Calcium carbonate prenucleation clusters: towards unification of classical and non-classical nucleation theory

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CALCIUM CARBONATE PRENUCLEATION CLUSTERS: TOWARDS UNIFICATION OF CLASSICAL AND NON-CLASSICAL NUCLEATION THEORY

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MSc – Marine Biogeochemistry

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Thesis declaration

I, Jonathan AVARO, certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University.
Abstract

Classical nucleation theory is a powerful and long-established concept that describes the formation of minerals in supersaturated solution based on thermodynamic concepts. However, recent findings have shown that the nucleation of calcium carbonate minerals does not conform to this theory and instead involves small and reactive hydrated nanoclusters called prenucleation clusters. The aim of this PhD was to use novel analytical methods to better characterise calcium carbonate prenucleation clusters and further understand their role in the nucleation process.

The combination of small angle X-ray scattering with a novel mixing device capable of triggering nucleation on very short time scales allowed monitoring of the size, shape, volume and concentration of prenucleation clusters under a range of saturation states and environmentally-relevant pH conditions. New evidence for the occurrence of liquid-liquid like phase separation during the early stage of precipitation, and information on prenucleation cluster growth processes, were also obtained. The impact of similar pH conditions on the short range order of prenucleation clusters was also investigated using X-ray absorption techniques. Results indicated calcium carbonate prenucleation clusters possess low coordination numbers and short range order presenting similarities with calcite, consistent with computer simulation models for these systems. Finally, the hydration state of prenucleation clusters was examined via small angle neutron scattering.
Interpretation of the neutron scattering curves and comparison with X-ray scattering results suggest the presence of hydrated nanoparticles containing around 25% water. Artefacts possibly associated with a liquid-liquid like phase separation were also observed with neutron scattering.

In conclusion, this study has better defined the impact of pH and saturation state on the formation of calcium carbonate prenucleation clusters and brings new insights toward the unification of classical and non-classical nucleation theories, while raising further questions about the definition of the solubility limit in this system.
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i. the SAXS/WAXS beamline at the Australian Synchrotron, Victoria, Australia;

ii. XAFS beamline at Elettra synchrotron, Italy;

iii. QUOKKA beamline at the BRAGG Institute, NSW, Australia.
List of abbreviations

ACCC – Amorphous calcium carbonate
CC – calcium carbonate
CN – Coordination number
CNT – Classical nucleation theory
EXAFS – Extended X-ray absorption fine structure
FFT – Fast Fourier transform
HEPES – (4 -(2-hydroxyethyl)- 1- piperazineethanesulfonic acid)
HPLC – High-performance liquid chromatography
IFFT – Inverse fast Fourier transform
NCNT – Non classical nucleation theory
PMMA – Poly(methyl methacrylate)
PNCs – prenucleation clusters
SANS – Small angle neutron scattering
SAS – Small angle scattering
SAXS – Small angle X-ray scattering
WAXS – Wide angle X-ray scattering
XAFS – X-ray absorption fine structure
XANES – X-ray absorption near edge stucture
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Chapter I

Introduction
Calcium carbonate minerals are ubiquitous in natural environments and exist in several forms, including three anhydrous crystalline phases (calcite, aragonite and vaterite, listed in decreasing order of thermodynamic stability under standard conditions), two hydrated crystalline phases (the hexahydrate ikaite, and the monohydrate monohydrocalcite) and one amorphous phase (Morse et al, 2007). Indeed, calcium carbonate minerals comprise 20% by volume of sedimentary rocks deposited since the Cambrian Era (547 million years ago) (Morse & Arvidson, 2002). Most of these calcium carbonate minerals can naturally precipitate abiotically in alkaline fresh and marine waters, and also represent the most common biominerals used by organisms for teeth, bone or shells with different shapes, sizes and properties (Pouget et al, 2009; Cartwright et al, 2012). Precipitation of calcium carbonate minerals is an important process for removal of carbon dioxide from the atmosphere, and directly affects the chemistry of the Earth’s oceans, atmosphere and climate (Morse & Arvidson, 2002; Gebauer et al, 2008).

Despite its importance in the environment and in various industrial processes, the formation of calcium carbonate minerals is still not well understood. In particular, the abiotic precipitation of calcium carbonate is atypical and does not appear to follow classical nucleation pathways. The classical view considers the formation of a crystal to occur via the attachment of basic building blocks (atoms, ions or molecules, depending on the crystal) (Hu et al, 2012). However, crystallisation of calcium carbonate appears to involve nanoparticles as the basic building block for the formation of larger crystals (Gebauer et al, 2008; Sear et al, 2012; Gebauer et al, 2014). The presence of these nanoparticles, which have been
called “prenucleation clusters” due to their appearance in both undersaturated and supersaturated conditions, appears inconsistent with Classical Nucleation Theory and a new model incorporating them into the nucleation processes needs to be developed (Gebauer et al, 2008).

Calcium carbonate prenucleation clusters, which are composed of calcium ions, carbonate ions and a varying number of water molecules, appear to form a very reactive phase. Each of these nanoclusters changes shape and size every few femtoseconds and forms through a sequence of consecutive, dynamic chemical equilibria (Kellermeier et al, 2013). Although the clusters are constantly forming and dissolving on very short timescales, their average properties remain constant over longer timescales. This unusual combination of thermodynamic and kinetic processes led to the development of an alternative model for nucleation known as Non-Classical Nucleation Theory, in which prenucleation clusters are the basic building block for mineral formation (Demichelis et al, 2011). Attempts to characterise prenucleation clusters have used a range of computer simulation, ultracentrifugation, electron microscopy and synchrotron based X-ray techniques (Gebauer et al, 2008, Pouget et al, 2009; Raiteri & Gale, 2010; Wolf et al, 2011; Demichelis et al, 2011; Cartwright et al, 2012; Wallace et al, 2013; Chao et al, 2014). However, these experiments have mostly been performed under high supersaturation and/or high pH and, to date, very little work has been done to understand the impact of saturation state, particularly under environmentally-relevant near-neutral to slightly alkaline pH conditions, on the formation and
properties of these nanoclusters. This thesis aims to better define the characteristics of PNCs and how these characteristics are affected by environmental parameters.
Research Objectives

The overall goal of this Ph.D. is to experimentally resolve details of the very early stages of homogeneous, abiotic calcium carbonate nucleation, including the processes leading to the formation of the very first nuclei, under environmentally-relevant conditions. This overall goal can be further divided into four main research objectives:

i. to develop experimental tools allowing acquisition of synchrotron based data with high temporal resolution.

ii. to determine the physical characteristics of calcium carbonate prenucleation clusters.

iii. to elucidate the pathways leading to the formation of prenucleation clusters and their importance in the nucleation process.

iv. to investigate the impact of pH and saturation state on prenucleation clusters and their formation processes under environmentally-relevant conditions.
This thesis is composed of eight chapters. Following this introduction, Chapter II provides a literature review of the discovery of calcium carbonate prenucleation clusters and the development of the Non-Classical Nucleation Theory. In particular, the evolution of knowledge has been described, and mistakes that were made with respect to the definition of PNCs over time have been highlighted and evaluated. Chapter III gives a brief description of the analytical methods used in this Ph.D. Chapter IV contains a detailed description of the development of a micro mixing device and characterisation of its mixing performance. Chapters V and VI describe the characterisation of calcium carbonate prenucleation clusters via Small Angle X-ray Scattering (SAXS) and Extended X-ray Absorption Fine Structure (EXAFS), respectively. Chapter VII describes attempts to characterise the hydration state of PNCs via Small Angle Neutron Scattering (SANS). Finally, the results and conclusions are summarised in Chapter VIII.
Chapter II

Toward a new nucleation theory: use of mechanistic concepts to describe homogeneous precipitation of calcium carbonate minerals in aqueous solutions
Nucleation, also called germination, describes the processes involved in the formation of the first crystalline structures of a mineral during the precipitation process. Homogeneous precipitation, in contrast to heterogeneous precipitation, occurs when crystallisation does not start on a pre-existing interface between the solution and another solid phase (i.e. particles) or vapour (i.e. air bubbles). The primary focus of this thesis, and thus the literature review presented in this chapter, strictly concerns homogeneous nucleation.

1. Classical nucleation theory

1.1. Introduction

Classical nucleation theory (CNT), which was first defined in the 1930s, is a powerful concept based on thermodynamics that describes the formation of a crystal from a solution, a liquid, or vapour. With reference to the solubility limit, i.e. the limit of how much solute will dissolve in a certain amount of solvent at a certain temperature and ionic strength, this theory describes the formation of a mineral in supersaturated solution (i.e. a solution containing high concentrations of the dissolved constituent ions of the mineral such that the saturation index $\Omega > 0$) as a function of the balance between the surface energy gain by the creation of an interface (which occurs upon formation of nuclei) and the free energy loss in the bulk solution due to the decrease in supersaturation. The saturation index $\Omega$ reflects the thermodynamic favourability of mineral formation: when $\Omega > 0$, mineral formation is favoured, whereas when $\Omega < 0$, mineral dissolution is favoured. For calcium carbonate minerals, this quantity is defined as:
\[ \Omega = \ln \left( \frac{[\text{Ca}^{2+}][\text{CO}_3^{2-}]}{K_{sp}} \right) \]

Equation 1

where \([\text{Ca}^{2+}]\) and \([\text{CO}_3^{2-}]\) represent the concentrations of calcium and carbonate ions, respectively, the product \([\text{Ca}^{2+}][\text{CO}_3^{2-}]\) is called the Ion Activity Product (IAP), and \(K_{sp}\) is the solubility constant.

CNT describes the formation of the first nuclei in supersaturated homogeneous solution as a one-step process (in the sense that there is one energy barrier to overcome, but consisting of many individual ion attachment events to overcome the barrier) driven by the balance between interfacial and bulk energy of the new phase. According to CNT, the difference between interfacial and bulk energy represents the only barrier to mineral formation (Figure 1). This energy barrier, which controls the formation of the very first nuclei, depends critically on the solution’s supersaturation state, and the kinetics of this reaction are only driven by the ambient pressure and temperature (Stumm and Morgan, 1969; Gebauer et al, 2009; Teng, 2013).

In other words, random collisions of ions in solution caused by thermal fluctuations and Brownian motion will create metastable ion clusters. As the unfavourable interfacial energy is dominant at small particle sizes, formation of an initial cluster is reversible before reaching the critical size \(r_{\text{crit}}\) and the nucleus will dissolve. At larger cluster sizes, the favourable bulk energy contribution compensates for the energy cost arising from creation of the interfacial surface. The inflection point where interfacial energy is compensated exactly by bulk energy defines the critical size from where any change towards a larger cluster radius will
render the system stable and lead to unlimited growth, while a change towards smaller cluster radius will render the system unstable and trigger dissolution of the nucleus. In that regard, small nuclei possess a positive excess in free energy, $\Delta G_{ex}$, that can be associated with the notion of a thermodynamic barrier to nucleation.

![Free Energy vs. Radius](image)

**Figure 1:** Size dependence of the energetics of the first nuclei described via CNT. Evolution of interfacial energy via changing particle area (blue line), bulk energy (red line) and sum of the two (green line) with cluster size. Critical radius ($r_{crit}$) at the inflection point of the green line, associated with positive free energy $\Delta G_{ex}$, define the first nuclei in the Classical Nucleation Theory. The figure is adapted from Gebauer & Cölfen (2011).

The rate of nucleation, $J$, can be expressed in terms of the thermodynamic barrier and the kinetic barrier as:

\[
J = A e^{-E_A/(k_B T)} e^{-\Delta G_{ex}/(k_B T)}
\]

*Equation 2*

where $A$ is a pre-exponential factor depending on the properties of the material, the term $-E_A/(k_B T)$ represents the kinetic barrier with an overall activation energy of $E_A$, the term $-\Delta G_{ex}/(k_B T)$ represents the thermodynamic barrier, $k_B$ is the
Boltzmann constant and $T$ the absolute temperature. To grow, the nucleus has to overcome the thermodynamic energy barrier. This last barrier can be estimated by assuming that the nuclei possess the same bulk structure and corresponding interfacial energy, and so behave exactly like, the macroscopic crystal. This assumption, called the capillary assumption, is the foundation of the CNT. On the other hand, the kinetic barrier is difficult to quantify and is therefore neglected in the CNT.

The bulk free energy of nuclei can be described as follows via the so-called affinity or chemical potential ($\phi$) term:

$$\phi = k_B T \ln \left( \frac{\text{IAP}}{K_{sp}} \right)$$

*Equation 3*

where IAP is the ion activity product of the component ions from which the nuclei are formed, and $K_{sp}$ is the bulk solubility constant of the nucleated phase (which is essential for the capillary assumption as described before). The quotient $\ln(\text{IAP}/K_{sp})$ reflects the degree of supersaturation when $> 0$ or undersaturation when $< 0$.

Equation 4 describes the excess free energy of the clusters at the maximum of the free energy curve shown in Figure 1:

$$\Delta G_{ex}(S) \sim \alpha \gamma^3 \phi^{-2}$$

*Equation 4*

where $\gamma$ is the size-independent interfacial free energy and $\alpha$ is a shape factor taking into account non-spherical nuclei. A mathematical expression for the critical radius,
\( r_{\text{crit}} \), can be extracted from Equation 4 assuming the critical cluster is spherical (and thus neglecting the term \( \alpha \), which is minimal for a sphere) and expressed as:

\[
r_{\text{crit}} \sim \gamma \phi^{-1}
\]

*Equation 5*

#### 1.2. Limits of the CNT

Based on the above discussion, and despite the fact that the kinetic barrier is neglected, it is theoretically possible using CNT to predict the nucleation rate of any crystal at a given degree of supersaturation. However, values calculated accounting only for the thermodynamic barrier differ by orders of magnitude from experimentally measured data. This divergence can have multiple origins due to difficulty in deriving the appropriate value of the interfacial free energy or in precise quantification of the bulk energy term.

Moreover, the capillary assumption can be flawed in a system where the phase present in the initial nuclei can have a different structure from the final crystallised product, as is the case with calcium carbonate. In such a system, the least stable (and often first to form) solid polymorph, called amorphous calcium carbonate (ACC), is described as a family of transient precursor species, possessing different levels of hydration and existing in several forms depending on the formation mode (biotic or abiotic) and different short to long range order (*i.e.* short or long atomic arrangement). The processes behind formation of crystalline forms from ACC in pure and natural systems are now well documented and can generally be described via a simplified pathway involving progressive dehydration and reorganisation of atomic nuclei driven by Ostwald ripening processes (dissolution
and re-precipitation of small crystals into larger crystals). At this stage, the following equation is generally accepted to describe precipitation of CC minerals:

\[
\text{CaCO}_3(s) \rightleftharpoons \text{Ca}^{2+}(aq) + \text{CO}_3^{2-}(aq), \quad K_{sp} = \frac{\{\text{Ca}^{2+}(aq)\}\{\text{CO}_3^{2-}(aq)\}}{[\text{CaCO}_3(s)]}
\]

*Equation 6*

where \{CaCO_3\}_s represents the activity of the first solid nuclei, and \{Ca^{2+}\}_aq and \{CO_3^{2-}\}_aq represent the activity of calcium and carbonate ions forming the nuclei, respectively.

However, assigning a solubility constant to the reaction shown in Equation 6 for all possible forms of ACC that is representative for the nucleated phases becomes extremely challenging as different forms of ACC may possess different hydration levels, different thermodynamic stability, and changing solubility with size. Therefore, strict evaluation of the level of supersaturation or an exact value of the solubility limit based on CNT might be difficult, if not impossible. Recent work by Nielsen *et al* (2014) has shown that direct precipitation of crystalline CC phases could occur via dissolution-reprecipitation from ACC. However, these results are controversial since the authors have recorded direct precipitation of aragonite, which normally forms at temperatures > 45°C, and it has been suggested that the observed dissolution-precipitation is instead most likely due to the beam intensity (Farhadi-Khouzani *et al*, 2016).

2. Toward a new nucleation theory

To understand current knowledge about nucleation through the atypical example of calcium carbonate systems, it is necessary to examine the key studies
involved in the development of a contradictory train of thought leading to the creation of an alternative nucleation theory.

In 2008, Gebauer et al described a new vision of mineral precipitation through a series of experiments focused on the early stages of nucleation processes and their implications for the formation of different ACC minerals (Gebauer et al, 2008). Measurements using a calcium ion selective electrode during titration of free calcium concentrations in solution at different pH presented discrepancies between theoretical and measured concentrations in conditions that were undersaturated with respect to ACC and early supersaturated conditions (Ω slightly larger than 0). While CNT defines the first stable particle as a particle possessing a critical radius and forming under supersaturated conditions, Gebauer et al (2008) demonstrated the presence of extremely small but stable ion clusters in both undersaturated and supersaturated solutions (with respect to ACC) via ultracentrifugation techniques and Wide Angle X-ray Scattering (WAXS) measurements. According to their results, the hypothetical size of these compounds, which were given the name prenucleation clusters (PNCs), should be around 2 nm, and correspond to an aggregate of approximately 70 Ca$^{2+}$ and CO$_3^{2-}$ ions. Larger clusters possessing a size of 4 nm to 6 nm were also identified. Furthermore, after nucleation these small clusters were no longer able to be identified.

