

In this study, ACC was precipitated in solution within a poly (acrylic acid) (PAA) matrix. AFM imaging revealed that the PAA-ACC nanoparticles are composed of ACC nanograins with embedded PAA. The influence of organic additives (glucose, aspartic acid, glutamic acid) and Ca^{2+} on the mineralization and stabilization of PAA-ACC nanoparticles was investigated by measuring the size of the nanoparticles as a function of time using Dynamic Light Scattering (DLS); the adsorption of organic additives to PAA-ACC nanoparticles was investigated using Quartz Crystal Microbalance (QCM). QCM results demonstrate that glutamic and aspartic acid have a stronger interaction with PAA-ACC nanoparticles than glucose, probably because of their net negative charge, but the three of them interact with the nanoparticles, and hence, they can intervene in the stabilization of the nanoparticles. DLS results show that calcium ions can accelerate the aggregation of PAA-ACC nanoparticles by mitigating the electrostatic stabilization provided by PAA, while glucose, glutamic acid and aspartic acid can neutrally influence, inhibit and enhance the aggregation process, respectively. In absence of calcium ions, glucose can promote aggregation of PAA-ACC particles, while glutamic acid has no significant effect on stabilization and aspartic acid will lead to disaggregation. In the presence of calcium ions, synergistic effects between calcium and the organic additives are revealed. The additives also affect the mineralization pathway of ACC, as demonstrated by AFM imaging. With higher concentration of glucose, ACC nanograins become smother and larger, and fill the space within the nanoparticle more efficiently. With higher concentration of aspartic acid, ACC nanograins become smaller and less compact. The study of this biomimetic system will help us understand better how calcium carbonate constituents can be chemically and structurally controlled by the biomineralization process and to be applied to other inorganic systems in advanced materials applications such as additives-control crystallization.

ACKNOWLEDGEMENTS

I will give my special thanks to Prof. Rosa M. Espinosa-Marzal and Yijue Diao as well as everyone else in the Smart Interfaces in Environmental Nanotechnology (SIEN) research group for their guidance and help in the research. I also thank Prof. Thanh Huong Nguyen for the use of the DLS instrument in her laboratory at UIUC.

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CHAPTER 1: INTRODUCTION

Biom mineralization is defined as the biogenic formation of mineral deposits¹. For example, many sea creatures, such like sea urchins mollusks, sponges, and ascidians² can create minerals that become the (hard) structural components in order to maintain shape and protect organisms. Most of these structures, (e.g. bones, teeth and shells) can utilize crystalline minerals to stiffen the tissue³, and the precipitated crystals often have unique shapes⁴. Biominerals usually contain a small amount of organic matter, including sugar, peptides and polysaccharides⁵⁻⁹.

Calcium carbonate is one of the most common minerals, and therefore, it has been widely investigated. It is engaged in many biological processes, including the formation of animal and protozoan shells and coral reefs¹⁰. There are six known forms of calcium carbonate, excluding high-temperature and high-pressure forms. Five of them are crystalline. The most widely biologically produced forms of calcium carbonate by far are calcite and aragonite. Vaterite and monohydrocalcite, although less stable, are formed by a limited number of organisms. Calcium carbonate hexahydrate, also known by its geological name ikaite, along with the other two high-temperature forms of calcium carbonate, are not yet known to be formed biologically. Finally, amorphous calcium carbonate (ACC) may be more widely distributed than crystalline calcium carbonate, but because it dissolves easily and is difficult to detect, the presence may have been overlooked in many mineralized tissues⁴.

It is possible that ACC has an important basic function in many calcium carbonate formation processes as a transient precursor phase of crystalline calcium carbonate^{8, 11}. Ihli, et al.¹² found that ACC can dehydrate before crystallization. ACC transforms with aging or heating into a less hydrated form, and with time crystallizes to calcite or aragonite¹³. Transformation and crystallization of ACC can follow an energetically downhill direction, from more metastable hydrated ACC to less metastable hydrated ACC, followed by biogenic anhydrous ACC, vaterite, aragonite, and then calcite. However, in a given reaction sequence, not all these transformations occur. The transformations involve a series of atomic ordering, dehydration, and crystallization processes.

An additive can also influence the relative energy barrier between each reaction step, which can alter the kinetics of the transformation pathway. In this way, an additive can induce and/or stabilize the precipitation of a less stable phase. The additives can be classified as polymer scale or low molecular-weight scale and have 9 types of actions to influence the crystallization of

crystals^{11, 14}. For example, polyacrylic acid (PAA) has been found to be a crystallization inhibitor by binding with free calcium ions and interacting with soluble calcium carbonate clusters¹¹. Lam, et al. ¹⁵ found that poly (aspartic acid) can inhibit the crystallization process of synthetic ACC. Orme, et al. ⁹ showed aspartic acid can inhibit crystallization only at high concentrations (i.e. 2000 times higher than the calcium concentration). In contrast, Gower ⁸ showed that aspartic acid can both accelerate the transformation of ACC into vaterite, as well as stabilize the vaterite phase. The principal structural changes occurring during solution-mediated recrystallization were identified to be expulsion of water from the structure and development of long-range order, while retaining the short-range structure around the calcium ions present in the first-precipitated ACC phase. They also found that additives such as magnesium, phosphate and organic macromolecules can cooperatively act to stabilize ACC in vivo. Finally, small molecules phosphoenolpyruvate and 3-phosphoglycerate, which are intermediates of the glycolic pathway, have also been found to be important in stabilizing ACC in the exoskeleton and gastroliths of crustaceans¹⁶.

In this work, we investigated the influence of selected organic additives (i.e. glucose, glutamic and aspartic acid) and calcium ions on formation and stabilization of colloidal ACC particles formed in a PAA matrix according to a synthesis protocol developed in reference ¹⁷. The size (hydrodynamic diameter) of ACC nanoparticles was measured as a function of time by Dynamic Light Scattering (DLS) to determine how inorganic and organic additives influence the kinetics of ACC formation and stabilization in a supersaturated solution. Atomic Force Microscopy (AFM) was used to image the microstructure of the ACC particles at nanoscale, which provided information about the mineralization pathway in the presence and the absence of additives. Further insight into the interaction between ACC and additives was gained by Quartz Crystal Microbalance (QCM).

CHAPTER 2: MATERIAL AND METHOD

Materials

The following chemicals were used without any further purification: calcium chloride dihydrate (Sigma-Aldrich, $\geq 99\%$), ammonium carbonate (Sigma-Aldrich, 99.95–100.05% dry basis), sodium chloride (EMD, min 99%), sodium hydroxide (Sigma-Aldrich, $\geq 97\%$), poly (acrylic acid) (PAA) solution (Polysciences, Inc., 30 000 g/mol, 30% wt in H₂O), L-aspartic acid (Sigma-Aldrich, $\geq 98\%$), L-glutamic-acid (Sigma-Aldrich, $\geq 99\%$), D-(+)-glucose (Sigma-Aldrich, $\geq 99\%$).

Synthesis

To prepare 2mM PAA solution, 5mM NaOH solution was first heated to 90 °C and stirred at 500rpm. Then, PAA solution was added to reach a final monomer concentration of 2mM and dissolved for 5 hours. The solution was cooled down overnight. The solution was then filtered using a 0.22 μ m filter (Millipore ExpressTM PLUS) then its pH was adjusted to 9.

Mineralization of PAA was achieved using Huang's protocol ¹⁷. First 0.1M CaCl₂ solution was added to 1mM PAA solution (diluted from the 2mM PAA solution), which formed spherical globules (as imaged by AFM) of PAA crosslinked by Ca²⁺ (labeled as Ca-PAA) in solution. After 24 hours 0.1M (NH₄)₂CO₃ was added to PAA-Ca solution at 1 mL/min. The final concentration of calcium carbonate in solution (before mineralization) is 5mM. The pH of all used solutions was adjusted to 9 using 2M NaCl solution and 1M HCl solution. The initial size of PAA-ACC spherical nanoparticles was 419 $\pm$ 45 nm before adding any additives, as determined by DLS. The observed standard deviation across different batches was attributed to the initial dissolution of PAA. The solutions were filtered using a 0.2 μ m Nylon filter (Fisherbrand) prior to use.

Glucose, aspartic acid and glutamic acid were selected to be the organic additives. The influence of additives on (a) stability and (b) mineralization pathway of PAA-ACC was investigated by adding additives at two different stages in the mineralization process:

- (a) To study the influence on PAA-ACC nanoparticle stabilization, the organic additive was added at 1 mL/min into the PAA-ACC solution while continuously stirring to achieve a final concentration of 2, 10, and 15 mM organic additive. The combined influence of organic additives and Ca²⁺ was also investigated. Organic additives were first added to the prepared PAA-ACC suspension to reach a final concentration of 20mM, then CaCl₂

was added to the solution to achieve 2, 5 and 10mM. Control tests were conducted when the additives were added in the inverse order.

- (b) To study the additive influence on the mineralization pathway, concentrated solutions of organic additives were added at 1 mL/min into the PAA-Ca solution, while continuously stirring to achieve a final concentration of 2, 10, and 15 mM. After complete mixing, the concentrated $(\text{NH}_4)_2\text{CO}_3$ solution was added to start mineralization. The measurements started after adding carbonate to the solution and 1 hour after, the solution was no longer stirred.