Thermodynamically, as these nanoclusters are considered stable with regard to ions in solution, they must possess an energy barrier lower than atoms in solution (Figure 2). Moreover, they seem to present different structural characteristics depending on pH. The change in the acid-base speciation of carbonate ions directly
impacts the stability of PNCs and leads to the precipitation of different crystalline phases (named ACC I and ACC II). The way these nanoclusters grow and their precise chemical (e.g. kinetics of nucleation, composition, thermodynamic stability, reactivity, stoichiometry, etc.) and physical properties (size, structure, etc.) cannot be explained via CNT and remains poorly understood. Even though the thermodynamic stability of the clusters was unable to be quantified because of the number of unknown variables, Gebauer et al (2008) suggested a theoretical composition for stable nanoclusters leading to an equilibrium equation as follows:

\[(\text{CaCO}_3)_x(\text{aq}) \rightleftharpoons x\text{Ca}^{2+}(\text{aq}) + x\text{CO}_3^{2-}(\text{aq}), \quad K_{sp} = \frac{\{\text{Ca}^{2+}(\text{aq})\}^x\{\text{CO}_3^{2-}(\text{aq})\}^x}{\{(\text{CaCO}_3)_x(\text{aq})\}}\]

\text{Equation 7}

where \{\{(\text{CaCO}_3)_x\}\}(\text{aq}) represents the activity of the PNCs, \(x\) the number of ions of each of \(\text{Ca}^{2+}\) and \(\text{CO}_3^{2-}\) in the cluster, and \{\text{Ca}^{2+}\}(\text{aq}) and \{\text{CO}_3^{2-}\}(\text{aq}) the activities of calcium and carbonate ions in solution, respectively.

A major difference to CNT comes from the fact that the spatial distribution of ions in solution is no longer homogeneous: since PNCs are considered as solute compounds, their formation and aggregation will generate a decrease in the degree of homogeneity.
**Figure 2:** Free energy of reaction ($\Delta G$) profile for the first calcium carbonate nuclei viewed through the prism of classical and non-classical nucleation theory as a function of the reaction coordinate. In the classical pathway (bold line), nucleation occurs via formation of metastable clusters when the critical nucleation energy barrier ($\Delta G^*$) is overcome. Following the non-classical nucleation theory (dashed line), stable clusters possessing a negligible activation barrier compared to thermal energy form. The exact structure and depth of the $\Delta G$ minimum as well as the activation barrier for nucleation remain unknown. Adapted from from Gebauer et al (2008).

Work conducted by Pouget et al (2009) further characterised the PNCs using cryo-TEM and recorded the size evolution of calcium carbonate nanoclusters over time. Their findings were consistent with the size of the PNCs identified in previous studies, i.e. between 0.6 and 5 nm. Whereas Gebauer et al (2008) assessed the presence of PNCs only in the early stage of nucleation due to the difficulty in differentiating free ions and PNCs at high concentration via ultracentrifugation, Pouget et al (2009) showed the persistence of these nanoclusters even after
nucleation and demonstrated that they remained present during all stages of the mineralisation process. The validity of these cryo-TEM findings has been partially disputed due to the resolution of the method failing to identify objects smaller than 2 nm. However, defining PNCs as stable with respect to dissolved ions in solution has raised some doubts. According to Nudelman et al (2010) and Sear (2012), neither equilibrium nor kinetic theory can explain why the clusters are still present in the solution after precipitation and, if so, why don’t they grow?

Analysis of the growth of PNCs has produced additional information on the processes involved and structural evolution that occurs during mineralisation. Although simulation previously indicated that ACC nanoparticles < 2 nm in size may already possess short-range local order different from all crystalline calcium carbonate polymorphs (Quigley et al, 2008), none of these structures were recorded at that time. The first internal structure recorded by Pouget et al (2009) in which crystallinity may have been present corresponded to a particle size range of 30 to 250 nm. These particles presented complex diffraction patterns, which could be attributed to vaterite-like profiles. After longer reaction times, larger particles presented atypical calcite and vaterite diffraction patterns, indicating a gradual development of crystallinity and use of PNCs as a feedstock for particle growth as they aggregate. More precisely, experiments employing light scattering and X-ray scattering (Liu et al, 2008; Liu et al, 2010) have demonstrated that development of ACC can be explained by a monomer addition model involving a short nucleation step followed by a long growth period. This mechanism thus distinguishes two phases: a first phase corresponding to formation of monomers and a second
corresponding to the growth of particles. Although the authors did not clearly speculate such, these monomers could correspond to PNCs.

Sear (2012) described two different processes that could stabilise PNCs. First, the presence of impurities could act as a support and thus stabilise nanoscale fluid clusters in both undersaturated and supersaturated conditions, thereby facilitating the formation of PNCs (Figure 3). The second stabilisation mechanism hypothesised by these authors refers to the charge of the nanoparticles. If both calcium and carbonate ions partition more strongly than other ions present in the solution, and thus decrease the homogeneity of the solution, the cluster should acquire a net charge. In such a cluster, with a volume $v$, Coulombic self-energy increases faster ($v^{5/3}$) than surface free energy ($v^{2/3}$) and bulk driving force from the solution ($v$), contributing to the cluster’s stability. Sear (2012) adds that due to their small size, the PNCs described by Gebauer et al (2008) shouldn’t exceed the Debye length (the size of the influence sphere of electrostatic forces). Therefore, charge stabilisation could create finite metastable clusters, positioned at a local minimum in free energy. This concept will be expanded on later.
Figure 3: Free energy of reaction ($\Delta G$) profile for homogeneous and heterogeneous precipitation. The presence of particles acting as favourable nucleation sites during heterogeneous nucleation implies a lower barrier ($\Delta G^*$) to nucleation and the formation of smaller stable nuclei.

While experimental studies have permitted collection of ongoing evidence regarding the existence of PNCs, acquiring direct information about their physical and chemical characteristics remains a major challenge. However, computer-based simulations, especially when coupled with experimental data, provide powerful and complementary tools for understanding nucleation processes. The first relevant study based on computer simulation, performed by Tribello et al (2009), demonstrated that the level of saturation has an impact on the growth rate of ACC minerals. However, their estimates of the solvation of calcium ions and calcium solubility were unrealistic and after reconsideration, it was admitted that this study
simulated a liquid-liquid like phase separation process occurring during the early stage of mineral precipitation and not in the PNCs themselves (Gebauer et al, 2014).

Quigley et al (2008) also investigated possible structural arrangements and the change in free energy of calcium carbonate nanoparticles (2 nm to 5 nm diameter), showing that nanoclusters could possess a minimum in free energy when in amorphous form. Regarding these results, small nanoparticles should not be stabilised by surface tension, as larger ACC particles are, but rather by kinetic processes, however this is currently not well known.

Quigley et al (2008) also demonstrated that a water molecule can be trapped between two nanoparticle aggregates, which seems consistent with the varying hydration level of ACC. Simulations of hydrated and anhydrous ACC nanoparticles have also shown that water integration into the crystalline structure generates a decrease in enthalpy (Raiteri & Gale, 2010). The presence of impurities may then not be necessary to stabilise these nanostructures as postulated by Sear (2012), as water molecules may effectively perform this role. Michel et al (2008) also demonstrated that under supersaturated conditions, growth may occur faster than water diffusion into the crystalline structure. This implies that, in addition to contributing to the stability of these nanoparticles, the hydration level might decrease toward the core, becoming a key factor to control the reactivity of such molecules.

On the basis of these findings, the definition of the PNCs has further evolved from that defined by Gebauer et al (2008) to incorporate water molecules in the clusters:

\[
[\text{Ca}_x(\text{CO}_3)_y(\text{H}_2\text{O})_z]^{2(x-y)+}(\text{aq}) \rightleftharpoons x\text{Ca}^{2+}(\text{aq}) + y\text{CO}_3^{2-}(\text{aq}) + z\text{H}_2\text{O}
\]
\[K_{sp} = \frac{\{\text{Ca}^{2+}(aq)\}^x\{\text{CO}_3^{2-}(aq)\}^y\{\text{H}_2\text{O}\}^z}{\left\{\text{Ca}_x(\text{CO}_3)_y(\text{H}_2\text{O})_z\right\}^{2(x-y)+}(aq)}\]

_Equation 8_

where \( x, y \) and \( z \) represent the number of ions of \( \text{Ca}^{2+}, \text{CO}_3^{2-}, \) and \( \text{H}_2\text{O} \) in each cluster and with \( 1 < z < 2 \) for ACC and values varying as a function of the amorphous nanoparticle’s size (Raiteri & Gale, 2010).

Demichelis _et al_ (2011) further added to the structural definition of PNCs by using molecular dynamics simulation of ions in solution on extremely short time scales (down to 70 ns) to show that PNCs exhibit a very dynamic structure initiated by ionic interactions. Random collisions of ions and/or ion pairs create linear, branched and ring-like ionic structures. These compounds, composed of calcium, carbonate and/or bicarbonate ions, held together by ionic bonds between calcium and carbonate ions, are stable with respect to solvated ions, despite constant bond breakage and rearrangement. The highest probability coordination number for calcium present in these dynamic compounds is two, meaning that chain-like structure are more likely to be formed (Figure 4).

Demichelis _et al_ (2011) termed these compounds Dynamically Ordered Liquid-Like Oxyanion Polymers (DOLLOPs) and defined their growth, lifetime and size as a function of of calcium and carbonate ion concentrations (the lifetime of a cluster will increase as the concentration decreases due to statistically fewer ionic collisions), concentration of bicarbonate ions and thus, indirectly, pH. The radius of gyration (_i.e._ the distance between the edge and the centre of gravity of the structure, corresponding to the effective radius of a fractal object) of these DOLLOPs
decreases in the presence of excess bicarbonate ions. As described by Gebauer et al (2008), the pH dependence of the properties of these PNCs can be ascribed to the competition between bicarbonate and carbonate ions being greater at low pH; since bicarbonate ions will create a weaker bond with calcium, this latter form will act as a chain terminator and limit the growth of a particular cluster.

**Figure 4:** Probability of occurrence of a given calcium-carbon coordination number in calcium carbonate PNCs as a function of pH. The highest probability is associated with the formation of chain like structures. pH does not seem to have any direct impact on the mean number of calcium-carbon bonds. Reproduced from Demichelis et al (2011).

This work gives a first clue to answer the conundrum exposed by Nudelman et al (2010) and Sear (2012): it appears that the PNCs are still present after nucleation, but don’t grow, because they possess a different structure than the macroscopic phase. Overcoming the energy barrier to nucleation, as described before, could imply a structural rearrangement leading to a phase that is more closely related to the macroscopic form. To go further, there should actually be two energetic barriers to nucleation: a first “low barrier” between solution and the first structural arrangement, and a second “larger barrier” corresponding to an internal
structural rearrangement and loss of hydration, leading finally to development of macroscopic-like structures.

An alternative pathway to mineralisation proposed by Gower *et al* (2008) and expanded by Wolf *et al* (2011) presents liquid-liquid like phase separation as a potential process for formation and aggregation of pre-critical hydrated complexes. Wallace *et al* (2013) simulated this mineralisation pathway for calcium carbonate, investigating cluster stability, structure and the nucleation pathway resulting from a liquid-liquid like phase separation mechanism. Calcium ion diffusion coefficients in clusters were higher than those expected for ACC, implying that PNCs could be droplets of a dense ion-rich liquid phase of hydrated calcium carbonate. Simulations of free energy as a function of the number of ions in a cluster indicated a general monotonic decrease, revealing a stable stage in the liquid-liquid separation. In other words, within the same liquid, two stable and distinct phases could coexist: a main “classical” phase containing ions homogeneously distributed in solution, and a second nanoscopic liquid phase characterised by an accumulation of ions. This alternative pathway is consistent with the change in the homogeneity of the mother liquid as described before.

Another key finding of this research was a smooth evolution in the coordination number of calcium by carbonate ions (evolution from 2 to 3 occurs with approximately 26 ions), indicating that the formation of a dense liquid does not mark an abrupt change in cluster character. Because the second liquid becomes increasingly supersaturated, it could potentially host different sized PNCs. These results are consistent with recent synchrotron-based Small Angle X-ray Scattering
(SAXS) results in which the authors identified two distinct sizes distributions during the early stages of nucleation (Chao et al 2014).

In a recent review paper, Gebauer et al (2014) synthesised and incorporated these new findings into the evolving picture of non-classical nucleation theory. The theoretical definition of liquid-liquid separation obeys the following equation:

\[ A^{z+}(\ell_1) + B^{z-}(\ell_1) \rightleftharpoons A^{z+}(\ell_2) + B^{z-}(\ell_2) \]

\textit{Equation 9}

where \( \ell_1 \) and \( \ell_2 \) represent the two liquid phases and \( A^{z+} \) and \( B^{z-} \) two ions in solution.

Because the second phase can contain and grow small and larger clusters, clusters previously observed by cryo-TEM in similar systems (silicate and silicate-stabilised calcium carbonate PNCs) could correspond to frozen droplets of the dense liquid or an amorphous solid. Such results have been previously illustrated in highly supersaturated silicate systems where a myriad of nanoclusters reversibly aggregate to form larger objects (20 to 30 nm in size) in a manner that is sensitive to pH change (Kellermeier et al, 2012). Even if no mechanistic pathways have been simulated, the progressive dehydration of the dense liquid and the aggregation of these nanodroplets strongly suggests that ACC nucleation from this dense liquid phase is possible.

A central conundrum when examining liquid-liquid like phase separation and the formation of PNCs is whether the formation of a dense liquid is triggered by the formation of PNCs or vice versa. At this stage, it is difficult to experimentally differentiate these two processes, as formation of PNCs could explain, from a
molecular point of view, the presence of nanoscopic dense liquid droplets, while phase separation via liquid-liquid like separation phase could conversely explain formation of PNCs (Gebauer et al, 2014).

One of the keys to understand the formation, stability and properties of PNCs is the balance needed between the free energy of hydration and the interaction of the constituent ions. This second consideration could partially explain the difference in precipitation of macroscopic-like phases between iron and calcium carbonate systems. In their attempt to combine non-classical and classical nucleation theory, Baumgartner et al (2013) focused on an analysis of the first thermodynamic barrier. As indicated in Equation 4, the magnitude of this barrier scales with the size of the clusters as well as the level of supersaturation. Their analysis showed that the sign of the excess free energy term, $\Delta G_{\text{ex}}$, can define whether the nuclei are metastable (if $\Delta G_{\text{ex}}$ is positive), and therefore nucleation produces an amorphous phase, or stable (if $\Delta G_{\text{ex}}$ is negative), in which case the nuclei directly form the crystalline phase. In the former pathway, metastable nuclei are less stable than ions in solution and are created via thermal fluctuations, providing a low barrier pathway to nucleation; in the latter pathway, nuclei are more stable than free ions and can then never form by surpassing the thermodynamic energy barrier (De Yoero et al, 2014). One of the issues in making this distinction in a real system is the extreme difficulty in attributing a correct value to the size dependence of the interfacial free energy. Indeed, configurations that maximise ion coordination and present a beneficial arrangement of hydration layers are more likely to exhibit local free-energy minima (Hu et al, 2012; De Yoreo et al, 2014). Thus, different nucleation pathways have to
be considered depending on the species that may potentially be present, whether the nuclei are on the most thermodynamically favourable path, and whether they may be stabilised by kinetic factors.

Different schools of thought thus consider PNCs either as either simple ionic complexes that constantly form sub-critical nuclei that spontaneously fluctuate in size (through ion addition or cluster aggregation) until they might reach the critical size, or as nanoclusters that form ion-by-ion and play no direct role in the nucleation process at low concentration (Gebauer et al, 2014). This second pathway could be rationalised due to the number and diffusivity of free ions being much greater than the number and diffusivity of the clusters, or because the kinetic barrier to form an ordered nucleus from ions in solution is much less than the kinetic barrier to ordering and desolvating the cluster (Gebauer et al, 2014). However, Hu et al (2012) concluded their study by pointing out the lack of experimental tools allowing the direct characterisation of PNCs under low concentration conditions, making distinguishing between these various possibilities difficult.

3. Defining PNCs, their formation and implications to the nucleation process

PNCs have thus been defined as “polynuclear solute ion association complexes exhibiting a highly dynamic character” (Gebauer et al, 2014, p. 2364), and must satisfy the following (Gebauer et al, 2014):

i. They must be composed of atoms, molecules, and/or ions of a forming solid, but can also contain additional chemical species.
ii. They must be small and thermodynamically stable solutes, without any phase boundary between the ambient phase and the clusters.

iii. They could participate directly or indirectly to the process of solid phase formation and be a direct or indirect precursor of mineral species.

iv. They must be very dynamic, changing configuration within hundreds of picoseconds.

v. They could have an internal structure resembling or relating to the structure of a crystalline polymorph.

Notably, point ii is the only criterion that distinguishes PNCs from a liquid.

Ultimately, PNC formation can be described by the following equation:

\[
[Ca_x(CO_3)_y(H_2O)_z]^{2(x-y)^+} (aq) \\
\cong xCa^{2+} (aq) + y CO_3^{2-}/HCO_3^- (aq) + z H_2O/OH^-/H_3O^+
\]

\[
K_{sp} = \frac{\{Ca^{2+} (aq)\}^x\{CO_3^{2-} (aq)\}^y\{H_2O\}^z}{\left[Ca_x(CO_3)_y(H_2O)_z\right]^{2(x-y)^+} (aq)}
\]

\textit{Equation 10}

Even if this theory is not universally accepted, the mechanistic pathways by which PNCs may be involved in mineral formation are described in Figure 5. It still remains unclear if the formation of PNCs is triggered by the formation of a second liquid phase, resulting in a local increase in saturation state, or if the aggregation of hydrated PNCs can initiate liquid-liquid like phase separation. Several essential variables, like the size and the shape of these PNCs, the presence (or otherwise) of a hydration layer, the kinetics of various reactions involved in the formation and
transformation of the PNCs, and the importance of parameters like pH or the calcium concentration, remain poorly understood and are the main focus of this PhD thesis.

**Figure 5:** Schematic representation of non-classical nucleation pathways. The prenucleation cluster pathway, the different phases and their interactions are still not well understood.

The choice of the analytical methods for this project is based on the literature review of characterisation of the properties of PNCs and the nanoscale environment in which they form. Use of synchrotron light sources presents many advantages compared to benchtop laboratory instruments, such as a very high signal to noise ratio due to their high brilliance. Combined with fast detectors, synchrotron and neutron radiation techniques are often the primary choices for the study of nano-scale objects. A more detailed presentation of the methods used in this project, including their advantages and limitations, is given in the following chapters.
Chapter III

Materials and methods
Characterisation of structures present during the early stages of calcium carbonate mineral formation was primarily performed using synchrotron-based Small Angle X-ray Scattering (SAXS) at the SAXS/WAXS beamline at the Australian Synchrotron (Australia), synchrotron-based X-ray Absorption Fine Structure (XAFS) spectroscopy at the EXAFS beamline at ELETTRA synchrotron (Italy), and Small Angle Neutron Scattering (SANS) at the Quokka beamline at ANSTO (Australia).

The use of synchrotron light sources presents many advantages compared to benchtop instruments for such measurements. In particular, the high brilliance of synchrotron radiation enables collection of data with a very high signal to noise ratio and, combined with fast detectors, rapid data acquisition. Thus, synchrotron radiation techniques represent the ideal choice for the study of nanoscale objects under dilute conditions and/or which form and transform at short timescales.

With the combination of these three different methods, I aimed to characterise the structures present during the early stages of calcium carbonate formation, potentially including prenucleation clusters, at different size scales. Specifically, XAFS allows characterisation of the average atomic structure around a calcium ion, while SAXS and SANS can potentially provide detailed information on the average size and shape of nanoscale structures such as calcium carbonate PNCs, and enable identification of possible liquid-liquid like phase separation processes.
1. Synchrotron radiation

A brief description of the formation of synchrotron radiation is presented below, followed by a brief introduction to each of the synchrotron and neutron based techniques used in this PhD.

Initially, low energy (90 keV) electrons are generated via a heated tungsten cathode called the “electron gun”. The electrons are accelerated first in a linear accelerator to 100 keV and finally in the booster ring where they reach 99.9% of the speed of light (3 GeV). The electron beam is then guided into a storage ring where the electrons circulate through short straight sections joined using bending magnets (quadrupole and sextupole magnets used to focus the beam, chromaticity and orbit corrections) (Figure 6). Synchrotron light, including high brilliance X-rays, is produced when electrons are forced to travel through a closed path by a strong magnetic field (typically induced by bending magnets, undulators or wigglers) (Figure 7). The synchrotron light produced is then used in a range of different beamlines situated around the storage ring. The synchrotron light is typically focused using different sets of monochromators and mirrors situated prior to and inside each beamline to tune the X-rays characteristics needed for a wide variety of analytical techniques such as spectroscopy, diffraction, scattering or imaging (Thompson et al, 2009).
Figure 6: Schematic representation of a synchrotron. 1 - Electron gun; 2 - Linear accelerator; 3 – Booster ring; 4 - Storage ring; 5 - Bending magnet; 6 - Undulator/wiggler; 7 - 8 - 9 Beamline with 7 - Monochromators and mirrors; 8 - Experimental hutch; 9 – Beamline control room. Reproduced from the general diagram of the Soleil Synchrotron (https://commons.wikimedia.org/wiki/File:Sch%C3%A9ma_de_principe_du_synchrotron.jpg).

Figure 7: Quadrupole bending magnet (blue) and undulator (orange) from the storage ring at the National Synchrotron Radiation Research Center, Taiwan (Author’s own image).
2. X-Ray Absorption Fine Structure (XAFS)

X-ray Absorption Fine Structure (XAFS) is a synchrotron based technique used to characterise the coordination environment around a central atom. It refers to the way X-rays are absorbed by an atom at X-ray energies near and above the core-level electron binding energy of that atom. XAFS spectra are particularly sensitive to the oxidation state, the nature of the surrounding atoms, their distances and coordination number. Commonly accepted as a reliable element-specific method, XAFS is commonly used to characterise bulk physical environment of a variety of systems in a wide range of scientific fields such as biology, materials science, catalysis research and environmental science.

The XAFS signal is generated by the absorption of light through the photo-electric effect and can be interpreted as the scattering of the photo-electron ejected from the absorbing atom by the photo-electric effect. X-ray absorption spectrum can be separated in two different energetic regime. While X-ray absorption near-edge spectroscopy (XANES), corresponding to an energy range close to the absorption edge of the absorbing atom, is sensitive to the oxidation state as well as the coordination chemistry (number and orientation of atomic bonds), extended X-ray absorption fine-structure spectroscopy (EXAFS), is used to determine the identity of the neighbouring atoms around the absorbing atom, the bond distance and coordination number (Newville, 2004) (Figure 8).
The EXAFS signal can be described by the following equation:

$$\chi(k) = \sum_j N_j f_j(k) e^{-2k^2\sigma_j^2} \frac{kR_j^2}{kR_j^2} \sin[2kR_j + \delta_j(k)]$$

*Equation 11*

where $\chi(k)$ is the overall wave function corresponding to the EXAFS signal, $f(k)$ and $\delta(k)$ represent the scattering amplitude and phase shift, respectively, induced by the atoms neighbouring the excited central atom, $N$ is the number of neighboring atoms, $e^{-2k^2\sigma_j^2}$ is known as the amplitude reduction factor, and $\sigma^2$ is the disorder in the vicinity of the neighbouring atom. Though somewhat complicated, the EXAFS equation allows determination of $N$, $R$, and $\sigma^2$ given the scattering amplitude $f(k)$ and phase-shift $\delta(k)$ resulting from the presence of particular neighbouring atoms.

XAFS constitutes an effective method to study the coordination environment of calcium carbonate minerals due to their intrinsic characteristics. However, it is important to consider that the EXAFS signal is representative of the average coordination environment, and heterogeneity or the presence of different structures...
(e.g. different mineral phases) within the same sample can greatly complicate data analysis. As a consequence, even if the data acquisition is relatively straightforward, the development of suitable samples to study PNCs presents a major challenge.

3. Small Angle Scattering (SAS) techniques

Over the last few decades, Small Angle Scattering has become a reference method for characterisation of nanoscale objects due to development of brighter synchrotron light sources with the ability to accommodate increasingly complex sample environments and development of faster detectors and more robust analytical tools for data processing.

3.1. Small Angle X-ray Scattering (SAXS)

Small Angle X-Ray Scattering (SAXS) is a non-destructive characterisation technique based on elastic coherent scattering of X-rays at low angles (0.1 to 10°) by inhomogeneities in nanometre-scale objects. Although SAXS can be considered a high-resolution technique, the amount of information for a given q range is highly dependent on the average microstructural characteristics of the sample. This high-resolution technique has been successfully applied to determine structural details and physical information of objects in the 0.5 to 100 nm size range for a myriad of different materials such as crystals, partially ordered materials, proteins and other biological molecules (Roe, 2000). Over the years, the development of more advanced detectors has permitted improvements in data collection through enhanced signal/noise ratio, accuracy of count rate correction, high dynamic range with short readout time and high frame rate. The high quality of SAXS data acquired during
this PhD are critically dependent on the high-quality detector available at the beamline.

The scattering intensity and pattern that results from the interaction of an incoming monochromatic X-ray plane wave with the electron cloud of individual atoms within a sample is typically recorded on a fast 2D detector. The scattering intensity is then normalised to the transmitted X-ray intensity and, for an isotropically scattering material, radially integrated. The radially-integrated scattering intensity, $I_q$, is then typically reported as a function of $q$, the scattering wave vector, defined as:

$$ q = \frac{4\pi}{\lambda} \sin 2\theta $$

*Equation 12*

where $\lambda$ is the X-ray wavelength and $\theta$ the scattering angle.

The scattering intensity is a function of the difference in scattering length density between the solvent and sample, and the size of the scattering object. The classical experimental apparatus and geometrical representation of the scattering vector is shown schematically in *Figure 9*.

The main advantages of SAXS are its broad range of application and ease of use. *In situ* and *ex situ* static and dynamic experiments can be performed on solid, liquid and gaseous compounds with crystalline, partially ordered or even randomly oriented structures. However, SAXS will provide information on the average objects. Accurate definition of scattering objects is accessible through modelling of SAXS scattering curves. However, a unique modelling approach does not exist, and each model considered can potentially provide different structural information about
the scattering objects. The choice of the model thus needs to consider the available information on the system considered and the characteristics that are sought. This step remains the critical component of data processing.

**Figure 9:** A: Classic experimental apparatus for a SAS experiment. A highly collimated X-ray (or neutron) incident beam will scatter on the sample generating secondary spherical waves. The interference pattern is detected and recorded on a 2D detector and the transmitted beam stopped by a beamstop. B: geometrical representation of the scattering vector \( q \) (Maric, 2014).

### 3.2. Small Angle Neutron Scattering (SANS)

The physical principle behind Small Angle Neutron Scattering (SANS) remains essentially the same as that for SAXS, with the difference being that secondary radial scattering is not be generated by X-rays but by elastic coherent scattering of neutrons. The neutrons are created in a nuclear reactor by fission or in more recent facilities via spallation. Both of these techniques will produce a wide range of slow to fast neutrons, and the appropriate wavelength needs to be selected, via their velocity, prior to any scattering experiment.
In neutron scattering, scattering nuclei don’t possess a size comparable to the wavelength of the probing radiation like in X-ray scattering but have to be considered as point particles. In contrast to X-rays, which are sensitive to the electron density (i.e. the number of electrons in the electron cloud) in the material, neutrons have zero charge and a negligible electric dipole, and therefore interact with matter via nuclear forces. As a result, neutron scattering depends on the strength of forces within the nucleus, and the scattering length density (i.e. scattering power of a material) of a particular atom will usually be quite different for neutrons than for X-rays (Figure 10).

*Figure 10: Relative scattering cross section (i.e. the effective area that is used to quantify a scattering event, expressed in barn with \(1 \text{ b} = 10^{-28} \text{ m}^2\)) of different elements with respect to X-rays and neutrons. As X-rays interact with electrons, the scattering cross section is directly related to atomic number Z. In contrast, the scattering cross section for neutrons depends on the size of the nucleus (From the NCNR NIST website http://www.ncnr.nist.gov/AnnualReport/FY2003_html/RH2/fig2.png).*

This property allows the use of neutron scattering to study light elements that would be very difficult to detect using X-ray scattering, and also allows contrast
matching experiments by varying the concentration of hydrogen and deuterium in the solvent of a sample. Because the scattering length density for deuterium is very different from that of hydrogen, by varying the ratio H\textsubscript{2}O/D\textsubscript{2}O it is possible to vary the scattering contrast of hydrogen-rich phases (water solvent, hydrated layer, proteins…). Materials or specific components of materials can then possess contrast matching points at given H\textsubscript{2}O/D\textsubscript{2}O ratios, depending on the hydrogen concentration of the material, at which they are effectively rendered invisible to neutrons.

Thus, SANS has similar advantages and disadvantages as SAXS, and is broadly used in a variety of scientific fields such as biology, materials science, and magnetism research. However, the flux of neutrons is usually quite small and the number of scattering events is far less than with X-ray scattering. Thus a much longer integration time is usually required for measurements of neutron scattering, such that studying dynamic systems can be difficult. However, contrast matching with this method is relatively straightforward, and is particularly useful to study properties of hydrated compounds and chemical processes involving mixtures of phases with different densities.
Chapter IV

Development of a micro-mixing device for the investigation of nucleation processes at short timescales
1. Introduction

Because the processes investigated in this PhD are highly kinetically dependent, mixing of reagent solutions to trigger nucleation processes under homogeneous conditions must be rapid compared to the timescale of the reaction(s) of interest, and must also result in a reaction mixture that can be easily observed to allow the acquisition of data with high temporal resolution.

Rapid mixing devices have previously been constructed for the purpose of studying fast reactions. Lin et al (2003) coupled a micromixer made of silica, operating at mixing times as short as 20 µs, with a freeze quench device with the aim of stopping reactions under investigation by rapidly freezing the mixed solution shortly after mixing. Similarly, Marmiroli et al (2009) designed a high velocity micromixer using deep X-ray lithography, allowing mixing times as short as 75 µs. Their micromixer was then coupled with SAXS to study the formation of calcium carbonate minerals, which inspired the experimental set up presented in Chapter V and Chapter VI.

In collaboration with The Australian National Fabrication Facility - South Australian Node, a micro-mixing device (“chip”) has been developed that:

- performs very fast diffusion-based mixing of two aqueous reagent solutions at low Reynolds number;
- results in a short residence time of the mixed solution inside the mixing chamber, with minimal opportunity for blockages due to particle formation;
allows the mixed solution to exit the chip as a free jet to enable positioning in front of an observation probe (e.g. X-ray beam) and/or collection in an appropriate way for off-line sample characterisation; and

- can operate at relatively high flow rates to produce sufficient mixed solution at a sufficiently high rate to meet analytical needs.

2. Fabrication process and characteristics

The mixing device was made from poly(methyl methacrylate) (PMMA). The mixing chamber was designed in such a way that the fluid streams introduced through the supply channels are repeatedly divided into thin strata and recombined at very low Reynolds number, allowing mixing via diffusion on very short length scales, and hence very short timescales. After a residence time of around 100 µs in the mixing chamber under typical operating flow rates (a few mL/min), the flow is convoluted due to the no-slip hydrodynamic boundary and surface topology, stretched and accelerated in the exit channel to a higher Reynolds number (around 10 to 100), at which point inertia contributes to the formation of a free jet as the fluid exits the device. Flow at this Reynolds number remains laminar, so mixing is still diffusion based (Figure 11).
Figure 11: Micro-mixing device made from poly(methyl-methacrylate) for the study of fast reactions. Three input channels can be used to drive reagent solutions into the mixing chamber. After mixing, the mixed solution exits the mixing chamber via a short outlet channel to form a free jet as it exits the chip. The scale bar is 5 mm in the upper image and 1 mm in the lower image.

3. Evaluation of the mixing performance

3.1. Villermaux-Dushman protocol description

The mixing performance of the device was evaluated following the Villermaux-Dushman protocol as described in Commenge et al (2011). This method is based on competition between two reactions: one involving neutralisation of an acid by borate (R1), and the other involving acid-catalysed comproportionation of iodate with iodide to form iodine (R2):
The principle for the determination of the mixing time of the mixing device is based on the reaction time of the reaction R2 being much slower than that of the first reaction R1, but still being fast enough to cover the same range of timescales as the mixing process. To determine the mixing time of the chip at different flow rates, two solutions are mixed in the device as follows. The first solution contains a mixture of iodide and iodate in boric acid buffer, while the second solution contains sulfuric acid as a source of protons for both reactions. In the case of ideal mixing, all protons are consumed by R1 and the second reaction cannot proceed. However, if mixing is not ideal, the presence of excess protons occurs heterogeneously within the fluid due to imperfect mixing of the reagents. In these “pockets” where excess protons persist, R2 will occur irreversibly to yield formation of iodine. The concentration of iodine formed in the final fluid mixture reflects the segregation state of the two liquids (i.e. mixing time). The key feature of this system is that iodine and iodide react following a quasi-instantaneous equilibrium to form triiodide ions. The concentration of triiodide can be measured by UV spectrophotometry at the wavelength of 353 nm according to the Beer-Lambert law.

\[ I_2 + I^- = I_3^- \]

**Equation 14**

The method described in Commenge et al (2011) proposes use of several different “concentration sets” for the reagents, which are designed to give measurable triiodide concentrations for a given range of mixing times. Because the
optical density (and hence the mixing time) will change depending on flow rate, the
chosen concentration set should aim for an optical density close to 1. The
concentrations proposed by Commenge et al (2011) have been established based on
an optical path length of 5 mm; thus if another path length is to be used, the optical
density needs to be normalised by the path length. The mixing time can then be
calculated from the optical density using the master curve given by:

$$t_m = 0.33(OD)[H^+]^{-4.55}[Kl^{-1.5}[KIO_3]^{5.8}[NaOH]^{-2}[H_3BO_3]^{-2}$$

Equation 15

where $t_m$ is the mixing time in seconds, OD the optical density per millimetre and
[X] represents the concentration of reagent X.