Dynamic Light Scattering (DLS)

The kinetics of PAA-ACC nanoparticle aggregation induced by the selected additives was studied by Dynamic Light Scattering (DLS) by measuring size and zeta-potential of PAA-ACC as a function of time. The hydrodynamic diameter and electrophoretic mobility (EPM) of PAA were examined with a Zetasizer (Malvern, ZS90, UK) at the wavelength of 633nm and the scattering angle of 90°. The zeta potentials of the polymers were calculated from the EPM following Ohshima's method¹⁸. To compare the zeta-potential regardless the influence of the particle size across conditions, the zeta potential was normalized by the square of the diameter, which is defined as "surface potential"¹⁹. All experiments were conducted at room temperature.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectroscopy is a rapid nondestructive method that has been applied to many biological systems. This technique is based on the principle that atoms in molecules are not held rigidly apart and, when subjected to infrared radiation (between 400 and 4000 cm^{-1}), the molecule will absorb the energy and the bond will be subjected to several different vibrations. Hence the absorption spectrum contains information regarding the molecular structure of the sample²⁰. The solution was filtered to get PAA-ACC nanoparticles as solid powder. The crystallinity of the powder was then studied by ATR-IR spectroscopy (Perkin Elmer, Frontier, USA and Pike Technologies, GladiATR, USA).

Quartz Crystal Microbalance (QCM)

Quartz crystal microbalance with dissipation monitoring (QCM-D) is used to measure molecular adsorption on various substrates in situ and in real time²¹. QCM-D records frequency and dissipation shifts (Δf and ΔD , respectively) upon adsorption occurring at the surface of an AT-cut piezoelectric quartz crystal²¹. These shifts are related to the mass and viscoelastic properties

of the layer in contact with the quartz crystal as well as to the surrounding liquid environment. Different approaches are available to model these shifts and to relate them to physical properties of the layer. Among them, the Sauerbrey equation establishes a proportional relationship between the frequency shift and adsorbed mass. It is valid for a rigid, thin, and evenly distributed adsorbed layer coupled to the quartz crystal ²².

To evaluate the adsorption of the organic additives to PAA-ACC, QCM measurements were performed with a Q-Sense E4 System (Biolin Scientific). The gold sensors were used. The baselines were established with saturated CaCO₃ solution for around 10 hours to reach equilibrium, followed by PAA-ACC adsorption to the gold-coated quartz crystal and then by the organic additives at a concentration of 20mM in saturated CaCO₃ solution. Saturated CaCO₃ solution was also used to rinse. Solutions were exchanged after equilibrium was achieved. The flow rate remained at 50μL/min. All experiments were conducted at room temperature.

Atomic Force Microscopy (AFM) Imaging

Silicon wafers were cleaned by piranha cleaning and thoroughly rinsed with milli-Q water. Wafers were then immersed in 0.2 wt% Poly (diallyldimethylammonium chloride) (PDADMAC, Sigma-Aldrich) solution for 30min. These PDADMAC-coated wafers were inertly placed on the interface between air and PAA-ACC solutions for 1 hour, thereby enabling the adsorption of the PAA-ACC nanoparticles while avoiding sedimentation of large aggregates. After the deposition, the wafers were rinsed gently with milli-Q water followed by ethanol, then dried with nitrogen gas before imaging in the air. An AFM (JPK, Germany) located in an acoustic chamber was used to collect images in tapping mode. Silicon tips (TAP300GD, Ted-Pella CA) with spring constant ~40 N/m and driving frequency ~300 kHz was used. The free amplitude was set to 20-30 nm and the set-point ratio to greater than 80%. At least 3 different spots per sample were imaged and the image size was selected to be 150 nm×150 nm and 500 nm×500 nm. The images are courtesy of Yijue Diao, who is a Ph.D. student in our research group.

CHAPTER 3: RESULTS

Synthesis and Characterization of PAA-ACC Nanoparticles

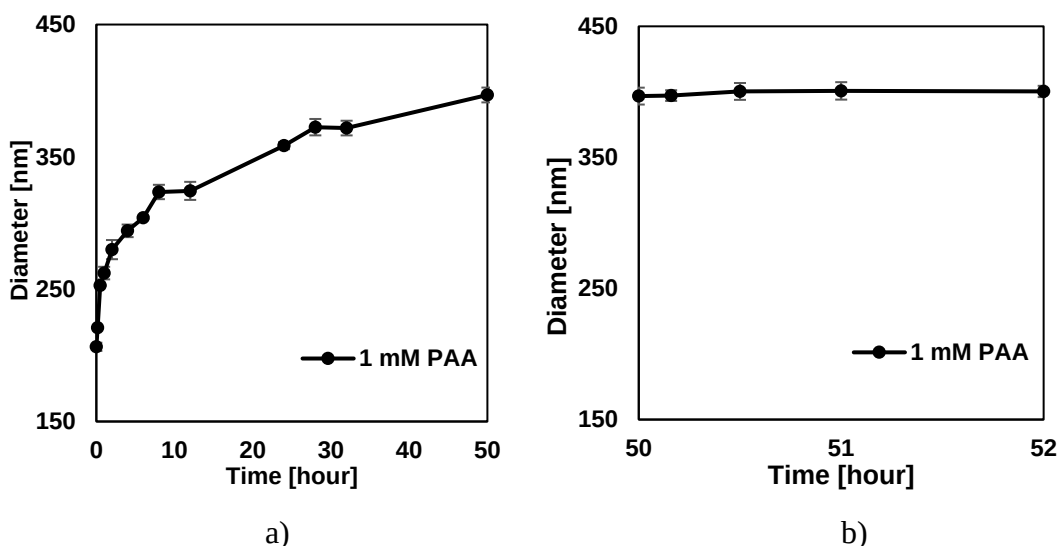


Figure 1 Size (diameter) of PAA growth as a function of time after a) adding CaCl_2 solution and b) adding $(\text{NH}_4)_2\text{CO}_3$

Figure 1 shows the variation of the size of a) PAA during the first 50 h of complexation with calcium and of b) PAA-ACC after addition of the carbonate solution. PAA globules began to grow after addition of CaCl_2 solution and kept growing over 50 hours with a decreasing growth rate. At pH of 9, PAA is negatively charged due to the presence of carboxylic groups, which can bind with calcium ions; Ca^{2+} ions mediate an ionic crosslinking between PAA-globules. The polymer chain began to crosslink because of Ca^{2+} bridging and form globules. After 50 hours, the diameter of PAA-Ca particles achieves 419 ± 45 nm, around 20 times larger than the initial size in the absence of Ca^{2+} (~ 22 nm). The size of the globules or spherical nanoparticles, as shown by AFM did not change upon addition of $(\text{NH}_4)_2\text{CO}_3$, at least during the first two hours. The low standard deviation indicated the good reproducibility of these measurements. Since mineralization happened during this step (as confirmed by FTIR), the carbonate is expected to bind to calcium but it does not crosslink across nanoparticles.

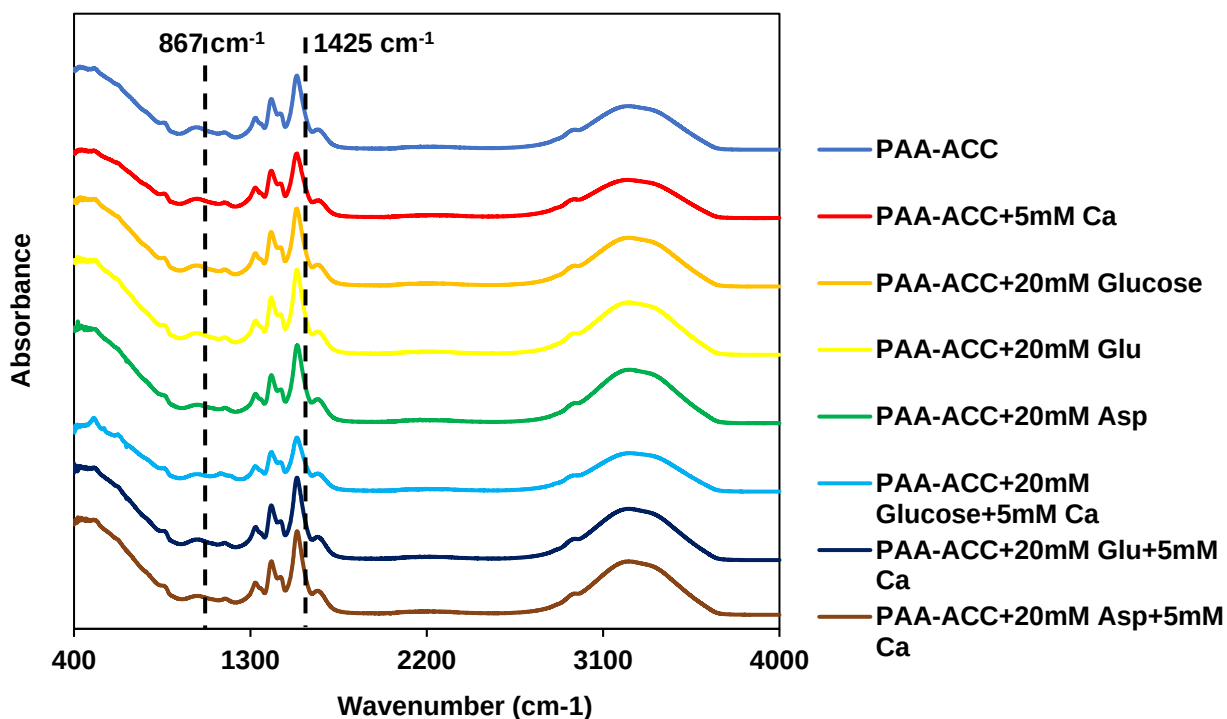


Figure 2 FTIR Spectra of PAA-ACC after addition of the selected additives

Twenty-four hours after adding additives, the solution was filtered to separate PAA-ACC powder and the collected powder was investigated by FTIR. Figure 2 shows the FTIR spectra of PAA-ACC nanoparticles after addition of the selected additives compared to a reference in the absence of additives. The ACC phase was identified by the peaks corresponding to the carbonate group appearing at 1425 cm^{-1} , 1075 cm^{-1} and 863 cm^{-1} and the disappearance of peaks at 747 cm^{-1} and 714 cm^{-1} , which represents crystalline vaterite and calcite, respectively¹². The peaks are consistent with literature data. The results show that ACC did not crystallize in the absence or presence of additives, during a period of 24 hours after filtering.

Influence of Additives on the Stability of PAA-ACC Nanoparticles

The influence of the sequence in the addition of glucose and calcium ions was also studied in control experiments (See Figure 15 and Figure 16 in appendix, respectively). Adding Ca^{2+} and then glucose makes the system very unstable. If Ca^{2+} is first added to the solution, it binds to the carboxylic group first and enhances ionic crosslinking across PAA-ACC nanoparticles. It may also influence the relative energy barriers between each reaction, thus altering the kinetics⁸. The following addition of glucose cannot compensate the complexation between calcium ions and PAA. Furthermore, it was concluded that stirring increases the turbulence of the system by

promoting the collision between the PAA-ACC particles, leading to enhanced aggregation. Hence, in the experiments discussed here, stirring was stopped after mixing the organic additives for one minute.

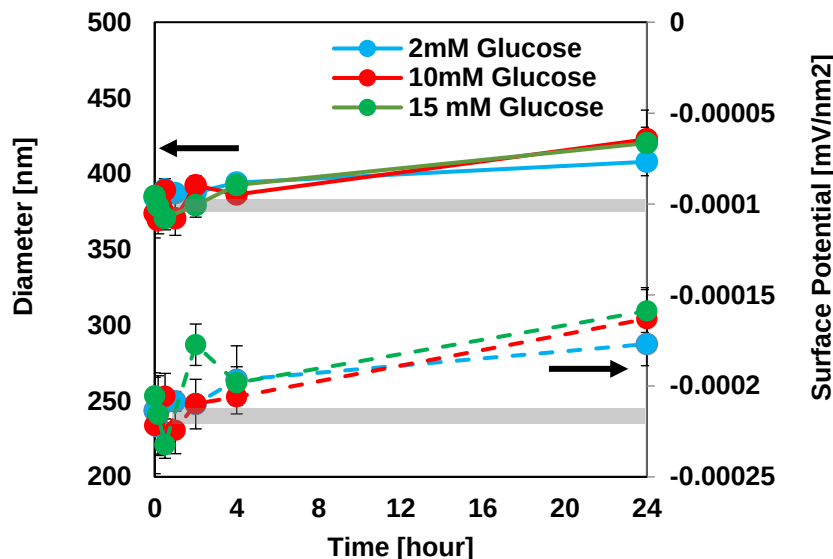


Figure 3 Change of size and surface potential of PAA-ACC nanoparticles over time as a function of glucose concentration

Figure 3 shows that glucose increases the size of PAA-ACC nanoparticles with time over 24h. The measured diameter of the reference PAA-ACC nanoparticles is 364 ± 5 nm (see grey line). In addition, the growth has not reached a plateau after 24 hours. The diameter of PAA-ACC nanoparticles increases from 364 ± 5 nm to 407 ± 9 , 423 ± 20 and 420 ± 10 nm, for 2, 10 15 mM glucose, respectively. The intensity distribution for the size of PAA-ACC shows an increase in size, as well, which confirms that the PAA-ACC nanoparticles slowly aggregate upon addition of glucose. On the other hand, glucose is neutrally charged at pH=9, but the surface potential of PAA-ACC nanoparticles became less negative over time, which is not only attributed to the increase of size, but also to the adsorption of (neutral) glucose to the nanoparticles. Based on Figure 3, a concentration of 2 mM leads to a smaller size than at the larger concentrations of 10 and 15 mM, and hence, an increase in concentration seems to enhance the mechanism of PAA-ACC aggregation. This supports that glucose (physically) adsorbs to PAA-ACC and that adsorption is enhanced with concentration, as expected for physisorption and chemisorption. The results suggest that the decrease in surface charge mediated by the adsorption of glucose decreases the electrostatic repulsion between the nanoparticles, which promotes aggregation.

Control experiments¹⁹ have shown that glucose slightly reduces the size of PAA globules in the absence of mineralization: PAA (1 mM)-Ca (7 mM) has a size of 400 nm, and the size is reduced to 300 nm upon addition of glucose at the same concentration (10 mM). This indicates that glucose leads to a decrease in crosslinking (either through release of calcium from PAA, or by release of PAA via binding to calcium ions in the globules). Perhaps, after mineralization, calcium is not released when glucose adsorbs to PAA-ACC nanoparticles. Further work is needed to understand the differences.

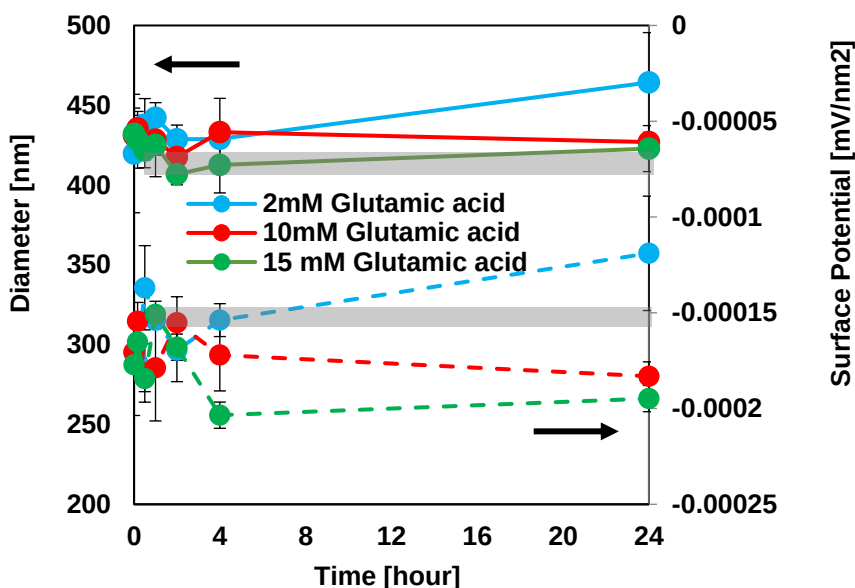


Figure 4 Size and surface potential of PAA-ACC nanoparticles over time as a function of changes glutamic acid concentration

Figure 4 shows the influence of glutamic acid on the size of PAA-ACC nanoparticles. The measured hydrodynamic diameter of the reference PAA-ACC is 419 ± 6 nm (see grey line). Addition of glutamic acid at different concentrations leads to a small change of the size of PAA-ACC nanoparticles at the concentration of 2mM, at which the diameter of the nanoparticles increases from 419 ± 6 nm to 464 ± 31 nm. At the concentrations of 10 and 15mM, the diameter of the nanoparticles increases slightly to 427 ± 4 nm and 422 ± 14 nm, respectively.

Meanwhile, the surface potential became more negative, which supports the glutamic acid adsorbs to the nanoparticles. Glutamic acid ($pK_{a1}=2.10$, $pK_{a2}=4.07$, $pK_{a3}=9.47$) has a negative net charge at pH=9, while the PAA-ACC nanoparticles have a negative surface potential. However, the interaction between PAA-ACC nanoparticles and the glutamic acid may be

possible either through electrostatic attraction between the positively charged amine group of the glutamic acid and the carboxylic groups of PAA or, more likely, through electrostatic binding to positively charged calcium and van der Waals interactions.

On the other hand, control experiments ¹⁹ have shown that glutamic acid remarkably reduces the size of PAA globules in the absence of mineralization: PAA-Ca at an ionic strength of 10mM NaCl has a size of 950 nm, and the size is reduced to 200 nm upon addition of glutamic acid at the same ionic strength (10 mM). This, again, shows a mitigation of crosslinking between the PAA globules induced by glutamic acid (like in the case of glucose), which differs from the results observed for PAA-ACC nanoparticles. This suggests that, after mineralization, the disaggregation of the nanoparticles is disfavored. However, the adsorbed glutamic acid, in contrast to glucose, maintains the size of the nanoparticles approximately constant, which is attributed to the stabilization of the nanoparticles by an electrostatic interaction as the surface potential significantly decreases; in fact, at a concentration of 2mM the less negative surface charge appears in concurrence to aggregation, supporting that stabilization of the PAA-ACC nanoparticles is a very relevant mechanism.

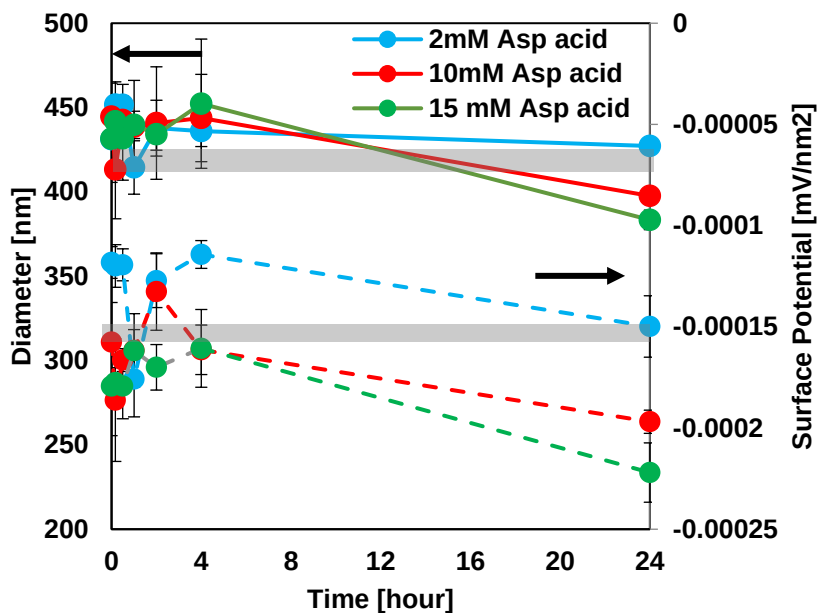


Figure 5 Size and surface potential of PAA-ACC nanoparticles over time as a function of changes aspartic acid concentration

The influence of aspartic acid on the size of PAA-ACC is different from those observed for glucose and glutamic acid, the size of PAA-ACC strongly decreases with time. The grey line

shows the initial PAA-ACC nanoparticle size before addition of additives, which is 419 ± 6 nm. When adding 2mM aspartic acid, the size of PAA-ACC nanoparticles did not significantly increase in 24 hours. Upon addition of 10 and 15mM, the size of PAA-ACC nanoparticles initially increases but after ~ 24 h the diameter has decreased to 398 ± 1 , 383 ± 6 nm respectively; these changes seem to be more remarkable with increasing concentration of aspartic acid.