3.2. Sample preparation

Boric acid ($H_3BO_3$; assay $\geq$ 99.0%) was purchased from Sigma-Aldrich and
sodium hydroxide ($NaOH$; assay $\geq$ 99.0%), potassium iodate ($KIO_3$; assay $\geq$ 99.0%),
potassium iodide ($KI$; assay $\geq$ 99.0%) and ultra-pure sulfuric acid ($H_2SO_4$) were
purchased from Ajax Finechem. A single stock solution containing 0.5 M $H_3BO_3$
and 0.5 M $NaOH$ was prepared and stocked in a Schott bottle prior to the experiment.
Stock solutions of $6 \times 10^{-2}$ M potassium iodate, $3.2 \times 10^{-1}$ M potassium iodide and
0.6 M ultra-pure sulfuric acid were prepared just before the experiment and stocked
in separate Schott bottles. Solutions S1 and S2 were prepared from these stock
solutions at concentrations corresponding to concentration set 1B from the
Villermaux-Dushman protocol, as presented in Table 1.
Table 1: Final concentration set used to characterise micromixer mixing efficiency.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagent</th>
<th>Final concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>H+</td>
<td>0.06</td>
</tr>
<tr>
<td>S2</td>
<td>KI</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>KIO3</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>NaOH + H₃BO₃</td>
<td>0.09</td>
</tr>
</tbody>
</table>

3.3. Experimental apparatus

Two HPLC pumps were used to drive solutions S1 and S2 through 0.15 mm internal diameter PTFE tubing to the mixing device at varying flow rates from 1 mL.min⁻¹ to 2.25 mL.min⁻¹ for each solution (total flow rates of 2-4.5 mL.min⁻¹ exiting the mixing device). Non-return valves were installed between the HPLC pump and the mixing device. The mixed solution exited the mixing device as a free jet with a 100 µm × 100 µm square cross-section (at the point of exit) and was collected in a glass beaker (Figure 12). The mixed solution was withdrawn from the beaker with a syringe and injected in a 1 m path length Liquid Waveguide Capillary Cell (LWCC; World Precision Instruments) that was coupled to a Cary 50 UV/Vis spectrometer equipped with a single source, full spectrum xenon pulse lamp. The absorbance of the solution was then measured at 353 nm. Ten replicates were obtained at four different flow rates to enable determination of the relationship between flow rate and mixing time.
4. Results

The measured relationship between flow rate and mixing time is presented in Figure 13. The minimum mixing time obtained was around 6 ms at a total flow rate of 4.5 mL.min\(^{-1}\), which is around two orders of magnitude slower than that achieved previously by Marmiroli et al \(2009\) where nucleation of calcium carbonate was characterised by SAXS at times from 75 µs to 770 µs after mixing. Despite the differences in mixing time, similarity between the results obtained in this Ph.D. project (see Chapter V) and those obtained by Marmiroli et al, \(2009\) suggest that the mixing time reached with our experimental apparatus was still sufficiently fast.
to allow homogeneous nucleation of calcium carbonate to occur under the investigated conditions.

Figure 13: Mixing time of the micro-mixing device as a function of total fluid flow rate through the device. Data points represent the mean, and error bars the standard error of the mean, from 10 replicate measurements at each flow rate. The solid line represents linear regression of the data (the Pearson product-moment correlation coefficient for the linear regression was $R^2 = 0.995$).

5. Conclusion

Micro-mixing devices such as that designed and fabricated here have proven to be efficient and flexible for the characterisation of relatively fast kinetic processes at the nanoscopic scale (Lin et al 2003; Marmiroli et al, 2009). Furthermore, the relatively simple fabrication process for the micro-mixing device described here could be easily modified to permit novel observation techniques; for example, adding a diamond window above the exit channel could allow reliable stopped flow data acquisition using X-ray based techniques only a few milliseconds after mixing. The transferable experimental tools developed here will thus potentially open the door to further work focusing on the characterisation of very early stages of
homogenous and heterogeneous nucleation processes and their kinetics under various experimental conditions, as well as potentially facilitate the characterisation of interactions between nanoscale compounds with organic and inorganic ligands at very short timescales.
Chapter V

Characterisation of calcium carbonate prenucleation clusters around the solubility limit using Small Angle X-ray Scattering
1. Introduction

Many recent studies have aimed to understand the role and characteristics of small nanoclusters that are present under conditions that Classical Nucleation Theory (CNT) fails to describe during the early stages of nucleation in the calcium carbonate system. However, ongoing development of Non-Classical Nucleation Theory, which is based on the formation of these prenucleation clusters (PNCs), is limited by the relatively small amount of information on the structural properties of PNCs.

Calcium carbonate PNCs have been identified in both under- and supersaturated conditions with respect to both ACC and calcite, and characterised as chain-like structures of cations and anions held together by ionic interactions via different techniques such as ultracentrifugation, cryo-Transmission Electron Microscopy (cryo-TEM) or Small Angle X-Ray Scattering (SAXS) (Gebauer et al, 2008; Pouget et al, 2009 and Chao et al, 2014). However, these studies have provided only limited information about the chemical and physical structural characteristics of calcium carbonate PNCs. While it has been established that their size lies between 0.5 and 5 Å, little is known about other characteristics like their shape, hydration level, and the influence of these and other factors on their formation and potential role in nucleation processes.

Calcium carbonate PNCs are hydrated structures that are stable with respect to solvated calcium and carbonate ions. They have been described via computer simulation on very short timescales (<70 ns) as constantly evolving hydrated linear, branched or ring-like compounds (Demichelis et al, 2011). The model developed by
Demichelis et al. (2011) provided the first concrete information on the shape of PNCs and highlighted the importance of kinetic parameters in the formation and stabilisation of such ionic complexes. While this framework successfully described previous experimental observations, a second pathway involving liquid-liquid like phase separation may also potentially explain the formation of PNCs and their essential characteristics at low levels of saturation (Wolf et al., 2008).

Due to the complexity and fast kinetics of processes related to the formation and transformation of PNCs, characterising their properties and role in the early stage of the nucleation process in calcium carbonate systems remains a challenge. Moreover, most previous studies have been performed under moderate to high levels of supersaturation and generally under high pH conditions, but little is known about the formation of PNCs in undersaturated conditions with respect to ACC and calcite, and/or at pH values closer to neutral that are more typical of most natural waters.

The aim of the present study was to characterise the chemical and physical properties of calcium carbonate PNCs around the solubility limits of ACC and calcite at pH values typical of natural waters via SAXS. Because the processes investigated in this study are highly kinetically dependent, reagent solutions were rapidly mixed using the micro-mixing device developed in Chapter IV and data acquisition was performed shortly after mixing. The formation of nanoparticles, even in undersaturated conditions, was expected to result in a possible independent hierarchy of structures representing particles forming at different stages of the nucleation process, each potentially possessing their own shape and size (Habraken et al., 2013). Because of its ability to probe multiple structural levels simultaneously
(Beaucage 1995), SAXS was therefore expected to be an ideal experimental tool to characterise the nanoscale structure of this system.

2. Research objectives

The research objectives for this chapter were to:

i. characterise the size and particle-scale structure of nanoparticles, potentially including PNCs, that might form during the early stages of calcium carbonate nucleation; and

ii. define the impact of pH and ion activity product (with respect to the solubility limit) on nanoparticle structure during the very early stages of calcium carbonate nucleation under environmentally-relevant conditions.

3. Experimental section

3.1. Chemical preparation

CaCl$_2$.2H$_2$O was purchased from Ajax Finechem. Na$_2$CO$_3$ (assay $\geq$ 99.0%) and HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) (assay $\geq$ 99.5%) were purchased from Sigma-Aldrich. Stock solutions of 1 M CaCl$_2$.2H$_2$O and 20 mM Na$_2$CO$_3$ plus 20 mM HEPES were prepared in ultrapure 18 M$\Omega$.cm resistivity milliQ water (hereafter referred to as MQ) obtained from a Millipore Academic water purification system. HEPES was used as a non-Ca complexing pH buffer in calcium carbonate solutions (Crowell et al, 1985), and was additionally favoured due to its fast response time and ability to maintain a quasi-steady-state pH after
about 10 ms (Schulz et al, 2006), which is appropriate for examination of calcium carbonate precipitation kinetics under the conditions investigated in this study. Although the influence of HEPES buffer on aggregation of PNCs is expected to be limited, it can’t be strictly excluded.

3.2. Equipment configuration

Small Angle X-ray Scattering measurements were performed at the SAXS/WAXS beamline at the Australian Synchrotron (Australia) in April 2015. Measurements were performed by mixing a solution of CaCl₂ (hereafter referred to as S1) and a solution of 20 mM NaCO₃ and 20 mM HEPES buffer adjusted just before the beginning of the scattering experiment to pH 7.5 or 8.5 (hereafter referred to as S2) in a 1:1 volume ratio using a continuous flow system (Figure 14). The pH was measured using a glass pH electrode calibrated by a three point curve with pH 4, 7 and 10 buffer solutions (pH 4 solution from Australian Scientific and pH 7 and 10 solutions from LabServ).

Two HPLC pumps drove solutions S1 and S2 through 0.15 mm internal diameter PTFE tubing to the mixing device described in Chapter IV at a rate of 2 mL.min⁻¹ for each solution (total flow rate of 4 mL.min⁻¹ exiting the mixing device). Non-return valves were installed between the HPLC pump and the mixing device. The mixed solution exited the mixing device as a free jet (100 µm x 100 µm). The X-ray beam was focused as close as possible to the exit of the mixing device in order to obtain measurements at the shortest possible time after mixing (calculated to be 9.2 ms).
Figure 14: Configuration of the experimental apparatus for SAXS experiments. Experimental solutions S1 (calcium chloride) and S2 (sodium carbonate + HEPES buffer) were continuously driven by two HPLC pumps to the mixing device where they mixed via diffusion. The mixed solution exited the mixing device as a free jet. The X-ray beam was focused as close as possible to the point of exit of the jet from the mixing device, corresponding to a reaction time of 9.2 ms after mixing of solutions S1 and S2.

One continuous run was conducted for each pH condition. The concentration of calcium in solution S1 was adjusted throughout each run by successive addition of concentrated calcium from the 1 M CaCl$_2$ stock solution by a remotely operated syringe pump into a known volume of MQ water contained in a Schott bottle covered with parafilm that was continuously stirred by a magnetic stirrer. The calcium concentration in solution S1 corresponding to each experimental condition was calculated by taking into account the initial volume of MQ in the covered Schott bottle (220 mL), the amount of calcium stock solution delivered into the Schott
bottle by the syringe pump at different times, and the rate at which the HPLC pump continuously withdrew the resulting solution S1 from a Schott bottle (2 mL.min\(^{-1}\), see below for further details). Solution S2 was stored in a Schott bottle covered with parafilm during experiments. Overall, 10 calcium concentrations were investigated ranging from 10\(^{-4}\) M to 10\(^{-1}\) M, which was estimated to span approximately one order of magnitude below to one order of magnitude above the solubility limit of calcite at pH 7.5 and supersaturation with respect to calcite at all calcium concentrations at pH 8.5 (Mucci \textit{et al}, 1983) (see Appendix I for details). Between each run (under each of the pH conditions), the whole system was rinsed with acidified ultrapure water and ultrapure water in succession.

\textbf{3.3. Small angle X-ray scattering data acquisition}

For each calcium concentration during an experimental run, 20 scans with an exposure time of 2.00 seconds each was acquired in “gapless mode” using an offset Pilatus 1M solid-state detector and a camera length of 3100 mm, corresponding to a \(q\)-range from 0.004 Å\(^{-1}\) to 0.325 Å\(^{-1}\) at an incident X-ray energy of 12 keV. The initial 20 scans of each run were acquired before adding any calcium to solution S1 and used as the background, then 20 scans were subsequently acquired for each calcium concentration. The background has been defined in this way to allow subtraction of any signal from non-calcium related structures, the pH buffer and possible micro-bubbles of outgassing CO\(_2\). All SAXS images were radially integrated to yield curves of the scattering intensity \(I(q)\) as a function of the scattering vector \(q\) using the ScatterBrain program available at the beamline (Kirby \textit{et al}, 2013). For each condition, the 20 resulting curves were summed and
background corrected by the subtraction of the scattering intensity prior to adding calcium to S1. Data were then calibrated using scattering from water as an absolute scattering standard, then normalised by beamstop intensity to yield scattering intensity as an absolute value.

### 3.4. Modelling of SAXS data

Small angle scattering analysis of polydisperse inorganic polymer systems usually results in the characterisation of independent structures possessing their own shapes and sizes (Glatter and Kratky, 1982, Beaucage et al, 1996, Rose et al, 2012). Such systems usually exhibit relatively featureless power law scattering on a log $I$ vs log $q$ plot. Such results were observed here and are further discussed in the Results section.

Such power law regions can generally be described as being composed of three associated regimes (**Figure 15**):

- a Porod regime characterised by a linear relationship between log $I$ and log $q$ over a particular $q$ range, where the negative of the slope (denoted $d$) relates to the dimensionality of the particles present;

- a Guinier regime characterised by a flat relationship between log $I$ and log $q$ (i.e. zero slopes) with an asymptotic value of $I$ at small values of $q$ denoted as $G$, i.e.:

\[
\lim_{q \to 0} I(q) = G
\]

**Equation 16**

- a transition regime characterised by an inflexion point of $I(q)$ related to the $z$-weighted radius of gyration of the particles (denoted $R_g$).
Figure 15: Idealised power law scattering curve on a log I vs log q scale showing the Guinier, transition, and Porod regimes.

These three regimes were fitted to each power law region in the data using Beaucage’s Unified Model (UM) (Beaucage, 1995) modified according to Hammouda (2010). As the measured scattering curves are the sum of the scattering contribution from (potentially) multiple structural levels, each of these parameters can be fitted for any individual structural level. Determination of parameters $d$, $G$ and $R_g$ for each structural level (denoted by $d_n$, $G_n$ and $R_{gn}$ for structural level $n$) thus allows evaluation of the contribution of each structural level to the overall scattering curve as follows:

$$I(q) = \sum_{n=1}^{N} I_n(q)$$

Equation 17

where $I(q)$ is the overall scattering intensity, $N$ is the total number of structural levels, and $I_n(q)$ is the scattering intensity contributed by the $n^{th}$ structural level.
Because of its empirical nature, the UM can be applied to any system exhibiting power low scattering regardless of the shape of individual particles. The UM is, therefore, valid for particles possessing a wide range of forms, including rod-like, spherical, or fractal, and a wide range of dispersity values, all of which influencing the dimensionality value of the power law without changing the property of power law scattering.

Fitting of parameters $d_n$, $G_n$ and $R_{gn}$ based on the UM allows reconstruction of the scattering curve resulting from each structural level over an infinite $q$ range. The total scattering mass of each structural level was calculated using the Porod Invariant ($Q_P$), which is related to the overall scattering power of samples taking into account the scattering intensity from all possible geometries. Practically, the Porod Invariant corresponds to the excess electron density of particles above the background within the $q$ range investigated. For each experimental condition, this value was calculated for each structural level by integrating Kratky plots ($q^2I(q)$ vs $q$) resulting from the reconstructed scattering function for each structural level over an infinite $q$ range. As the value of the Porod Invariant remains constant only over a particular $q$ range (structural level), we can consider the evolution of the scattering mass above the background for each structural level individually. This approach of calculating separate $Q_P$ functions for different structural levels has been previously used by Beaucage (1996).

Values of $d_n$, $G_n$ and $R_{gn}$ and $Q_{Pn}$ were used to determine the average particle volume by weight, $V_{Pn}$, and number density of particles (number of particles per volume of solution), $C_{Pn}$, for particles corresponding to structural level $n$ (Rose et
al, 2014). Similarly to the Rose et al, (2014) approach, relationships between these parameters can be used to extract information about the mechanism of particle formation and transformation.

3.5. **Unified Model fitting and constraints**

The UM model expression modified by Hammouda (2010) is given by:

\[
I_n(q) = C_n e^{\left(\frac{-q^2 R_{gn}^2}{3}\right)} + G_n d_n (q R_{gn})^{-d_n} \left[ \frac{6 d_n^2}{(2 + d_n)(2 + 2d_n)} \right]^{d_n/2} \text{erf} \left[ \frac{q R_{gn}}{\sqrt{6}} \right]^{3d_n} \text{G} \left[ \frac{d_n}{2} \right] e^{\left(\frac{-q^2 R_{gn}^2}{3}\right)}
\]

*Equation 18*

where erf\([x]\) is the error function and \(\text{G}[x]\) is the gamma function.

Best fit parameter values were determined by nonlinear least-squares fitting weighted by \(I^2\) using GraphPad Prism software. Only data between \(q = 0.007\ \text{Å}^{-1}\) and \(q = 0.16\ \text{Å}^{-1}\) were considered during modelling as anisotropy artefacts were present below \(q = 0.007\ \text{Å}^{-1}\) (which were attributed to refraction of the X-ray beam from the side of the jet) and data at \(q > 0.16\ \text{Å}^{-1}\) exhibited poor signal/noise ratio. Models with \(N = 1, 2\) or \(3\) were considered; however, models with \(N = 1\) and \(N = 3\) failed to satisfactorily fit the scattering curves (as indicated by lower values of the weighted sum of squares for the model fit to the data, \(r^2\), and the relatively high standard deviation of residuals). The presence of more structure levels can’t be strictly excluded (as two structural levels presenting similarly close fitted parameters would be difficult to clearly distinguish), but statistically, the two fitted structural levels contributed to the majority of measured scattering.
Because the $n = 2$ (i.e. low $q$) structural level did not exhibit a Guinier regime or transition regime, $G_2$ was arbitrarily set to 10,000 (i.e. the condition $G_2 \gg G_1$ was enforced) and the value of $R_{g2}$ allowed to vary subject to the constraint that $R_{g2} \gg R_{g1}$. The model was then used to fit $d_2$ independently of $G_2$ or $R_{g2}$.