At pH of 9, the aspartic acid ($pK_{a1}=2.10$, $pK_{a2}=3.86$) has a net negative charge, while the PAA-ACC nanoparticles have a negative surface potential. However, the interaction between PAA-ACC nanoparticles and the aspartic acid may be possible either through electrostatic attraction between the positively charged amine group of the aspartic acid and the carboxylic groups of PAA or, more likely, through binding to calcium ions. Adsorption of aspartic acid to ACC is also possible based on van der Waals and perhaps, electrostatic interactions. The decrease in size cannot be explained by the increase in the ionic strength upon addition of aspartic acid based on control experiments at 10mM NaCl (See Figure 14 in appendix). We note that both a decrease in size and adsorption of aspartic acid can contribute to the increasing negative surface potential.

Control experiments¹⁹ with PAA and additives have shown that aspartic acid remarkably reduces the size of PAA globules in the absence of mineralization: PAA-Ca at an ionic strength of 10mM NaCl has a size of 950nm, and the size is reduced to 200nm upon addition of aspartic acid at the same ionic strength (10mM). This supports that addition of aspartic acid reduces the Ca^{2+} -bridging between PAA nanoparticles, as it is also observed for the PAA-ACC nanoparticles. Based on Figure 5, a concentration of 2mM is not sufficient to disaggregate the nanoparticles, while an increasing concentration from 10 to 15mM, as adsorption increases, seems to enhance the proposed mechanism for PAA-ACC disaggregation. Hence, the decrease in size is attributed to a partial disaggregation of PAA-ACC nanoparticles, as also supported by the increase in count rate in concurrence to the decrease in size. Although mineralization of PAA mitigates the effect of the organic additives that was observed in ref. ¹⁹, the interaction between aspartic acid and PAA-ACC seems to be strong enough to compete with Ca^{2+} bridging and induce disaggregation of the mineralized nanoparticles.

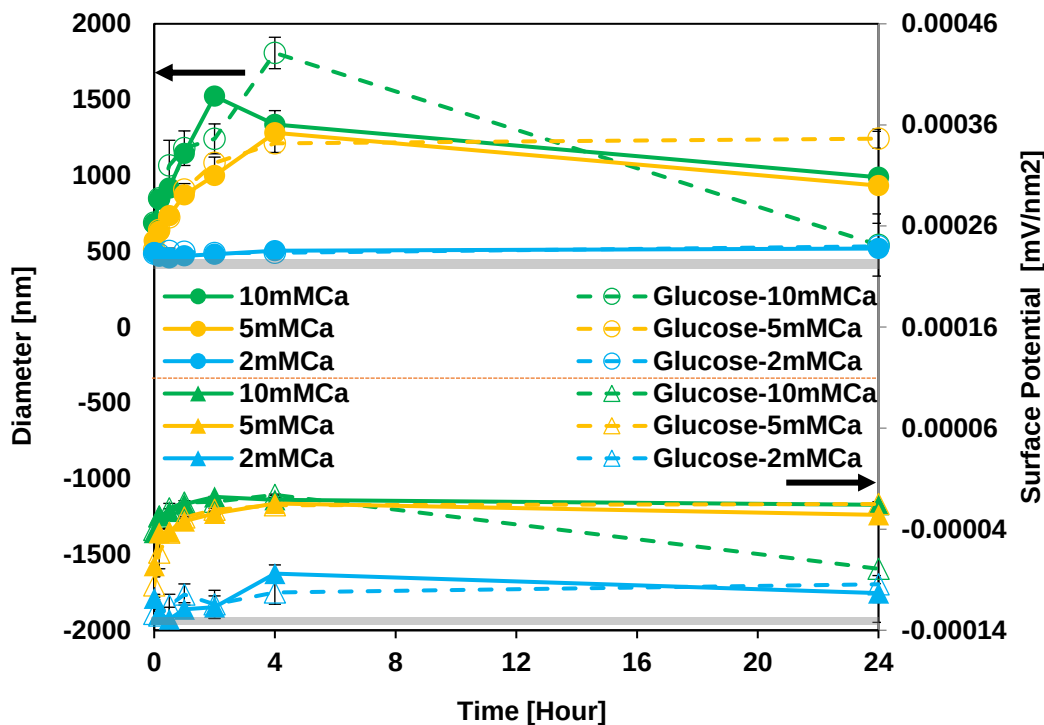


Figure 6 Combined influence of calcium ions and glucose on size and surface potential of PAA-ACC nanoparticles. Such competitive effects were investigated in a different set of experiments. Figure 6 shows the combined influence of Ca^{2+} and glucose on size and surface potential of PAA-ACC nanoparticles. When the CaCl_2 concentration is 2 mM, the diameter of the PAA-ACC nanoparticles increases from 476 ± 9 to 532 ± 38 nm. When the concentration is 5 mM, the diameter first increases to 1211 ± 59 nm at the initial 4 hours but has a slight decrease after 24 hours. When the concentration is 10 mM, the diameter first increase to 1807 ± 103 nm after 4 hours and it finally decreases to 541 ± 205 nm. An inspection of the count rate shows that it decreases, which reflects the decrease in concentration of nanoparticles and it proves that the increase followed by the decrease in size is caused by aggregation and sedimentation of the nanoparticles. Thus, this demonstrates that calcium can effectively induce aggregation of the mineralized nanoparticles, most probably by electrostatic binding to the carboxylic groups in PAA, which eliminates the electrostatic stabilization of the nanoparticles and after aggregation, it keeps them stabilized by ionic crosslinking.

Glucose, however, can delay aggregation at low calcium concentration. However, if the concentration of Ca^{2+} is high (i.e. 10mM), the effect of calcium prevails and sedimentation

occurs after 4 hours. This indicates that at low concentration, glucose first adsorbs to the nanoparticles, which decreases the subsequent binding of Ca^{2+} to the nanoparticles.

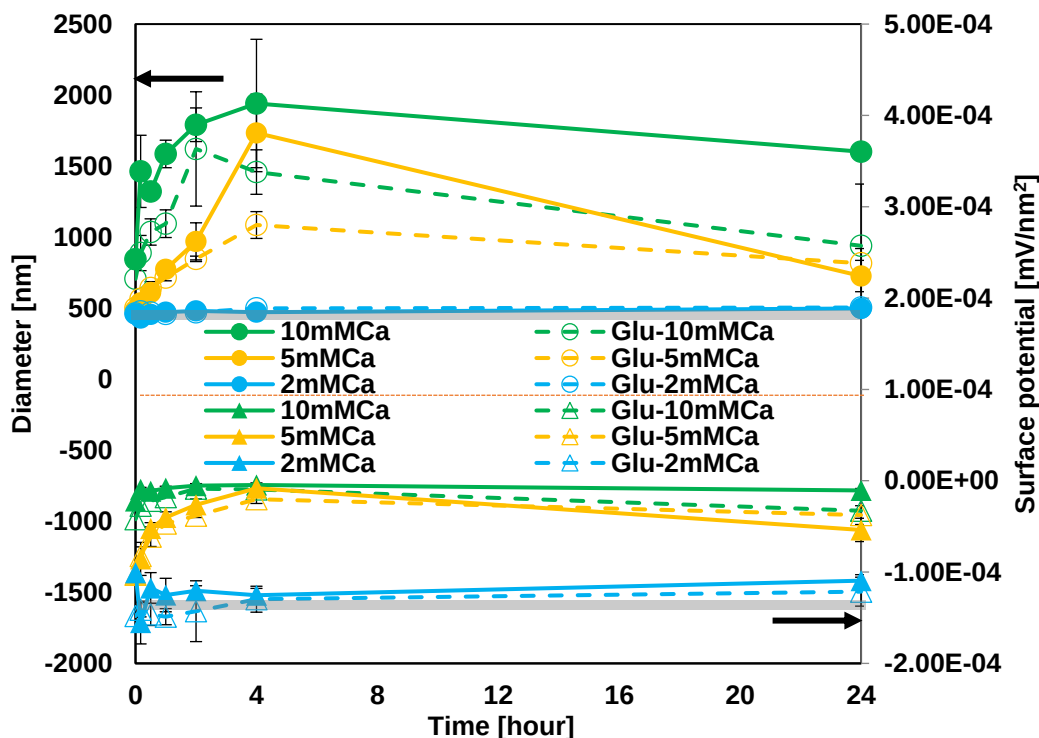


Figure 7 Combined influence of Ca^{2+} and glutamic acid on size and surface potential of PAA-ACC nanoparticles
Figure 7 shows the combined effect of Ca^{2+} and glutamic acid on size and surface potential of PAA-ACC nanoparticles. Glutamic acid was first added to PAA-ACC suspension and reached a final concentration of 20mM. Then concentrated CaCl_2 solution was added to reach a final concentration of 2, 5 and 10mM, respectively.

Similar to the previous results in Figure 6, when the CaCl_2 concentration is 2mM, the diameter of PAA-ACC nanoparticles exhibits a slight increase from $476 \pm 9 \text{ nm}$ to $505 \pm 23 \text{ nm}$. When the concentration is 5mM, the diameter first increases to $1085 \pm 94 \text{ nm}$ after the initial 4 hours but it decreases to $816 \pm 103 \text{ nm}$ after 24 hours, which means the sedimentation occurs after 4 hours. When the concentration is 10mM, the diameter first increase to $1458 \pm 156 \text{ nm}$ after 4 hours and it finally decreases to $939 \pm 434 \text{ nm}$. The size of PAA-ACC nanoparticles increases with increasing concentration of Ca^{2+} , which suggests Ca^{2+} can promote aggregation of PAA-ACC nanoparticles by reducing their electrostatic stabilization.