The Porod Invariant is given by:

$$Q_{pn} = \int_{0}^{\infty} q^2 l_n dq = \phi_{pn}(1 - \phi_{pn})(\Delta \rho_n)^2$$

Equation 19

where $\phi_{pn}$ is the fraction of the sample volume occupied by particles corresponding to power low region $n$, and $\Delta \rho_n$ is the contrast of these particles i.e. the scattering length density of particles in excess of that due to the background solvent (Rose et al, 2014). As the value of $\Delta \rho_n$ has not previously been reported for calcium carbonate mineral precursor species, the average value given by Liu et al (2010) of $\Delta \rho_n = 5.19 \times 10^{-4}$ nm$^{-2}$ for ACC particles studied over longer time frames was used. According to modelling, PNCs possess a higher level of hydration and potentially much lower physical density than ACC (Demichelis et al, 2011). These differences could result in the scattering length density for the observed scattering objects being closer to that of the solvent than to that of ACC. This will be further addressed in the Discussion section.

The value of the Porod Invariant was calculated for $n = 1$ (the smallest structural level) only, as the scattering curve corresponding to $n = 2$ could not be reconstructed since only the value of $d_2$ could be constrained by the UM fit in this region. The Porod Invariant was then used to determine the mean particle volume
$V_{p1}$, and the number density of particles (number of particles per volume of solution) $C_{p1}$, using the relationships:

\[ V_{p1} = \frac{2\pi^2 G_1}{Q_{p1}} \]

*Equation 20*

\[ C_{p1} = \frac{(Q_{p1})^2}{4\pi(\Delta\rho_1)^2G_1} \]

*Equation 21*

As described in the literature, formation of calcium carbonate nanoparticles is only driven by ionic interactions (Demichelis *et al*, 2011), whereby the random collision of ions will break or recreate three-dimensional objects. In order to verify if the growth processes described via computer simulation were experimentally observed, an analysis of the logarithmic relationship between $G_1$, $R_{g1}$ and $V_{p1}$ was used to attempt to differentiate whether a monomer addition mechanism or a Smoluchowski-type cluster-cluster addition mechanism was dominant (Liu *et al*, 2008). In the case of a cluster-cluster addition mechanism, the slope of log $G_1$ vs log $R_{g1}$ should be equal to the slope of log $G_1$ vs log $V_{p1}$. In the case of a monomer addition mechanism, the slope of the former curve should only be half that of the latter. Removing $R_{g1}$ from the equation by plotting log $G_1$ vs log $V_{p1}$ enables direct comparison between the two mechanisms, with a corresponding slope of 2 for a monomer addition mechanism and a slope of 1 for a cluster-cluster addition mechanism. This correlation is also valid for a polydisperse distribution of particles sizes as long as the nature of the distribution does not change with increasing particle size and volume (Liu *et al*, 2010). This type of analysis was established and validated for kinetic data, while the experiment conducted here involved a titration
of Ca concentration at fixed reaction times. However, as putative PNCs are expected to form only via the random collision of calcium ions with (bi)carbonate ions, it was assumed that the rate of putative PNC formation should increase in proportion to the Ca concentration such that increasing Ca concentration should be a proxy for greater reaction progress. This and other assumptions behind this analysis are discussed further in the Discussion section.

3.6. Modification of Unified Model to account for a diffuse interface

In some cases, fitting of the UM resulted in values of $d > 4$. According to Schmidt (1991), values of $d > 4$ are possible when there is a diffuse (i.e. not sharp) interface between the scattering object and the solvent. Schmidt (1991) demonstrated that the UM could still be used to model such scattering objects, resulting in values of $d > 4$. However, his formulation assumed that the excess electron density decreased from that of the bulk scattering object at its surface to that of the solvent at some finite distance from the surface according to a power law decay function, resulting in a maximum permissible value of $d = 6$.

Therefore, in order to better rationalise the observation of values of $d > 6$ in this study, a different approach was used to describe the change in electron density near the object’s surface. A negative deviation from Porod’s law can be induced by the presence of a gradient in electron density between the nominal surface of the scattering object and the solvent. Fitting of such diffuse interface can be approached by considering a smooth evolution of electron density around a spherical scattering object (Figure 16).
Figure 16: Models of variation in electron density near the surface of a scattering object for an ideal electron density profile $\rho_{e, id}(r)$. A: Electron density model for a sharp boundary. B: Sigmoidal electron density gradient model for a diffuse interface with a smoothing function. C: Electron density profile corresponding to model B. Adapted from Guo et al (2013).

Following the approach used by Guo et al (2013), the electron density for a two phase system possessing a diffuse interface layer, $\rho_e(r)$, can be expressed as
the convolution of the electron density in an ideal two phase system separated by a
sharp interface, $\rho_{e, id}(r)$, with a smoothing function $h(r)$, as follows:

$$\rho_e(r) = \rho_{e, id}(r) \otimes h(r)$$

Equation 22

where $r$ is the distance along an arbitrary vector perpendicular to the boundary
surface inside the scattering volume, $\otimes$ is the convolution operation and $h(r)$ the
smoothing function. Assuming a sigmoidal smoothing function, $h(r)$ is defined as:

$$H(q) = (2\pi\sigma^2)^{-1/2}e^{-r^2/(2\sigma^2)}$$

Equation 23

where $\sigma$ is the standard deviation of the smoothing function $h(r)$.

The relationship between the scattering intensity function for an object with
a diffuse interface and the scattering intensity function for an object with a smooth,
sharp interface (i.e. an object obeying Porod’s law, with $d = 4$) is then given by (Guo
et al, 2013):

$$I(q) = I_{\text{Porod}}(q).H^2(q)$$

Equation 24

where $I(q)$ is the scattering intensity function for the object with the diffuse interface,
$I_{\text{Porod}}(q)$ represents the scattering intensity function for an object with a sharp
interface ($d = 4$) and $H(q)$ is the Fourier transform of the electron density gradient
function. For the sigmoidal electron density gradient function above, this is given
by:

$$H(q) = e^{-(\sigma q)^2/2}$$

Equation 25
The UM was modified to incorporate this sigmoidal electron density gradient model by multiplying the intensity of the structural level possessing high dimensionality values by the Fourier transform of the smoothing function $h(r)$ as follows:

$$I(q)_T = I(q)_1 e^{-(qC)^2} + I(q)_2$$

*Equation 26*

where $I(q)_T$ is the total scattering intensity, $I(q)_1$ is the scattering intensity of the first structural level present at high $q$ values and $I(q)_2$ is the intensity of the second structural level present at low $q$ values. This approach is based on the assumption that $I(q)_1$ is then the scattering from an object with a smooth, sharp interface (i.e. $d_1 = 4$). This modified version of the UM model was fit to the data in the same way as the original UM, except that $d_1$ was set to a constant value of 4.

The thickness of the diffuse boundary layer, $E$, is then defined as (Guo et al, 2013):

$$E = 2 \sqrt{3} \sigma$$

*Equation 27*

4. Results

$I(q)$ vs $q$ plots for each of the ten calcium concentrations examined under each pH condition are shown (after background subtraction) in Figure 17.
Figure 17: I(q) vs q curves for all experimental conditions investigated. Solid lines represent experimental data corresponding to different calcium concentrations as shown on the graph. Dashed lines represent the best fit of the UM (panel A) or modified UM (panel B) to the data. A: pH 7.5. B: pH 8.5.
For all conditions investigated, the scattering curves exhibited two power law regimes separated by a single transition regime and a single Guinier region over the considered $q$-range. For each of the pH conditions, the intensity of the scattering signal between $q = 0.01$ Å and 0.1 Å increased with increasing calcium concentration. Similar trends were present and even more pronounced at higher pH, suggesting the presence and the formation of a higher concentration of nanoparticles and/or particles possessing different volume and/or density.

The presence of the coupled power law regime, transition regime and a flat Guinier region at high $q$ suggests that a complete structural level of nanoparticles was observed in this $q$ range. The presence of a power law regime in the low $q$ region suggests the presence of larger scattering structures. Due to the absence of a Guinier region and transition regime at low $q$, it was not possible to fully define the characteristics (including particle size) of this structural level. Low scattering intensity at high $q$ values also prevented extraction of possible information on diffraction peaks that might be expected with the presence of a repetitive crystalline structure, if such a feature was present; therefore, these data were not investigated further.

Information about the size and the dimensionality of these nanoparticles were extracted via the UM or modified UM including diffuse interface correction as described before. For each condition, good fits of the UM were obtained (Pearson product-moment correlation coefficient for the non-linear regression of $r^2 > 0.990$ in all cases), as illustrated in Figure 17.

4.1. Scattering at pH 7.5
Parameter values obtained from the fitting the UM at pH 7.5 are presented in Figure 18.

**Figure 18**: Parameter values extracted from fitting of the UM to experimental data at pH 7.5. **A**: Radius of gyration, $R_g$. **B**: Dimensionality, $d$, for structural level $n = 1$ (black circles) and structural level $n = 2$ (white circles). Symbols represent the best fit value and error bars the standard error from the UM model fit. Vertical red lines indicate calculated solubility values for various calcium carbonate polymorphs (see Appendix 1 for details of calculations); $\Omega_C$ corresponds to calcite solubility, $\Omega_V$ corresponds to vaterite solubility and $\Omega_{ACC}$ corresponds to the solubility of amorphous calcium carbonate.
4.1.1. Size and dimensionality of nanoparticles

At pH 7.5, scattering objects were observed under and above the solubility limit of all crystalline and amorphous forms of calcium carbonate. These scattering objects presented an initial $z$-weighted radius of gyration ($R_{g1}$) of 35 Å, which increased with calcium concentration up to 51 Å at 0.02 M Ca$^{2+}$, then increased much more gradually up to 52 Å at 0.05 M Ca$^{2+}$. The dimensionality value ($d_1$) of these objects remained constant regardless of the calcium concentration with a mean value of 2.1 ± 0.1. In a monodisperse system a dimensionality value of 2.1 ± 0.1 is very close to a Euclidian dimensionality and usually characteristic of planar/plate-like morphology. Nevertheless, the same value could be also characteristic of a polydisperse ensemble of fractal or non-fractal objects (Beaucage, 2012). However, the limited $q$ range investigated here does not allow a definite conclusion on the exact shape of the scattering objects. Regardless, the consistency of the $d$ values over the range of Ca concentrations investigated suggests the presence of nanoparticles possessing similar shapes with increasing calcium concentration.

The second dimensionality value characteristic of the low $q$ structural level, $d_2$, exhibited a value close to 4 (mean value of 3.9 ± 0.1) that remained constant with calcium concentration. A $d_2$ value very close to 4 suggests the presence of a strong interface forming within the first few milliseconds after reagent mixing.

4.1.2. Mean particle volumes and concentrations

Determination of the mean particle volume, $V_{p1}$, and number density of particles (number of particles per volume unit), $C_{p1}$, has been possible through the
calculus of the Invariant, $Q_{\text{pI}}$ (**Figure 19**). At pH 7.5, the mean volume of the nanoparticles increased with calcium concentration from $3.9 \times 10^2$ nm$^3$ to $1.4 \times 10^3$ nm$^3$, while the number of particles per unit volume remained fairly constant with a mean value of $(4.8 \pm 0.3) \times 10^{14}$ particle mL$^{-1}$ (± SEM). The combination of these two parameters suggests that the number of particles remains relatively constant with increasing calcium concentration, but they increase in volume.

**Figure 19**: Evolution of particle volume and concentration at pH 7.5 with increasing calcium concentration. **A**: mean nanoparticle volume and **B**: $C_{\text{pI}}$ (number of particles per unit volume)

### 4.2. Scattering at pH 8.5

Fitted parameter values obtained from the fitting the UM at pH 8.5 are presented in **Figure 20**.
Figure 20: Parameter values extracted from fitting of the UM to experimental data at pH 8.5. A: Radius of gyration, $R_g$ (black circles) and thickness of the diffuse interface (white polygons), total size (Radius of gyration + diffuse interface) (white squares). B: Dimensionality, $d$, for structural level $n = 1$ using the original UM, i.e. before accounting for the diffuse interface (black circles), and structural level $n = 2$ (white circles). The value of $d_1$ was fixed at 4 when accounting for the diffuse interface. Symbols represent the best fit value and error bars the standard error from the UM model fit. Vertical red lines indicate calculated solubility values for various calcium carbonate polymorphs (see Appendix 1 for details of calculations): $\Omega_C$ corresponds to calcite solubility, $\Omega_V$ corresponds to vaterite solubility and $\Omega_{ACC}$ corresponds to the solubility of amorphous calcium carbonate.
4.2.1. Size and dimensionality of nanoparticles

As with pH 7.5, scattering objects were observed at pH 8.5 under the solubility limit of amorphous calcium carbonate and above the solubility limit of both crystalline and amorphous forms. Under this pH condition, all dimensionality values, $d_1$, obtained from fitting the original form of the UM were greater than 4, suggesting a diffuse interface (Guo et al, 2013). Fitting the modified version of the UM that assumes a sigmoidal electron density gradient near the surface of the scattering object suggested that the diffuse interface thickness was relatively constant at $(29 \pm 0.8)$ Å as the Ca$^{2+}$ concentration increased from $10^{-4}$ M to $5 \times 10^{-4}$ M, then increased to 41 Å at $5 \times 10^{-3}$ M Ca$^{2+}$. The $z$-weighed radius of gyration, $R_{g1}$, remained fairly constant regardless of calcium concentration, with a mean value of $(28 \pm 2.0)$ Å.

4.2.2. Mean particle volumes and concentrations

The mean volume of particles at pH 8.5 remained fairly constant at $269 \pm 29$ nm$^3$ regardless of Ca$^{2+}$ concentration. However, the particle concentration under this condition increased from $1.6 \times 10^{13}$ particle mL$^{-1}$ at $10^{-4}$ M Ca$^{2+}$ to a plateau at around $7.8 \times 10^{13}$ particle mL$^{-1}$ at $5 \times 10^{-4}$ M Ca$^{2+}$ (Figure 21).
**Figure 21:** Evolution of particle volume and concentration at pH 8.5 with increasing calcium concentration. **A:** mean nanoparticle volume and **B:** \( C_{p1} \) (number of particles per unit volume).

### 4.3. Mechanism of particle formation and growth

At pH 7.5, the relationship between \( \log G_1 \) and \( \log V_{p1} \) was consistent with the expected outcome if nanoparticle formation is controlled by a monomer addition mechanism (Figure 22). The monomer could represent ions in solution or any particles smaller than the analytical window. However, at pH 8.5, the relationship observed was only consistent with the expected outcome for either a monomer addition or cluster-cluster addition process for the last three calcium concentrations, suggesting that the assumptions behind the analysis were not valid. This is discussed further in the Discussion section.
Figure 22: Analysis of the primary particle formation mechanism based on log $G_1$ vs log $V_{p1}$. **A**: pH 7.5. **B**: pH 8.5. The solid line represents the best fit for linear regression of log $G_1$ vs log $V_{p1}$, while the annotated numbers represent the slope with standard error and Pearson product-moment correlation coefficient ($R^2$) value for the regression line.

5. Discussion

5.1. Size and structure of nanoscale (high q) scattering objects

At pH 7.5, the reaction mixture was characterised by a high concentration of nanoparticles presenting a small volume, increasing in size with calcium concentration but maintaining planar/sheet like morphology. A similar value for $d_1$ could also be characteristic of a polydisperse ensemble of fractal or non-fractal objects (Beaucage, 2012), but in the absence of additional information, it is impossible to differentiate between the two possibilities from SAXS data alone. However, low pH increases the competition between carbonate and bicarbonate ions, with the latter species creating a weaker bond to calcium and thus acting as a chain terminator (Demichelis et al, 2011). Under bicarbonate dominant conditions, the complexity of the nanoparticles is therefore limited and the occurrence of a polydisperse system seems less likely. Furthermore, according to the model used by
Guo et al. (2013), the presence of a diffuse interface manifests as a negative deviation from Porod’s Law. It is therefore also impossible to know if non-Euclidian dimensionality values < 4 represent Euclidian objects possessing a diffuse interface or the presence of (mass or surface) fractal structures. In contrast, at higher pH (8.5) the particle size remained relative constant over the entire range of calcium concentrations, although the particle structure evolved with increasing calcium concentration due to formation of a diffuse interface. In this case, the size of the particle core remained relatively constant with increasing calcium concentration, but the size of the diffuse interface increased. This population of nanoparticles exhibited a greater volume, but were present at a 6-fold lower concentration, than the nanoparticles at pH 7.5.

The clear presence of scattering structures at calcium concentrations several orders of magnitude above and below the solubility limit of ACC (and, at pH 7.5, below the solubility limit of calcite) is consistent with the expectation that calcium carbonate PNCs initially form in undersaturated conditions, but remain present even at a high degree of supersaturation (Gebauer et al., 2008; Pouget et al., 2009). Moreover, the nanoclusters present at high q-values persisted over the range of pH and saturation state conditions examined. Furthermore, the nanoparticles observed here are similar in size and shape to calcium carbonate PNCs described by NCNT (Demichelis et al., 2011; Gebauer et al., 2014). The average size of these putative PNCs acquired with our apparatus is comparable with previous SAXS data but slightly larger than sizes determined using cryo-TEM and ultracentrifugation techniques (Gebauer et al., 2008; Pouguet et al., 2009). Additionally, the difference
in the sizes of the putative PNCs at pH 7.5 and 8.5, could be associated with the absence of a diffuse interface at pH 7.5. Similar nanoparticulate structures in a silicate stabilised calcium carbonate system have also been characterised via cryo-TEM. In this case, larger entities possessing a diffuse boundary (20 to 50 nm) were reported and appeared to be loosely aggregated single nanoclusters (Kellermeier et al, 2012). The aggregation process observed at pH 7.5 is concordant with a monomer addition process driving PNC formation, with monomer being dissolved ions randomly colliding with PNCs (Gebauer et al, 2014). Therefore, it seems likely that the structures observed in the high q range are PNCs.