The size of PAA-ACC nanoparticles is smaller when glutamic acid is added to the solution. Glutamic acid shows a retardation of aggregation, likely by adsorbing to PAA-ACC, as shown in

Figure 4 as well, and thereby, increasing the electrostatic stabilization, and or mitigating the adsorption of Ca^{2+} to the nanoparticles. However, if the concentration of Ca^{2+} is high enough (i.e. 10mM), the effect of calcium prevails and sedimentation occurs after 2 hours, as indicated by the decrease in size of the nanoparticles, which is also proved by a decrease in count rate.

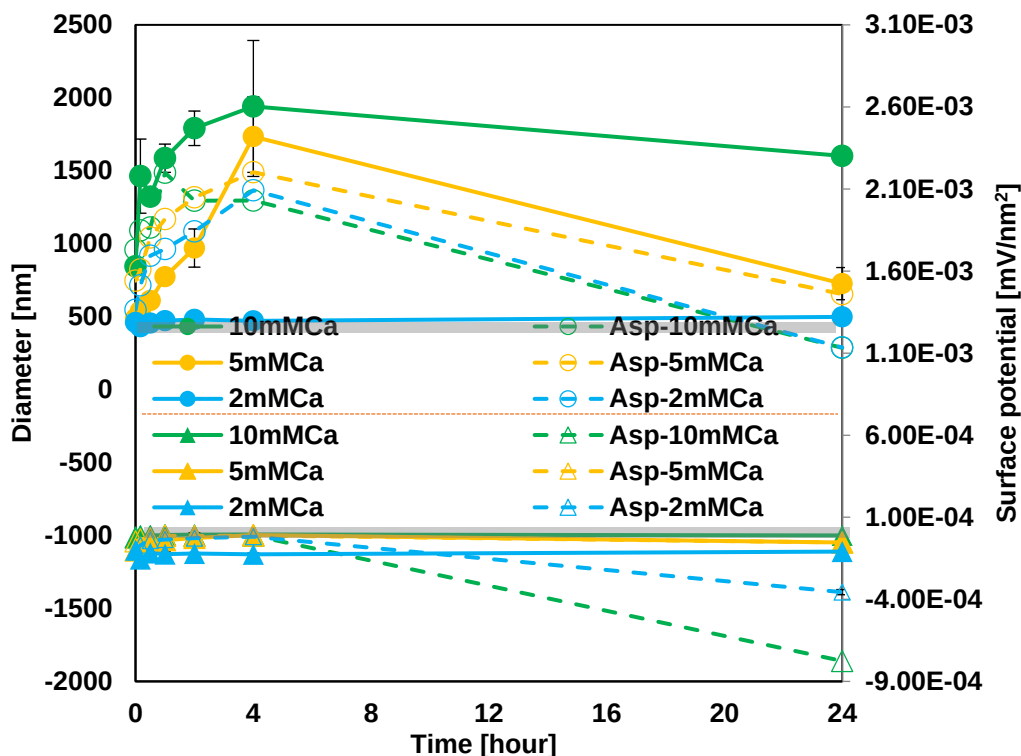


Figure 8 Combined influence of Ca^{2+} and aspartic acid on size and surface potential of PAA-ACC nanoparticles
Figure 8 shows the combined influence of Ca^{2+} and aspartic acid on size and surface potential of PAA-ACC nanoparticles. Aspartic acid was first added to PAA-ACC suspension to achieve a final concentration of 20mM. Then concentrated CaCl_2 solution was added to reach a final concentration of 2, 5 and 10mM, respectively.

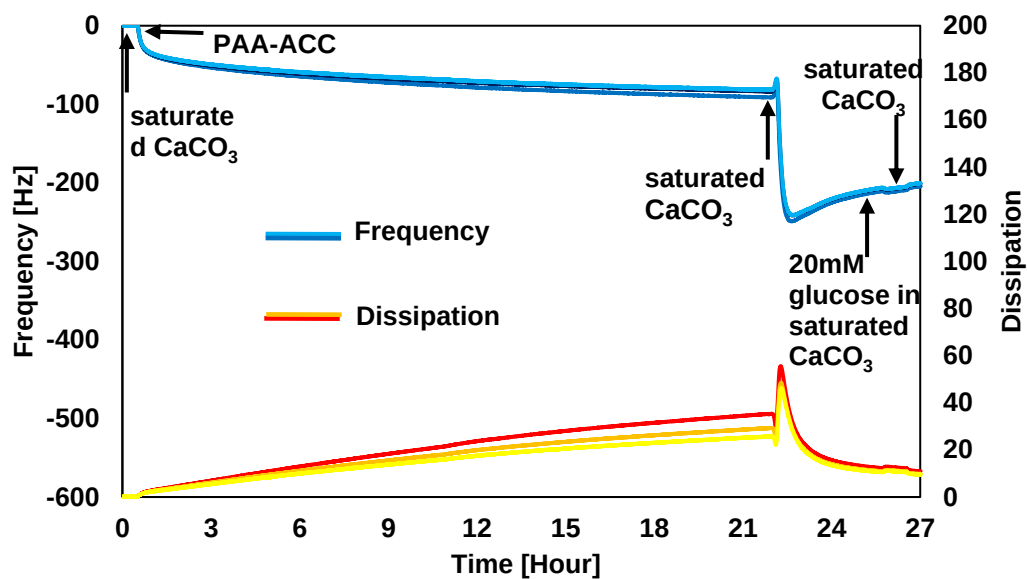
When the CaCl_2 concentration is 2 mM, the diameter of PAA-ACC nanoparticles exhibits an increase from 476 ± 9 to 1366 ± 213 nm first at the four hours, and then, it decreases to 289 ± 59 nm, When the concentration is 5 mM, the diameter first increases to 1489 ± 169 nm at the initial 4 hours but has a decrease to 654 ± 183 nm after 24 hours. In both cases, the size begins to decrease, suggesting the sedimentation occurs after four hours, which is also proved by a decrease in count rate. When the concentration is 10mM, the diameter first increase to 1486 ± 116 nm after 1 hours and finally decreases to 286 ± 210 nm.

Interestingly, the size of the PAA-ACC nanoparticles is larger with aspartic acid than in its absence, which indicates the synergistic effect of calcium and aspartic acid: when acting cooperatively, aspartic acid can promote the aggregation of PAA-ACC nanoparticles, while in the absence of Ca^{2+} , aspartic acid causes a disaggregation of the nanoparticles. This suggests that calcium can bind to the aspartic acid adsorbed to the nanoparticles, and it provides additional sites for calcium binding.

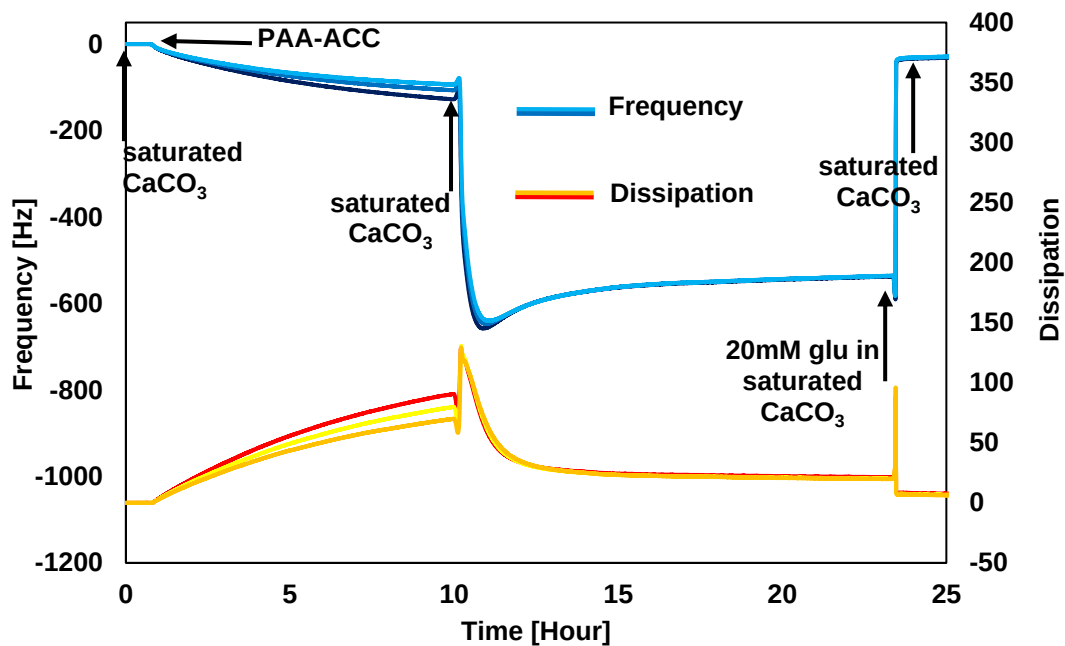
For lower calcium concentration (i.e. 2 mM), the effect is obvious because the Ca^{2+} cannot lead alone to a significant aggregation. Although the effect is less significant at higher concentration, significant aggregation and sedimentation are still observed. The surface charge significant decreases at the calcium concentrations of 2mM and 10 mM, which supports a strong binding of aspartic acid to PAA-ACC. This contrasts with the results for glutamic acid, perhaps because of the weaker interaction between glutamic acid and PAA-ACC nanoparticles, which is consistent with the results in Figure 4.

Insight into Interaction between Additives and PAA-ACC Nanoparticles

The baseline was taken using saturated CaCO_3 solution for around 10 hours to reach equilibrium. Then, adsorption of PAA-ACC nanoparticles on the QCM sensors was measured and finally the organic additives were injected at a concentration of 20mM. Saturated CaCO_3 solution was also used to rinse. Solutions were exchanged after equilibrium was achieved. The flow rate remained at 50 $\mu\text{L}/\text{min}$. All experiments were conducted at room temperature.