Values of \( d_1 > 4 \) at pH 8.5 have been ascribed here to a diffuse interface forming around these putative PNCs. However, it is worth considering that this deviation from the Porod Law may instead reflect a partial form factor for a distribution of monodisperse spheres. This seems very unlikely, however, as no periodicity in the high q region of the scattering signal was observed. Based on Bolze et al (2002), in the case where spherical nanoparticles with a well-defined monodisperse distribution were observed, as a rough approximation the wavelength of the first oscillation should occur at \( R_g l / \pi \approx 3 \text{ nm}^{-1} \). Such oscillations, if present, should be clearly visible in Figure 17. A more precise approach was also considered by attempting to fit the scattering curves with a spherical model using all form factors available in SaSView software package, however there was no improvement to the fit quality. This supports the notion that the values of \( d_1 > 4 \) are likely due to a diffuse interface.

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The presence of a diffuse interface at the higher pH condition suggests that the hydration state of these nanoparticles is a major factor influencing their structure and evolution. The first stage of ACC mineral development is known to involve the formation of hydrated mineral phases that subsequently dehydrate (Addadi et al., 2003; Cartwright et al., 2012; Bots et al., 2012). Nielsen et al. (2014) further showed that under similar pH and reactant concentrations to those used in this work, a possible multi-step process linked to the surrounding liquid layer led to the formation of ACC. Smooth evolution of the hydration state of the nanoparticles is also consistent with computer simulations performed by Wallace et al. (2012), where the evolution of the coordination number (i.e. complexity) of PNCs was not observed to result in a substantial change in the cluster character (i.e. there was no strong change in the free energy).

The results of this study at pH 8.5 support this processes. The total size of the scattering objects (radius of gyration of the core + thickness of the hydrated interface layer) at pH 8.5 remained fairly constant with calcium concentration at around 61 ± 3.7 Å (mean ± SEM). However, the size of the core appeared to shrink slightly while the hydrated layer grew with increasing calcium concentration, suggesting an ongoing expulsion of water from the core and thus dehydration. These features are, however, not visible at pH 7.5, possibly due to the shape of the nanoparticles. As putative PNCs observed under low pH conditions are expected to possess low coordination number and chain/planar/sheet-like structures, water will probably only be bound in the first hydration shell and therefore the diffuse interface may not
have a sufficient surface area/volume ratio to generate a negative deviation from Porod’s law that is large enough to be observed via SAXS (Demichelis et al, 2011).

The presence of a hydrated layer at pH 8.5 suggests that larger droplets are characterised by a complex and nested network favoring encapsulation of water molecules, increasing unfavorable enthalpy and thus potentially limiting their growth (Zahn et al, 2015). This also suggests that these nanodroplets must be composed of small sub-units of calcium carbonate surrounded by water. This would be consistent with the SAXS study performed by Chao et al (2014), who observed two populations of nanoparticles with mean radii of 0.9 nm and 9.7 nm at similar pH and Ca concentration to that used in this study. However, in view of their data resolution, accurate determination of two structural levels must be considered with caution. The amount of water surrounding each of these sub-units would be expected to increase with calcium concentration but decrease moving towards the core of the nanoparticle. This would suggest that the nanoparticles are likely to be highly dynamic, since the interaction between hydrated sub-units separated by multiple layers of water would be purely electrostatic (i.e. due to electrostatic interaction of bicarbonate ions and water molecules in the periphery of the cluster) (Kellermeier et al, 2012). Development of hydrated layers could lead to a rise in entropy and explain the absence of an observable diffuse interface around the PNCs at low pH (Sinn et al, 2004). In this latter case, the size of the nanodroplets should be limited by size as such interfaces have never been seen before in such systems. As recently suggested by Zahn (2015), this size limitation can only be explained by the size-dependent term for the free energy of the bulk phase. Incorporation of water into the
structure of the putative PNCs would increase unfavourable enthalpy and limit their growth (Rateri & Gale 2010).

The particle concentrations observed here under the range of conditions examined are much higher than those reported in other studies performed on similar calcium carbonate systems (1.75 × 10^{10} particles mL^{-1} by Bolze et al (2002) and 2.32 × 10^{11} particles mL^{-1} by Liu et al (2010)). Part of this discrepancy could be due to using the contrast (Δρ) value, reported by Liu et al, (2010) for ACC, based on the assumption that Δρ for PNCs is similar to that for ACC. PNCs are known to be less dense and highly hydrated, as supported by this study, and could possess a contrast value closer to that of the solvent (Demichelis et al, 2011). Dehydration of the particle core, as observed at pH 8.5, would result in an increase in scattering contrast leading to an artificial increase in the calculated particle concentration, since this value is calculated through the invariant (Equation 19) and is proportional to (Δρ)^2. Similarly, as the volume is inversely proportional to the contrast, an increase in (Δρ)^2 would result in an apparent decrease of the calculated mean particle volume. The discrepancy in the calculated concentrations of nanoparticles in this study compared to previous studies can also be attributed, in part, to the faster mixing time, resulting in highly homogeneous reaction conditions, and lower pH conditions used in this study. The results of this study indicate that the particle concentration was around 6-fold higher at pH 7.5 compared to pH 8.5, suggesting competition between carbonate and bicarbonate ions is likely to strongly influence the concentration of nanoparticles. This would be consistent with the lower particle concentrations
observed by Bolze et al (2002) and Liu et al (2010), given that both these studies were conducted by mixing calcium with pure carbonate solutions.

5.2. **Structure of low q scattering objects**

The presence of a sharp interface in the solvent at low $q$ values (i.e. $d_2$ value around 4) strongly suggests a significant change in the electron density. Furthermore, this feature was present under all conditions examined, implying that although the nanoparticles observed at high $q$ possess different characteristics depending on pH and calcium concentration, the structure (and potentially the processes leading to formation) of this strong interface at low $q$ values do not strongly depend on pH or calcium concentration. A possible explanation for such a scattering pattern could be degassing of nanosized CO$_2$ bubbles. However this hypothesis seems unlikely as the solution used for background subtraction also contained the same carbonate concentration. Furthermore, similar features have also been observed in previous time resolved SAXS studies of calcium carbonate formation but at lower intensity (Marmiroli et al, 2009; Liu et al, 2010; Chao et al, 2014). Using a free jet micromixer approach similar to that used in this study, Marmiroli et al, (2009) observed this feature at times as low as 75 µs after first contact between the reagent solutions, although at higher pH than in this this study. At similar calcium concentrations to those used in this study but at pH ~10, Liu et al (2010) observed a similar power law regime at low $q$ at the shortest reaction time of 40 ms, but this regime evolved to yield a clear Guinier region at longer reaction times. Chao et al (2014) used “fast controlled precipitation”, in which CO$_2$ degassing from calcium bicarbonate solutions that are supersaturated with CO$_2$
generates in an increase in pH that triggers nucleation, at similar pH and calcium concentrations to those used in this study. In addition, artifacts caused by the geometry of the free jet that were present in the raw scattering data at the lowest $q$ values were corrected by application of radially oriented mask to the scattering image by the beamline scientists during data acquisition. The consistency between these studies despite use of different approaches therefore strongly implies that the observed low $q$ scattering is a result of the chemistry and not an artifact resulting from use of the free jet.

The observed sharp interface could thus be associated with either a population of large particles (e.g. aggregates) such that only the Porod regime associated with the structural level is present in the analytical window, or formation of a second liquid phase within the primary solvent, possessing a different scattering length density. The latter hypothesis would suggest the presence of two liquid phases at the same time within the solvent, consistent with suggested mechanisms for calcium carbonate nucleation. Hypothetically, aggregation or formation of large hydrated nanoclusters, as observed at high $q$-values, could create a second ion-rich liquid phase. Alternatively, a liquid-liquid like phase separation via binodal or spinodal demixing could also generate two liquid phases within the solvent, with one liquid phase possessing a high concentration of ions needed to generate a significant change in the physical density (Figure 23). This has previously been observed via ex-situ TEM and NMR, where a dense liquid phase was characterised (Wolf et al, 2012; Bewernitz et al, 2012). Further evidence for liquid liquid-like phase separation in calcium carbonate systems has also been very recently observed
via terahertz spectroscopy, reinforcing its potential importance in CC nucleation processes (Sebastiani et al, 2017).

![Figure 23: Liquid-liquid like phase separation representation obtained via spinodal decomposition (left) or binodal demixing/nucleation (right). Both processes lead to the formation of two liquid phases separated from each other by a strong interface. Figure reproduced from García-Ojalvo et al (1998).](image)

The present data set does not allow determination of whether the formation of hydrated nanoparticles leads to a phase demixing process, or phase demixing triggers the formation of nanoparticles by inducing local supersaturation. However, it would seem plausible to consider the formation of this sharp interface at low $q$ values to be linked to the nature of the putative PNCs at high $q$ regimes. In particular, it is notable that nanoparticles observed at high $q$ at both pH 7.5 and 8.5 appear to maintain their identities as separate structural levels despite the existence of the low $q$ feature. It is unclear if the nanoparticles observed at pH 7.5 would maintain their short range structure during aggregation due to their highly hydrated and dynamic nature. In particular, it may be hypothesised that such dynamic structures would rapidly lose their short range structure as a result of aggregation, such that the high $q$ features would no longer be apparent. In contrast, the nanoparticles observed at pH 8.5 would seem more likely to maintain their short range structure during
aggregation due to the formation of a more stable core-shell structure. Observations that (i) the low $q$ feature at pH 7.5 appears to coexist with the high $q$ feature, and; (ii) the low $q$ regime appears to be fully developed even at the lowest calcium concentrations and lowest pH values where the kinetics of particle aggregation would be expected to be minimum both in this study and in the study by Marmiroli et al (2009) just 75 µs after mixing, might thus favour the hypothesis of a liquid-liquid like phase separation as the first step, followed by development of the high $q$ features within the ion rich phase. These possibilities could potentially be resolved by investigating the scattering intensity over a broader $q$-range and accessing even shorter timescales in order to verify formation of a population of nanoparticles either before or after formation of this sharp liquid interface. Chao et al (2014) recorded similar low $q$ scattering patterns during calcium carbonate nucleation when using a 1.5 mm diameter quartz capillary rather than a free jet, but did not comment on or analyse this feature in their study. Similar scattering patterns have also been recorded during phase separation of organic compounds, associated with larger structures not accessible over the $q$ range investigated here (Kulkarni & Beaucage, 2005; Takenaka et al, 2007; Kulkarni et al, 2012).

It is therefore difficult to reach a definitive conclusion about the nature of the low $q$ regime feature. However, in any case, use of the Beaucage model as presented and discussed before allows for differentiation and discrete analysis of each scattering level, such that the conclusions relating to the high $q$ regime remain valid regardless of the cause of the low $q$ feature.

5.3. Formation mechanisms of scattering objects
Analysis of the aggregation mechanism indicated different apparent behaviour at the different pH values examined. At pH 7.5, the relationship between log $G_1$ and log $V_{p1}$ appeared to conform well to a monomer addition model of nanoparticle formation, with the monomer being either ions in solution or smaller nanoparticles present outside the analytical window. In either case, the mechanism involves these “monomers” randomly colliding with the existing nanoparticles through Brownian motion, and thus following an ion-by-ion addition process as described for ACC by Liu et al (2010). Resolving the true identity of the monomer could be possible by increasing the scattering mass and improving the quality of the data in the high $q$-range. The consistency of these results with the theoretical model for the pH 7.5 condition also supports the assumption that calcium concentration can be considered a proxy for the kinetics of the reaction under these conditions.

However, at pH 8.5, analysis of log $G_1$ vs log $V_{p1}$ did not conform to the expected relationship for either of the aggregation models described by Liu et al (2008). This is most likely due to violation of the assumption that titration of calcium concentration is a proxy for the reaction kinetics, and/or the assumptions behind the Liu et al (2008) models that (i) monomers must possess the same scattering contrast (i.e. similar physical density) as polymers for the monomer addition model; and (ii) coalescence between similar sized particles dominates the aggregation process, and the interaction potential among particles does not vary with particle size, for the coagulation (i.e. cluster-cluster aggregation) model. The assumption that the calcium titration will act as a proxy for reaction kinetics should be valid provided that the reaction mechanism is the same at each Ca concentration and proceeds in a
relatively linear manner. However, the assumption would be expected to fail if the reaction products (i.e. putative PNCs) undergo further reaction with each other or with other species present the system. This appears to be the case at pH 8.5, given the likelihood that the nanoparticles are undergoing progressive dehydration with increasing calcium concentration. Therefore, while it may also be the case that formation of nanoparticles occurs via a monomer addition mechanism at pH 8.5, it is not possible to confirm or refute this hypothesis based on the available data.
6. Conclusion

By use of in situ time resolved SAXS employing a free jet with rapid reagent mixing, the shape, size and formation/growth mechanism of calcium carbonate nanoparticles as a function of calcium concentration and pH have been extracted using the UM. The nanoparticles observed through these experiments exhibited similar size and shape as calcium carbonate PNCs described by NCNT, and could potentially be formed by the aggregation of highly hydrated sub-units, based on evolution of the core-shell structure observed at pH 8.5. In particular, these putative PNCs were clearly distinguishable at calcium concentrations both above and below the saturation state of calcite, as in previous studies of calcium carbonate PNCs (Gebauer et al, 2008; Pouget et al, 2009). The presence of these nanoparticles was associated with a strong interface at low q, likely associated with a liquid-liquid like phase separation process or due to the aggregation of highly hydrated nanoclusters, consistent with expectations from recent computer simulations (Wallace et al, 2014; Kellermeier et al, 2016). The results also suggest that the processes leading to the formation of such a sharp interface occur much faster than the shortest observation time accessible in these experiments, and that the fundamental processes seem to be relatively independent of the saturation state and pH over the range of conditions examined.

The results presented therefore provide further evidence for the multi-step processes linking non-classical nucleation theory and formation of PNCs with classical nucleation theory. However, it still remains unclear which processes link prenucleation clusters to the formation of the first amorphous solid phase; this
threshold could be part of a more complex system where both non-classical and
classical nucleation theory co-exist and overlap over a certain kinetic and
thermodynamic range. Further studies aiming to better characterise the influence of
pH over a wider range are thus necessary to more fully explore the phase diagram
of PNCs. Slowing the kinetics of the reactions involved by further decreasing the
concentration of calcium used and increasing the number and/or duration of SAXS
scans to compensate for the associated decrease in scattering intensity could allow
the characterisation of the very early stages of nucleation and potentially
differentiate the formation of the very first prenucleation clusters from the
appearance of the sharp interface observed at low $q$. 
Chapter VI

Characterisation of the calcium coordination environment in calcium carbonate prenucleation clusters
1. Introduction

In the previous chapter, Small Angle X-Ray Scattering was used to gain new information on the mean size, structure and aggregation processes of calcium carbonate PNCs at the nanoscale, as well as the impact of pH and calcium concentration on these properties. In this chapter, X-ray absorption spectroscopy is used to investigate how these variables impact on the structure of the nanoclusters at the atomic scale.

At present, the only information available on the atomic structure of calcium carbonate PNCs is derived from computer simulations. The first attempt to simulate the coordination environment of calcium during the early stages of calcium carbonate nucleation was performed by Di Tommaso et al (2008) using density functional theory (DFT). These simulations examined the behaviour of single calcium ions in roughly fifty water molecules on timescales of up to only a few picoseconds, which is smaller than the average residence time of water in the first solvation shell of calcium ions (Gebauer et al, 2014). Furthermore, only the behaviour of single calcium ions with varying bicarbonate and carbonate ions was considered; the study did not consider the interactions of calcium ions with each other and, as such, formation of PNCs.

The first study examining the coordination environment of calcium in PNCs came from Tribello et al (2009) who performed force-field simulations showing formation of ACC clusters possessing local order similar to vaterite and aragonite. However, their estimates of the solvation of calcium ions and calcium solubility were unrealistic and after reconsideration, it was admitted that this study simulated
a liquid-liquid like phase separation process occurring during the early stages of mineral precipitation and not the PNCs themselves (Gebauer et al, 2014).

Demichelis et al (2011) moved towards much more realistic simulation of the atomic structure of PNCs. These simulations, performed over a range of concentrations in the supersaturated domain, demonstrated formation of a combination of linear chains, rings and occasionally branched structures composed of calcium and (bi)carbonate ions. The main characteristic of these structures, which are held together only via ionic interactions, is the relatively low coordination number of calcium ions by carbonate; in these structures, the mean calcium to carbon and calcium to calcium coordination numbers varied from 1 to 3 (calcium-oxygen coordination was not directly considered in their simulation, this last parameter reflecting more the hydration state of the PNCs rather than the structure of the PNCs themselves).

Experimental characterisation of the atomic structure of PNCs is particularly challenging as it requires the ability to stop the nucleation process during its very early stages. Furthermore, PNCs must be isolated from the solvent and be sufficiently well defined for analysis (Gebauer et al, 2016). While several experimental studies using X-ray diffraction, total scattering, X-ray absorption, nuclear magnetic resonance and infrared spectroscopy have focused on the characterisation of various ACC phases, surprisingly little information on the structure of ACC has been obtained (Michel et al, 2008; Gebauer et al, 2010; Goodwin et al, 2010; Gebauer et al, 2016). Several anhydrous and hydrated forms of ACC possessing similarities in short range order (i.e. bond distances and
coordination number) with crystalline forms of aragonite and calcite have been observed, however development of a general structural model describing ACC phases remains challenging.