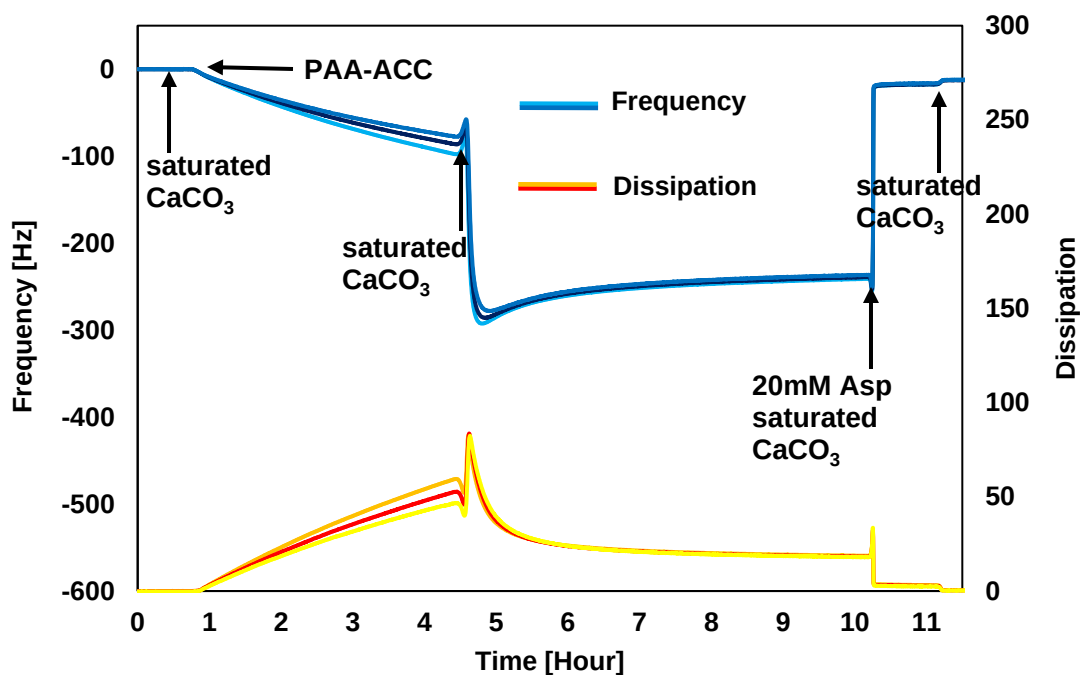


a)



b)

Figure 9 Adsorption of a) glucose b) glutamic acid c) aspartic acid on PAA-ACC nanoparticles measured by QCM



c)

Figure 9 (cont.)

Figure 9 shows the frequency and dissipation for the adsorption of PAA-ACC followed by that of the additives, a) glucose, b) glutamic acid and c) aspartic acid, measured by QCM. In a), after introducing PAA-ACC into the QCM cell, the frequency goes down slowly to -100Hz after around 4 hours, which indicates a slow adsorption of PAA-ACC is taking place on the surface. Then saturated CaCO₃ solution was also used to rinse. The frequency first drops due to swelling and reaches the equilibrium at -208 Hz. After introducing 20mM glucose solution, the frequency has a change of less than 3 Hz, which means the adsorption of glucose to PAA-ACC nanoparticles corresponds to ~ 1 monolayer²². In b), after introducing 20mM glutamic acid, the frequency has a sharp increase to -28Hz, which reflects either the desorption of PAA-ACC, indicating a strong interaction between PAA-ACC and the glutamic acid, or the collapse (due to the increase in ionic strength), or a combination of both. It is confirmed by AFM images (See Figure 17 in the appendix) that glutamic acid can remove PAA-ACC nanoparticles from the surface, and hence, the collapse seems to be a secondary factor. In c), after introducing 20mM aspartic acid, the frequency has a sharp increase to -15Hz, which reflects the desorption of PAA-ACC, thereby indicating the strong interaction between PAA-ACC and the aspartic acid, which agrees with DLS.

Influence of Additives on the Mineralization Pathway of PAA-ACC Nanoparticles

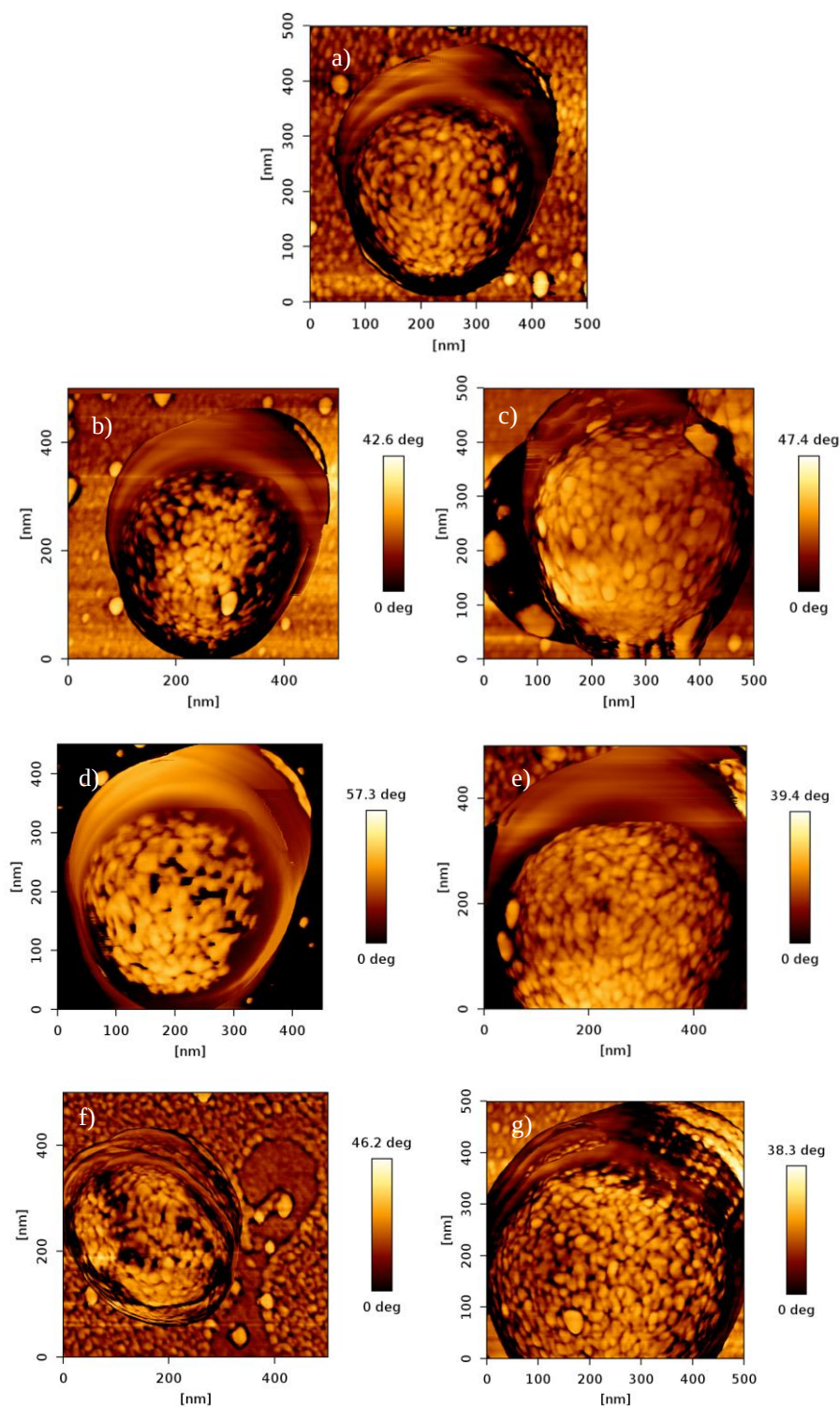


Figure 10 Atomic Force Microscopy (AFM) images of ACC in tapping mode for a) no additives b) 2 mM glucose, c) 10 mM glucose, d) 2 mM glutamic acid, e) 15 mM glutamic acid, f) 2 mM aspartic acid, g) 15 mM aspartic acid

Figure 10 shows AFM images of PAA-ACC nanoparticles taken in tapping mode. The nanoparticles were chosen randomly on the PDMAC coated substrate. Figure 10 a) shows PAA-ACC nanoparticle in the absence of any additives. It has been found that stable prenucleation clusters size can form in solution for crystallization of CaCO_3 , and can further aggregate to form amorphous nanograins and nanoparticles²³⁻²⁵. The clusters were formed in the PAA- CaCl_2 solution because of mineralization. The typical size of ACC is $\sim 10\text{-}30\text{ nm}^{24}$, which is consistent with the size observed in AFM images. Comparing Figure 10 b) 2 mM glucose and c) 15 mM glucose, the nanograins became smoother and bigger with increasing concentration, thereby filling the space in the nanoparticle. Figure 11-Figure 13 show the influence of the additives on size and surface potential of PAA-Ca nanoparticle. Diameter and surface potential of PAA-ACC nanoparticles is not affected by the addition of glucose, as shown in Figure 11. Aspartic acid may lead to a less compact microstructure with looser nanograins, as shown in Figure 10 f) and g). Diameter and surface potential of PAA-ACC nanoparticles is not affected by the addition of aspartic acid, as shown in Figure 13.