Titration of calcium with carbonate through to calcium carbonate precipitation implied a link between bond strength in PNCs and the type of ACC formed, suggesting that PNCs might already possess short range order (Günther et al, 2008; Wolf et al, 2008; Gebauer et al, 2008; Cartwright et al, 2012; Gebauer et al, 2016). However, there have been few attempts to characterise the atomic scale structure of PNCs, and typically those attempts have failed to give accurate information due to formation of multiple phases within the sample or difficulty in isolating and analysing the clusters (Oaki et al, 2008; Kellermeier et al, 2012). In this chapter, I aim to overcome these limitations by coupling rapid mixing of reagents (using the mixing device described in Chapter IV) with a freeze quench and freeze drying technique to produce a stable sample for analysis by conventional X-ray absorption spectroscopy techniques.
2. Research objectives

In this chapter, I aimed to use X-ray Absorption Fine Structure (XAFS) to:

- characterise the coordination environment of calcium ions in the early stages of calcium carbonate nucleation;
- define the impact of pH and ion activity product on the short range order of calcium carbonate PNCs; and
- compare the experimentally-determined coordination environment with that predicted from molecular dynamics simulations.

3. Experimental section

3.1. Chemical preparation

CaCl$_2$.2H$_2$O was purchased from Ajax Finechem. Na$_2$CO$_3$ (assay $\geq$ 99.0%) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (assay $\geq$ 99.5%) were purchased from Sigma-Aldrich. Stock solutions of CaCl$_2$.2H$_2$O (4 $\times$ 10$^{-4}$ M, 4 $\times$ 10$^{-3}$ M and 4 $\times$ 10$^{-2}$ M) (hereafter represented as S1) and Na$_2$CO$_3$ (2 $\times$ 10$^{-2}$ M) with HEPES (3 $\times$ 10$^{-2}$ M) (hereafter represented as S2) were prepared in ultrapure 18 M$\Omega$.cm resistivity milliQ water (hereafter referred to as MQ) obtained from a Millipore Academic water purification system and stored in closed Schott bottles. The pH of S2 was set at either 7.5 or 8.5 immediately before preparation of each sample (as described below) by addition of small amounts of concentrated HCl or NaOH. The pH was measured using a glass pH electrode calibrated by a three-point curve with pH 4, 7 and 10 buffer solutions (pH 4 buffer solution from Australian Scientific; pH 7 and pH 10 pH buffer solutions from LabServ).
Additionally, a calcite standard was prepared by mixing a solution of 1 M CaCl$_2$.2H$_2$O with a solution of 1 M Na$_2$CO$_3$ (both prepared in MQ) in a 1:1 volume ratio in a Schott bottle, immediately forming a white precipitate. The suspension was stirred using a magnetic stirrer for 1 h and then filtered. The precipitate was dried in an oven at 125°C for 2 days until a fine white powder remained. The identity of the standard was confirmed as calcite via XRD using a Co K-alpha source (Bruker D4 Endeavour XRD) at a wavelength of $\lambda = 1.541$ Å, from 5 to 80° 2$\theta$ with 0.05° 2$\theta$ step size and 2 s counting time per step. The XRD patterns were evaluated using EVA software (DIFFRAC-plus evaluation package, Bruker AXS, Germany).

3.2. Sample preparation

Samples were prepared for XAFS analysis based on a modified version of the method described by Lin et al (2003) using a coupled micro mixing and freeze-quenching device (Figure 24). Mixing of S1 (three different Ca concentrations) with S2 (two different pH values) as described below produced six different samples with varying Ca concentration and pH. A solution of calcium chloride (S1) pumped at 2 mL.min$^{-1}$ was rapidly mixed with a solution of sodium carbonate containing HEPES pH buffer (S2) also pumped at 2 mL.min$^{-1}$ using the micro mixing device described in Chapter IV in the same manner as described previously for the SAXS experiment (Chapter V). However, in this experiment, the free jet exiting the mixing device was directed into a 50 mL polypropylene centrifuge tube filled with and immersed in liquid nitrogen such that the distance between the exit from the mixing device and surface of the liquid nitrogen in the tube was 5.5 cm. Upon contact with liquid nitrogen, the free jet was nebulised into small droplets, resulting in rapid freezing of
the reaction mixture. Assuming that the freezing in liquid nitrogen is instantaneous, and taking into account a mixing time of 8.7 ms (as calculated in Chapter IV for a total flow rate of 4 mL.min$^{-1}$), a travel time of 0.5 ms between the exit of the mixture from the mixing chamber and the exit of the jet from the chip, a jet cross-sectional area of 10$^{-8}$ m$^2$, jet velocity of 6.7 m.s$^{-1}$, and a distance of 5.5 cm between the exit point of the jet from the mixing chip and the surface of the liquid nitrogen (corresponding to a travel time of 8.3 ms), the mixed solution was estimated to have been frozen 17.5 ms after initial contact between S1 and S2 in the mixing chamber.

The centrifuge tubes containing the frozen materials were stored frozen at 193.3 K prior to being freeze dried for 2 d until all solvent water was evaporated and a fine powder or a paste remained. Subsequently, freeze dried samples were stored in new, capped 50 mL polypropylene centrifuge tubes at room temperature prior to XAFS analysis. As demonstrated by the results (presented in detail later), this method appears to preserve the short order hydration state of the material while removing the water present in solvent (Abdelwahed et al, 2006).
Figure 24: Experimental design for XAFS sample preparation. The point at which the free jet exited the mixing device and surface of the liquid nitrogen bath were 5.5 cm apart.

3.3. XAFS data acquisition

XAS measurements at the Ca K-edge (4.0381 keV) were undertaken at ELETTRA Synchrotron (Italy), operating at an energy of 2 GeV and current of 300 mA, at the XAFS beamline in December 2015. At the beamline, lyophilised samples were ground in an agate mortar then diluted with polyvinylpyrrolidone (PVP) powder and compacted into thin pellets. An optimal sample thickness of ~300 µm
and optimal Ca concentration of 0.8 M was calculated for absorption at the Ca K-edge using the X-ray absorption calculator ABSORBIX, yielding a theoretical edge jump of $\Delta(\mu x) = 0.91$ (Michalowicz et al., 2009). Full scans of the X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) were collected in steps of 5 eV in the pre-edge region (from 3738.43 eV to 4028.53 eV) and 0.2 eV in the edge region (from 4061.51 eV to 4061.71 eV), gradually increasing to 2.6 eV in the post-edge region (4061.71 eV to 4589.30 eV). Each scan lasted 96 min in duration. When the signal/noise ratio was sufficiently high, two scans per sample were collected in transmission mode using an FMB OXFORD ion chamber detector and then averaged. For samples where the signal/noise ratio was poor in transmission mode, a silicon drift detector was used to collect four scans per sample in fluorescence mode, which were then averaged. Sample acquisition modes are reported in Table 2. In both modes, samples mounted on a copper sample holder were positioned at 45° with respect to the incident beam and maintained at a temperature of 80 K in a liquid N$_2$-Cryostat to minimise potential beam damage and thermal disorder in the samples. Energy calibration was performed based on the first inflection point of a CaF reference pellet.
Table 2: XAFS acquisition mode for the standard and samples.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Acquisition mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcite standard</td>
<td>Transmission</td>
</tr>
<tr>
<td>pH 7.5, [Ca] = 2 \times 10^{-2} M</td>
<td>Transmission</td>
</tr>
<tr>
<td>pH 7.5, [Ca] = 2 \times 10^{-3} M</td>
<td>Transmission</td>
</tr>
<tr>
<td>pH 7.5, [Ca] = 2 \times 10^{-4} M</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>pH 8.5, [Ca] = 2 \times 10^{-2} M</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>pH 8.5, [Ca] = 2 \times 10^{-3} M</td>
<td>Transmission</td>
</tr>
<tr>
<td>pH 8.5, [Ca] = 2 \times 10^{-4} M</td>
<td>Transmission</td>
</tr>
</tbody>
</table>

3.4. Fitting of XAFS data

Merging of scans, alignment, energy calibration, and de-glitching were performed using standard features of the ATHENA software package (Ravel & Newville, 2005). Normalisation and background removal was performed identically for each sample. $E_0$ was fixed at exactly half the edge jump and the $R_{bkg}$ value for the algorithm used for background removal of high frequency oscillations was set at 1.1 (Newville, 2001). Due to the use of thin samples and low concentrations of calcium, self-absorption was minimised and no correction was applied to fluorescence scans.

EXAFS data were analyzed according to standard procedures using the ARTEMIS software package (Ravel & Newville, 2005). The EXAFS relationship, given by the equation below, was used to generate the theoretical phase and amplitude function for single and multiple scattering pathways of calcium with oxygen, carbon, and calcium neighbors:
\[
\chi(k) = \sum_j S_0^2 N_j f_j(k) e^{-2R_j/\lambda(k)} e^{-2k^2\sigma_j^2} \frac{1}{kR_j^2} \sin[2kR_j + \delta_j(k)]
\]

*Equation 28*

where \(f(k)\) represents the amplitude and \(\delta(k)\) the phase-shift of the scattered wave vector from the surrounding atoms, \(N\) the number of neighbor atoms (also called coordination number), \(R\) the interatomic distance between the central atom and the neighbors, and \(\sigma^2\) the mean square of disorder in the neighbor atoms (usually called the Debye-Waller factor). The amplitude reduction factor, \(e^{-2R_j/\lambda(k)}\), and the passive electron reduction factor, \(S_0^2\), account for the discrepancy between predicted and experimentally measured amplitudes.

The first shell was initially fit by generating single scattering pathways of Ca-O based on crystallographic data for calcite, aragonite, vaterite, monohydracalcite and ikaite (Michel *et al*, 2008; Fulton *et al*, 2008; Wang *et al*, 2009). Subsequently, the same method was applied for fitting of the second (Ca-C), third (Ca-O) and fourth shells (Ca-Ca). Based on Fulton *et al* (2003), fitting of Ca-H shells was also attempted, but ultimately ignored as this failed to improve the fit. The inclusion of each additional shell was validated only if both:

- fitting with an additional shell was at least 20% better (from Artemis goodness of fit parameters) than the fit without the additional shell; and

- the difference of the amplitude in \(\text{Re}[\chi(k)]\) between the fit without and the fit with the additional shell was greater than the amplitude of
the noise level obtained via Inverse Fast Fourier Transform (IFFT) of the EXAFS oscillations between $k = 15$ and $20 \, \text{Å}^{-1}$.

Analysis of single scattering pathways accounting for all coordination shells (Ca-O$_1$, Ca-C, Ca-O$_2$ (O$_2$ as a second shell containing oxygen atoms) and Ca-Ca) was then undertaken from 1.3 to 4.25 Å in $R$ space on Fourier transformed $k^3$ weighted data from $k = 1$ to 10 Å$^{-1}$. After having determined the amplitude reduction factor ($S_0^2$) and difference from the energy origin ($\Delta E_0$) in the calcite sample using crystallographic data, a two stage approach was used to obtain the best fit of single shells in each sample and multiple scattering pathways:

1. A first stage where the amplitude reduction factors ($S_0^2$) were set at 1 as per fitting of the calcite sample and the coordination numbers set at values from 1 to 9, allowing the difference from energy origin ($\Delta E_0$), difference from bond distance input value (as given by the crystallographic data) of each shell ($R$), and mean square disorder of the distances (Debye–Waller factors, $\sigma^2$) to vary; and

2. A second stage aiming to improve the fit where $S_0^2$ was set at 1 and the coordination number allowed to vary as a fitting parameter. The contribution of single scattering pathways was then analysed by varying $\Delta E_0$, the coordination number, $R$, and the Debye–Waller factors within each of their appropriate ranges for each couple of neighboring shells until the best fit was obtained.
3.5. *Estimation of multiple scattering pathways*

Due to the lack of atomic scale structural information on such systems, estimation of multiple scattering pathways is extremely difficult. In this study, we attempted to characterise these pathways using the structure of a cluster obtained via computer simulation from Demichelis *et al* (2011). A cluster is composed of four atoms of calcium, four atoms of carbon and surrounded by water molecules. The structure of the first four shells (Ca-O$_1$, Ca-C, Ca-O$_2$ and Ca-Ca) with the appropriate atomic bond distances was simulated in EXCURV98 (Binsted, 1989).

Three dimensional structure of the Ca coordination environment (i.e., bond angles in addition to bond lengths) is required for the calculation of multiple scattering. This was approach by using hypothetical model complexes with C$_1$ symmetry as given by computer simulations (Demichelis *et al*, 2011; Robbins *et al*, 2015). Four different structures derived from a bigger cluster simulated by Demichelis *et al*, (2011) were recreated in EXCURV98 and classified depending on the number of oxygen atoms in the first shell. These simple structures exhibited linear, branched or ring like shapes depending on the number of oxygen atoms coordinated in the first shell of calcium. Theoretical EXAFS signals were then simulated with multiple scattering enabled or disabled. The phase-shifts and potentials were calculated in the small atom (plane-wave) approximation (Moon & Peacock, 2012) and the phase-shift functions used in the curve fitting were derived by *ab initio* methods in EXCURV98 using Hedin–Lundqvist potentials (Hedin & Lundqvist, 1970) and von Barth ground states (Peacock & Sherman, 2004). The difference between the two EXAFS signals (with and without multiple scattering)
was first compared in $k$ space to evaluate the potential importance of multiple scattering pathways for each type of structure.

4. Results

4.1. EXAFS analysis of single scattering pathways

EXAFS in $k$ space and Fourier transformed EXAFS in $R$ space for the calcite standard and the samples are presented in Figure 25. The fitted model is also shown. As in any standard EXAFS analysis, the minimum possible error in the coordination number, established by taking into account the error in determination of $S_0^2$ and background subtraction, was estimated at 10% for the first shell, 15% for the second and third shells, and 20% for the fourth shell. The error in the radial bond distance was estimated at 0.03 to 0.04 Å as it is defined in standard EXAFS analysis (Newville, 2005).

An example of the validation procedure for inclusion of additional shells in the fit for the sample with pH 8.5 and [Ca] = 2 × 10$^{-4}$ M is presented in Figure 26. The difference between values of $k^2[\chi(k)]$ for the fit with 4 shells and for the fit with 3 shells demonstrates that including the additional shell enabled decreasing the residual by 20%. While this difference is not necessarily immediately obvious, comparison of the residuals resulting from subtraction of the fit with 3 shells from the fit with 4 shells with the noise level shows a statistically relevant improvement of the fit by inclusion of the fourth shell.
Figure 25: Ca K-edge EXAFS spectra (left panel) and calculated Fourier transform magnitudes (right panel) for a calcite standard and samples containing 10 mM total carbonate, calcium concentrations varying from $2 \times 10^{-4}$ M to $2 \times 10^{-2}$ M, and pH 7.5 or 8.5. The best fit model for the samples and calcite standard are shown as a red dashed line. Radial distances are not corrected for phase shift.
Figure 26: Example fits with a different number of shells for the sample with pH 8.5 and [Ca] = 2 × 10^{-4} M. **A:** Comparison of $k^2 \chi(k)$ (in Å$^{-3}$) for the fit to experimental data (red) based on modelling of 3 shells (orange) or 4 shells (blue). **B:** Comparison of the noise level (purple) with the residual after subtraction of the fit based on modelling of 3 shells from the fit based on modelling of 4 shells. The fact that the residual has higher amplitude than the noise indicates that the fit is significantly improved by inclusion of the fourth shell in the model.

Based on the SAXS data from Chapter V, nanoscale structures possessing similar characteristics to PNCs are expected to be present in all samples (i.e. at all calcium concentrations and both pH 7.5 and 8.5). However, due to the intrinsic properties of EXAFS, which measures the average local coordination geometry,
accurate characterisation of individual bond distances in PNCs at higher calcium concentrations is difficult with this method since the stoichiometry implies that a substantial proportion of the Ca will not be associated with carbonate. The broadness of peaks in the Fourier transformed data can be explained by a relatively broad distribution of bond distances for the same shell (i.e. Ca-O) and/or the co-existence of various structures for a given condition. Due to the poor data quality past \( k = 8 \) Å\(^{-1}\) for some of the samples and the presence of a glitch at \( k = 9.2 \) Å\(^{-1}\), fitting of higher order shells was difficult and must be interpreted with care.

The potential appearance of different structures with variable atomic distances at the highest calcium concentration also appears to flatten the Fourier transformed data. Flattening of the EXAFS signal is generally associated with heterogeneity of mineral phases (Günther et al, 2005; Michel et al, 2008; Gebauer et al, 2010). Thus, it is expected that the EXAFS for the samples with low calcium concentrations may be most indicative of the short-range structure of PNCs. Therefore, while the EXAFS results on all samples are presented below, particular attention is given to the samples containing the lower calcium concentrations.

The fitted values of coordination number and radial distance for each shell are presented in Figure 27. Structural parameters derived from the fits to the calcite standard and the experimental data from this work are presented in Table 3, while structural parameters for standards reported in other published work are compiled in Table 4. For each condition, a fit of the first four shells containing atoms of oxygen, carbon, additional oxygen and calcium, respectively, was obtained. With the coordination number of each shell fixed based on crystallographic data, the
Fourier transformed EXAFS of the calcite standard gave almost identical bond distances as reported in the literature, with six atoms of oxygen at 2.36 Å; six atoms of carbon at 3.24 Å; an additional six atoms of oxygen at 3.49 Å; six atoms of calcium at 4.05 Å; and a low Debye-Waller factor ($\sigma^2 < 0.0225$) (Michel et al, 2008).
Figure 27: Fitted coordination number (black circles) and radial distance (red vertical bars) at pH 7.5 and 8.5 for each fitted shell. 