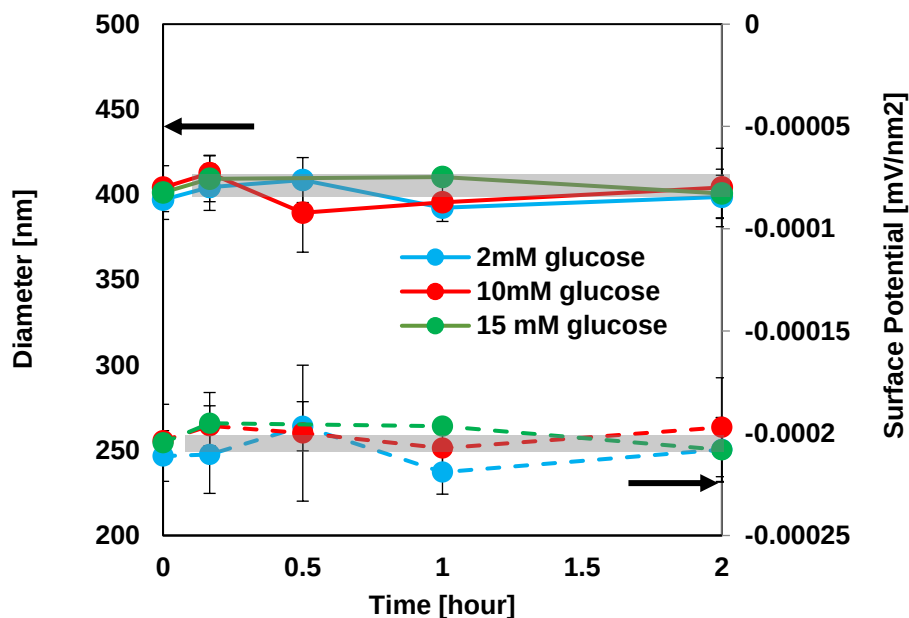


Figure 11 Size and surface potential of PAA-Ca nanoparticles changes over time as a function of glucose concentration

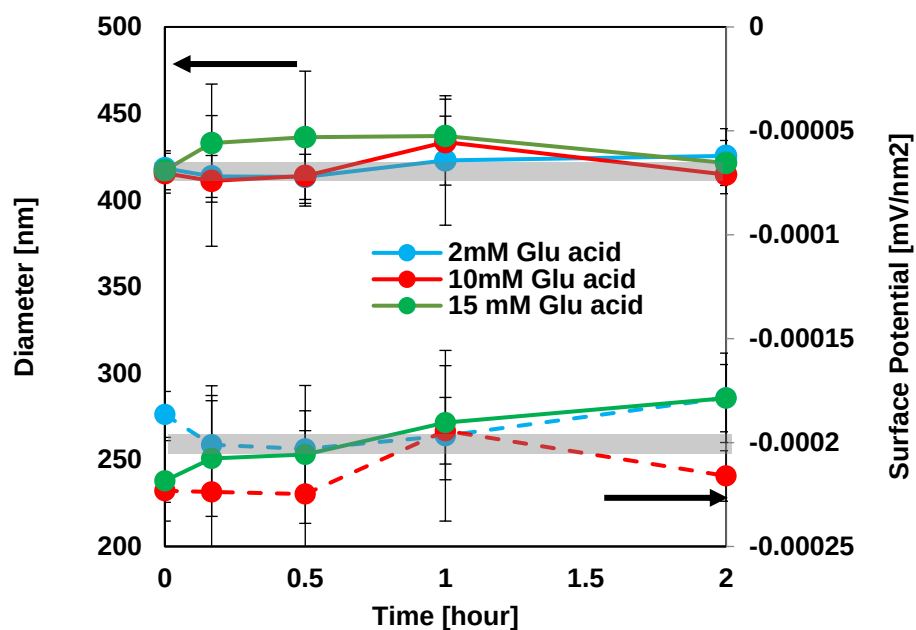


Figure 12 Size and surface potential of PAA-Ca nanoparticles changes over time as a function of glutamic acid concentration

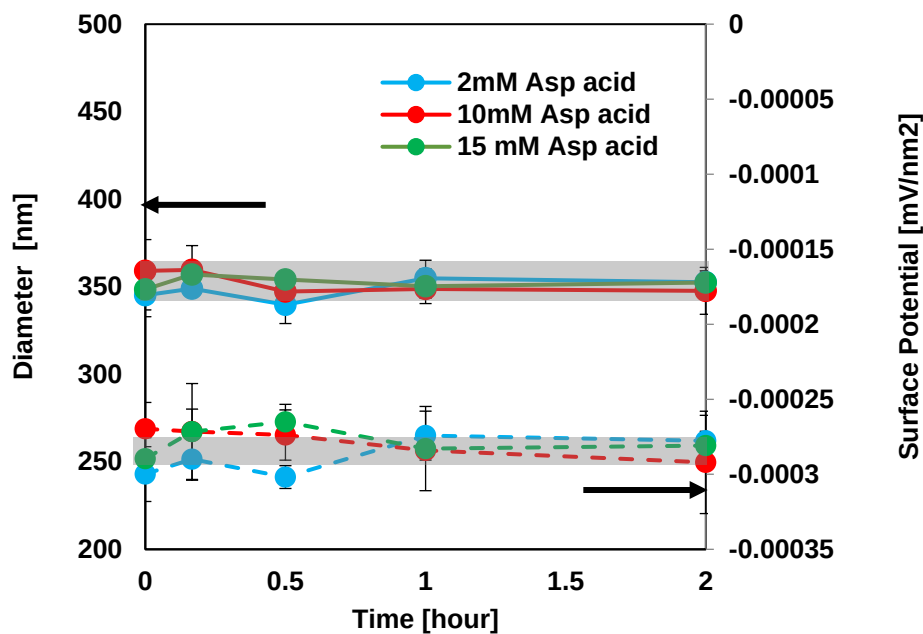


Figure 13 Size and surface potential of PAA-Ca nanoparticles changes over time as a function of aspartic acid concentration

The additives were added before mineralization and did not affect the size of PAA-Ca. As shown in Figure 1, the size of PAA-Ca remained constant during mineralization in the absence of additives, and the additives did not change this behavior.

CHAPTER 4: DISCUSSION

The influence of soluble organic additives (i.e. glucose, glutamic acid and aspartic acid) and calcium ions on mineralization pathways and stabilization of ACC nanoparticles was investigated by combining nanoscale imaging, stabilization, and adsorption measurements. Results show that the mineralization pathways and stability of ACC are not equally affected by the additives, which suggests that the function of additives is different in altering the mineralization process or (de)stabilizing nanoparticles.

reveals the interaction between glucose and PAA-ACC nanoparticles is much weaker than that of glutamic acid and aspartic acid. One possible influencing factor is that at the selected experimental condition (pH=9), glucose is neutrally charged, while the amino acids are negatively charged, and hence, they bind electrostatically to calcium ions. This was also observed in a previous study in the absence of mineralization¹⁹. Figure 3-Figure 5 show the influence of organic additives alone on PAA-ACC stabilization. Glucose and aspartic acid make PAA-ACC nanoparticles aggregate and disaggregate, respectively, while glutamic acid shows no significant effect. It is suggested that glucose can be adsorbed to PAA-ACC nanoparticles and the surface potential decreases. As a result, the repulsion force is smaller, which promotes the aggregation of nanoparticles. In the case of aspartic acid, the results are like those observed for PAA (in the absence of mineralization. It is possible that either Ca^{2+} ions providing crosslinking of the mineralized nanoparticles are released to interact with aspartic acid, or that aspartic acid binds to Ca^{2+} in the nanoparticles and cause disaggregation.

Ca^{2+} ions mediate aggregation of PAA-ACC nanoparticles. This is most probably caused by electrostatic binding of the calcium ions to the carboxylic groups in PAA, which eliminates the electrostatic stabilization of the nanoparticles and after aggregation, it keeps them stabilized by ionic crosslinking.

Competitive adsorption of glucose and Ca^{2+} hinders aggregation to a small extent, but only at low calcium concentrations. In contrast, the effect of glutamic and aspartic acid is more significant. Both glutamic acid and aspartic acid have a high affinity to Ca^{2+} in solution. However, glutamic acid can retard the aggregation of PAA-ACC nanoparticles, while Figure 8 shows that aspartic acid can accelerate the aggregation of ACC. This is indicative of synergistic effects. It is possible that glutamic acid binds to Ca^{2+} in the solution, which would effectively reduce the calcium concentration that can crosslink PAA-ACC nanoparticles. This is consistent

with the lesser retardation effect of glutamic acid at higher calcium concentrations. Based on the strong affinity of glutamic acid to PAA-ACC nanoparticles shown in QCM, as well as the decrease in surface potential observed when glutamic acid is used alone (Figure 4), it is, however, more likely that glutamic acid adsorbs to PAA-ACC nanoparticles. Hence, the additional negative charge via the adsorption of glutamic acid is the most probable factor that can retard aggregation mediated by Ca^{2+} ions. In contrast, the results for aspartic acid suggest that the additional negative charge mediated by aspartic acid adsorption enhances calcium crosslinking. Their different actions in influencing the stability of additives are probably attributed to molecular size²⁶. This is of particular interest with respect to the structures of aspartic acid and glutamic acid, which merely differ by one CH_2 group. These results further underline the importance of side chain configuration, in addition to chemical functionality²⁷.

If the additives are added before the mineralization, they seem to influence the mineralization pathways by changing the size and shape of ACC nanograins and the way they aggregate, whereas the stabilization of the PAA-ACC nanoparticles is not affected. This demonstrates that the organic additives are directly involved in the ACC formation, while they do not affect the role of PAA in providing an electrostatic stabilization of the nanoparticles.

It has been revealed by molecular dynamics simulations that there are four key factors that determine the ability of an additive to modulate crystal nucleation, including strength of interaction with the solute, disruptive ability of crystals (which may be based on steric, entropic, or energetic effects), interfacial properties, along with its ability to serve as a template for nucleation²⁶. Although it has been shown that glucose promotes nucleation⁵, our results rather suggest that either it promotes either growth or inhibits nucleation, or a combination of both, since the nanograins become bigger in size but they occupy the same volume (Figure 10).

Solute-philic additives, whose molecular size is larger than that of the solute, inhibit nucleation. Our results, in contrast, show that the nanograins become less compact and looser with higher concentration of aspartic acid. This indicates that either nucleation or the growth (or a combination of both) is retarded by aspartic acid.

This work reveals the influence of additives on stabilization of ACC nanoparticles, while the influence of additives on ACC mineralization pathway is not very clear yet, and future work is needed. It is proposed that ultraviolet–visible spectroscopy (UV-vis) is used to quantify the mineralization kinetics, which will provide information about the nucleation and growth kinetics.

In future, the combined effect of other inorganic additives, like magnesium ions with soluble organic additives should also be investigated. In seawater environment, magnesium ions are even more abundant, compared to calcium ions⁸. Because of their similarity as free ions and in carbonated salts, magnesium ions can certainly effect calcium carbonate precipitation.

CHAPTER 5: CONCLUSIONS

Additives are involved in biomineralization process and can influence the mineralization pathway and stabilization of biominerals. In this work, the investigation is based on a biomimetic system, which was inspired by nature. It is shown that organic additives (i.e. glucose, glutamic acid and aspartic acid) and calcium ions influence the mineralization pathway and stabilization of ACC nanoparticles. In addition, different behaviors among additives and their roles in different stages of biomineralization were observed. Many results can be explained through the electrostatic stabilization of the mineralized nanoparticles that is mediated by the polymer matrix, PAA, and modified by the additives. However, subtle differences between the effects of the additives seem to rely on their molecule structure, and more work is needed to elucidate these aspects at different experimental conditions

Calcium carbonate mineralization is central to many natural processes occurring at or near the Earth's surface. Complex organic matrix and soluble organic or inorganic additives are involved in the mineralization process and stabilization. While nature is even more complex and surface chemistry is over simplified in our work, our work provides a hint to understand the effect that additives can have on mineralization and stabilization with implications on diverse fields ranging from designing of biomimetic materials to better understand biomineralization.

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APPENDIX

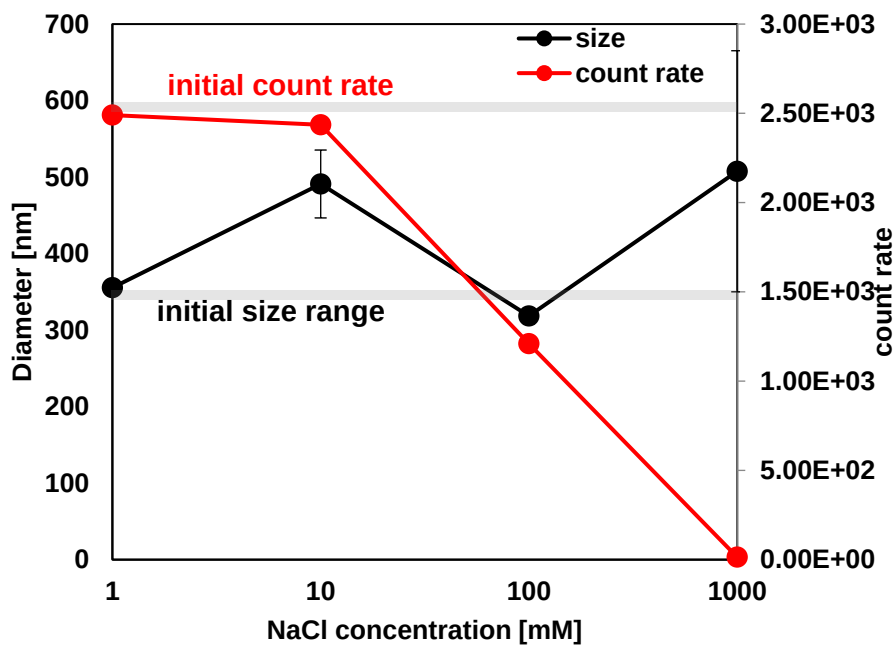


Figure 14 Size and count rate of PAA-ACC at different concentration of NaCl solution. At a concentration less than 10mM, the size of PAA-ACC nanoparticles increases and has a decrease at 100mM, then increase again at 1000mM

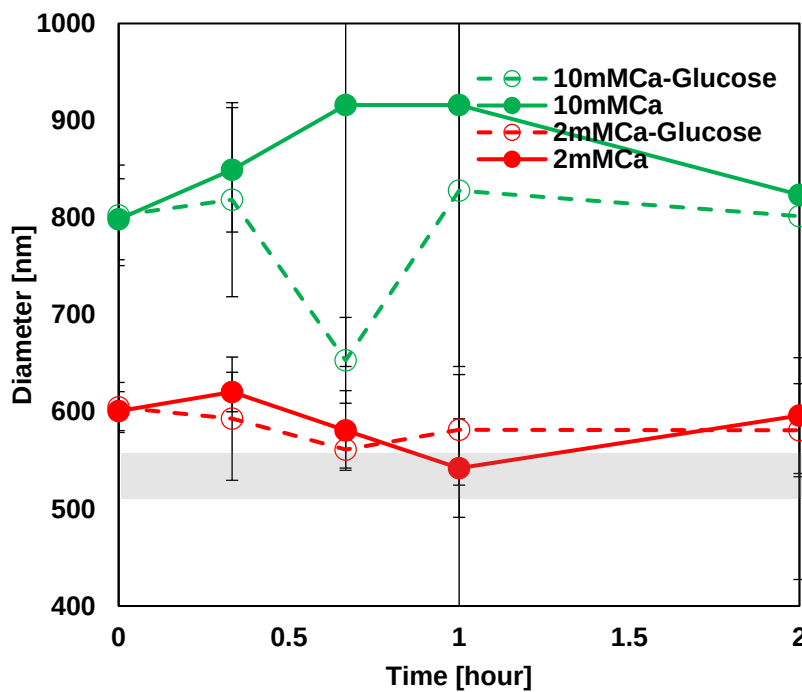


Figure 15 The influence of Ca^{2+} and glucose on size of PAA-ACC nanoparticles. CaCl_2 solution at concentration from 2 to 10mM was first added to synthetic PAA-ACC solution and then 20mM glucose was added

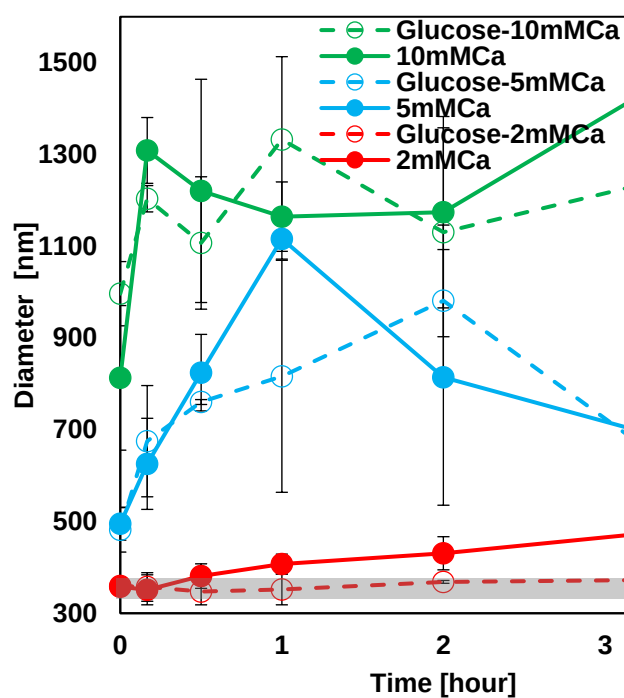


Figure 16 The influence of Ca^{2+} and glucose on size of PAA-ACC nanoparticles. CaCl_2 solution at concentration from 2 to 10mM was first added to synthetic PAA-ACC solution and then 20mM glucose was added. The solution was stirred during the entire time

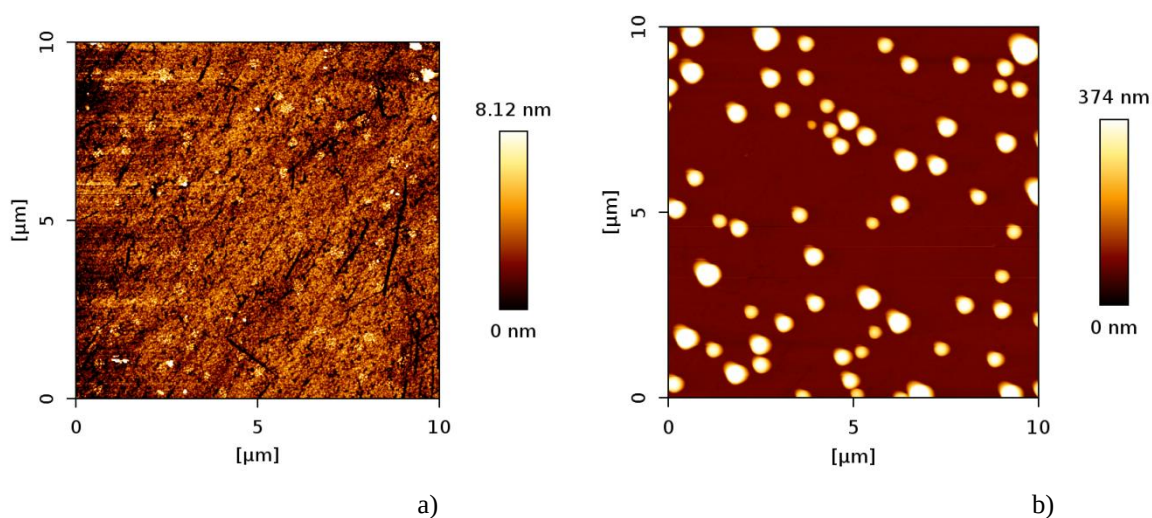


Figure 17 AFM tapping mode images for QCM gold sensors a) with b) without introducing glutamic acid