A: Ca-O₁ shell. B: Ca-C shell. C: Ca-O₂ shell. D: Ca-Ca shell. The error in the CN was estimated at ±10% for the first shell, ±15% for the second and third shells, and ±20% for the fourth shell. The error in the radial distance was estimated at ±0.04 Å for all samples.
Table 3: Best fit parameter values from EXAFS analysis of the first four shells for samples at pH 7.5 and pH 8.5 and the calcite standard. Estimated errors for N and R are the same as given in Figure 27.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acquisition mode</th>
<th>Scatterer</th>
<th>N</th>
<th>R (Å)</th>
<th>$\sigma^2$ (Å$^2$)</th>
<th>$\Delta E_0$</th>
<th>k-weighting</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcite</strong></td>
<td>Transmission</td>
<td>Oxygen</td>
<td>6</td>
<td>2.36 ± 0.04</td>
<td>0.0084</td>
<td>-1.762</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbon</td>
<td>6</td>
<td>3.24 ± 0.04</td>
<td>0.0141</td>
<td>0.787</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>6</td>
<td>3.49 ± 0.04</td>
<td>0.0190</td>
<td>-4.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>6</td>
<td>4.06 ± 0.04</td>
<td>0.0139</td>
<td>-1.318</td>
<td></td>
</tr>
<tr>
<td><strong>pH 7.5</strong></td>
<td>Transmission</td>
<td>Oxygen</td>
<td>4.9 ± 0.5</td>
<td>2.40 ± 0.04</td>
<td>0.0131</td>
<td>0.441</td>
<td></td>
</tr>
<tr>
<td>[Ca] = 2 × 10$^{-2}$ M</td>
<td></td>
<td>Carbon</td>
<td>2.0 ± 0.3</td>
<td>3.20 ± 0.04</td>
<td>0.0047</td>
<td>-2.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>1.5 ± 0.2</td>
<td>3.71 ± 0.04</td>
<td>0.0121</td>
<td>-0.321</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>1.4 ± 0.3</td>
<td>4.38 ± 0.04</td>
<td>0.0230</td>
<td>3.951</td>
<td></td>
</tr>
<tr>
<td><strong>pH 7.5</strong></td>
<td>Transmission</td>
<td>Oxygen</td>
<td>5.3 ± 0.5</td>
<td>2.37 ± 0.04</td>
<td>0.0075</td>
<td>-0.632</td>
<td></td>
</tr>
<tr>
<td>[Ca] = 2 × 10$^{-3}$ M</td>
<td></td>
<td>Carbon</td>
<td>1.6 ± 0.3</td>
<td>3.23 ± 0.04</td>
<td>0.0001</td>
<td>-0.556</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>2.0 ± 0.3</td>
<td>3.69 ± 0.04</td>
<td>0.0075</td>
<td>3.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>4.1 ± 0.8</td>
<td>4.19 ± 0.04</td>
<td>0.0138</td>
<td>-3.001</td>
<td></td>
</tr>
<tr>
<td><strong>pH 7.5</strong></td>
<td>Fluorescence</td>
<td>Oxygen</td>
<td>5.5 ± 0.5</td>
<td>2.37 ± 0.04</td>
<td>0.0086</td>
<td>0.095</td>
<td></td>
</tr>
<tr>
<td>[Ca] = 2 × 10$^{-4}$ M</td>
<td></td>
<td>Carbon</td>
<td>1.6 ± 0.3</td>
<td>3.32 ± 0.04</td>
<td>0.0008</td>
<td>6.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>4.6 ± 0.7</td>
<td>3.62 ± 0.04</td>
<td>0.0165</td>
<td>-5.331</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>2.5 ± 0.5</td>
<td>4.21 ± 0.04</td>
<td>0.01</td>
<td>-0.68</td>
<td></td>
</tr>
<tr>
<td><strong>pH 8.5</strong></td>
<td>Fluorescence</td>
<td>Oxygen</td>
<td>5.3 ± 0.5</td>
<td>2.36 ± 0.04</td>
<td>0.0100</td>
<td>-3.588</td>
<td></td>
</tr>
<tr>
<td>[Ca] = 2 × 10$^{-2}$ M</td>
<td></td>
<td>Carbon</td>
<td>1.6 ± 0.3</td>
<td>3.06 ± 0.04</td>
<td>0.0061</td>
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<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>2.9 ± 0.4</td>
<td>3.74 ± 0.04</td>
<td>0.0096</td>
<td>-0.346</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>1.8 ± 0.4</td>
<td>4.12 ± 0.04</td>
<td>0.0169</td>
<td>-4.714</td>
<td></td>
</tr>
<tr>
<td><strong>pH 8.5</strong></td>
<td>Transmission</td>
<td>Oxygen</td>
<td>5.7 ± 0.5</td>
<td>2.40 ± 0.04</td>
<td>0.012</td>
<td>1.296</td>
<td></td>
</tr>
<tr>
<td>[Ca] = 2 × 10$^{-3}$ M</td>
<td></td>
<td>Carbon</td>
<td>2.3 ± 0.4</td>
<td>3.24 ± 0.04</td>
<td>0.0158</td>
<td>5.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>1.2 ± 0.2</td>
<td>3.33 ± 0.04</td>
<td>0.0095</td>
<td>-5.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>1.9 ± 0.4</td>
<td>4.35 ± 0.04</td>
<td>0.0148</td>
<td>2.46</td>
<td></td>
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<tr>
<td><strong>pH 8.5</strong></td>
<td>Transmission</td>
<td>Oxygen</td>
<td>6.4 ± 0.6</td>
<td>2.38 ± 0.04</td>
<td>0.0134</td>
<td>0.346</td>
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</tr>
<tr>
<td>[Ca] = 2 × 10$^{-4}$ M</td>
<td></td>
<td>Carbon</td>
<td>2.0 ± 0.3</td>
<td>3.24 ± 0.04</td>
<td>0.0096</td>
<td>6.469</td>
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<td></td>
<td></td>
<td>Oxygen</td>
<td>1.4 ± 0.2</td>
<td>4.02 ± 0.04</td>
<td>0.0212</td>
<td>5.008</td>
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<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>2.7 ± 0.5</td>
<td>4.33 ± 0.04</td>
<td>0.015</td>
<td>3.03</td>
<td></td>
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</tbody>
</table>
Table 4: Reported literature values of coordination number and radial distances for various calcium carbonate minerals.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference</th>
<th>Scatterer</th>
<th>N</th>
<th>R (Å)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcite</td>
<td>Michel et al (2008)</td>
<td>Oxygen</td>
<td>6</td>
<td>2.36</td>
<td>Ca octahedron</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbon</td>
<td>6</td>
<td>3.31</td>
<td>Monodentate</td>
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<td></td>
<td></td>
<td>Oxygen</td>
<td>6</td>
<td>3.46</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>6</td>
<td>4.05</td>
<td>Single corner sharing</td>
</tr>
<tr>
<td>Ca(OH)₂</td>
<td>Michel et al (2008)</td>
<td>Oxygen</td>
<td>6</td>
<td>2.38</td>
<td>Ca octahedron</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>6</td>
<td>3.58</td>
<td>Edge sharing</td>
</tr>
<tr>
<td>CaCl₂₂(H₂O) (solid)</td>
<td>Fulton et al (2003)</td>
<td>Oxygen</td>
<td>2</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorine</td>
<td>4</td>
<td>2.74</td>
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</tr>
<tr>
<td>CaCl₂₆(H₂O) (solid)</td>
<td>Fulton et al (2003)</td>
<td>Oxygen</td>
<td>3</td>
<td>2.45</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺-solvated</td>
<td>Fulton et al (2003)</td>
<td>Oxygen</td>
<td>7.2</td>
<td>2.44</td>
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<tr>
<td>Monohydrocalcite</td>
<td>Michel et al (2008)</td>
<td>Oxygen</td>
<td>4</td>
<td>2.37</td>
<td>Average Ca-O distance = 2.41 Å</td>
</tr>
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<td></td>
<td></td>
<td>Oxygen</td>
<td>4</td>
<td>2.44</td>
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<tr>
<td></td>
<td></td>
<td>Carbon</td>
<td>2</td>
<td>2.69</td>
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<td>Calcium</td>
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<td>Edge sharing</td>
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<td>Oxygen</td>
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<td>3.92</td>
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<tr>
<td></td>
<td></td>
<td>Calcium</td>
<td>2</td>
<td>3.92</td>
<td></td>
</tr>
</tbody>
</table>
At pH 7.5 and \([\text{Ca}^{2+}] = 2 \times 10^{-4} \text{ M}\), the Ca-O\(_1\) shell was isolated, then back-transformed and initially fitted as described before, assuming 6 atoms of oxygen surrounding calcium as for the calcite standard. The second fitting stage allowed better definition of the coordination number, yielding to an average CN of 5.5 ± 0.6 (± 10%) at \(R_{\text{Ca-O}1} = 2.37 \pm 0.04 \text{ Å}\) with a Debye-Waller factor of 0.0085 ± 0.0012 Å\(^2\). Using the same two-step process, the second (Ca-C) shell was initially fitted with 6 atoms of carbon based on the structure of defined crystalline phases, then refined to yield to an average CN of 1.6 ± 0.2 (± 15%) at \(R_{\text{Ca-C}} = 3.32 \pm 0.04 \text{ Å}\) with a Debye-Waller factor of 0.0008 ± 0.0003 Å\(^2\). Similarly, fitting of the third (Ca-O\(_2\)) shell yielded an average CN of 4.8 ± 0.7 (± 15%) at \(R_{\text{Ca-O}2} = 4.11 \pm 0.04 \text{ Å}\) with a higher Debye-Waller factor of 0.0063 ± 0.0034 Å\(^2\). Fitting of the fourth (Ca-Ca) shell yielded an average CN of 3.7 ± 0.5 (± 20%) at \(R_{\text{Ca-Ca}} = 4.28 \pm 0.04 \text{ Å}\) with a Debye-Waller factor of 0.0114 ± 0.0047 Å\(^2\).

At pH 8.5 and \([\text{Ca}^{2+}] = 2 \times 10^{-4} \text{ M}\), the first (Ca-O\(_1\)) shell exhibited the same bond distance as at pH 7.5. The mean number of oxygen atoms increased to a CN of 6.4 ± 0.7 (± 10%) with a Debye-Waller factor of 0.0134 ± 0.0053 Å\(^2\) but the difference was not statistically different to that at pH 7.5. The mean number of atoms within the second (Ca-C) shell increased to 2.0 ± 0.3 (± 15%), again not statistically different from that at pH 7.5, at a bond distance of \(R_{\text{Ca-C}} = 3.24 \pm 0.04 \text{ Å}\) (Debye-Waller factor of 0.0096±0.0047 Å\(^2\)). Contrary to pH 7.5, the third (Ca-O\(_2\)) shell exhibited a mean CN of 1.4 ± 0.2 (± 15%) atoms at \(R_{\text{Ca-O}2} = 4.02 \pm 0.04 \text{ Å}\) with a Debye-Waller factor of 0.0212 ± 0.0064 Å\(^2\). The fourth (Ca-Ca) shell exhibited a similar mean CN that at pH
7.5 of 2.7 ± 0.5 (± 20%) at $R_{Ca-Ca} = 4.34 \pm 0.04 \text{ Å}$ with a Debye-Waller factor of 0.0150 ± 0.0053 Å$^{-2}$.

With increasing calcium concentration, the CN of the first shell (Ca-O$_1$) decreased from 5.5 ± 0.6 to 4.9 ± 0.5 at pH 7.5 and more significantly from 6.4 ± 0.6 to 5.3 ± 0.5 at pH 8.5 (Figure 27). Radial distances remained similar at around 2.40 ± 0.04 Å. For the second shell (Ca-C), the CN remained relatively constant at 1.8 ± 0.2 (mean ± SEM from all Ca concentrations) at both pH 7.5 and 8.5. While radial distance decreased with increasing calcium concentration at pH 7.5 from 3.32 ± 0.04 Å to 3.20 ± 0.04 Å, at pH 8.5 it was relatively constant for the two lowest calcium concentrations (mean ± SEM of 3.26 ± 0.04 Å for these Ca concentrations) before decreasing to 3.06 ± 0.04 Å at the highest calcium concentration. The third shell, which is attributed to a second group of oxygen atoms, presented the largest variations in atomic bond distance and coordination number. At pH 7.5, the CN decreased from 4.6 ± 0.7 to 1.6 ± 0.2 and the radial distance monotonically increased from 3.61 ± 0.04 Å to 3.72 ± 0.04 Å with increasing calcium concentration. However at pH 8.5, the CN remained constant at around 1.2-1.4 (± 0.2) for the two lowest Ca concentrations then increased to 2.9 ± 0.4 for the highest calcium concentration. Radial distances decreased from 4.02 ± 0.04 Å to 3.33 ± 0.04 Å as the Ca concentration increased from $2 \times 10^{-4}$ M to $2 \times 10^{-3}$ M, then increased to 3.74± 0.04 Å with $2 \times 10^{-2}$ M Ca. The last shell (Ca-Ca) was particularly difficult to fit and presented the highest fitted $\Delta E_0$ values, potentially due to multiple scattering (MS) interferences. At pH 7.5, the CN decreased between the lowest and highest Ca concentrations, but increased slightly at the intermediate concentration, while at pH 8.5 the CN decreased monotonically with increasing Ca
concentration. The radial distance remained relatively constant, with a mean value of 4.27 Å ± 0.11 (mean ± SEM from all Ca concentrations at both pH values).

4.2. Simulation of multiple scattering pathways

The atomic structure of clusters recreated from the simulations of Demichelis et al (2011) with EXCURV98 are presented in Figure 28 and classified based on the number of oxygen atoms in their first shell. The simulated EXAFS signals for each of these clusters with and without accounting for multiple scattering are compared in Figure 29.
**Figure 28**: Simulated clusters from Demichelis et al (2011). All non-bound water molecules surrounding the cluster have been removed to aid visibility. Calcium, carbon, oxygen and hydrogen atoms are represented respectively in blue, black, red and white. **A**: Full cluster simulated by Demichelis et al (2011). **B**: Four different atomic structures extracted from the full cluster are illustrated and classified depending on the central calcium coordination number. These four atomic structures served as models to characterise multiple scattering effects using EXCRUV98. Hydrogen atoms were ignored due to their negligible contribution to the EXAFS signal.
Figure 29: Estimation of the contribution of multiple scattering pathways to the EXAFS signal for the structures shown in Figure 28. Multiple scattering contributions were determined accounting for four coordination shells in k-space (Ca-O₁, Ca-C, Ca-O₂ and Ca-C) for structures with a first shell CN = 5, 6 or 7 for the central calcium atom in the cluster. Red curves represent simulated EXAFS spectra ignoring multiple scattering and blue curves EXAFS spectra accounting for multiple scattering. A: Ca_{CN=5}. B: Ca_{CN=6}. C: Ca_{CN=7-1}. EXAFS were also simulated for cluster Ca_{CN=7-2} (possessing 27 atoms and 7 oxygen atoms in its first shell), however due to a possible unresolved bug with EXCURV98, the generated EXAFS signal with multiple scattering enabled was unrealistic and the cluster is not considered further in this study.

The effect of multiple scattering was found to vary depending on the structure considered. For the least coordinated structures, MS seemed to induce a slight increase in amplitude, sharpening of peaks and a slight dephasing at higher wavenumber values. With more highly coordinated clusters, MS seemed to increase
peak amplitudes, and result in shoulders and secondary peaks that were not present when MS was ignored. This suggests that ignoring multiple scattering could potentially lead to an underestimation of the amplitude of the EXAFS signal and thus the CN of each shell for the lower coordinated simulated cluster. In contrast, the impact of MS on structures possessing a higher coordination number for oxygen in the first shell was radically different, resulting in artificial oscillations in $k$-space and a slight dephasing, which could again contribute to a substantial error in estimation of the coordination number and atomic radial distance of furthest shells.

Multiple linear regression analysis was used to compare experimental and simulated EXAFS in $k$-space for clusters with first shell coordination numbers of 5, 6 and 7. The calculated EXAFS contribution of simulated clusters to the observed (experimental) EXAFS are presented in Figure 30 and Table 5. While the results suggest a higher contribution of more highly coordinated structures ($Ca_{CN=7-1}$), multiple regression analysis failed to produce a fit with a high Pearson product-moment correlation coefficient ($r^2$) value for all conditions investigated.

Because comparison of $k$-space scans was not conclusive, a similar multiple linear regression analysis was also performed to fit the experimental EXAFS in $R$ space (Figure 31 and Table 6). It is important to note that such analysis is not usually performed on the filtered data, given that the back Fourier Transform is a frequency filter analysis and $R$ space is thus a filtered representation of the true $k$ space signal. However, as the simulated clusters and experimental clusters present similar shell arrangements, comparison of EXAFS in $R$ space should allow evaluation of the contribution of clusters with each coordination number to the
observed (experimental) EXAFS. Multiple linear regression fitting of the experimental EXAFS in $R$ space resulted in much higher correlation coefficients than in $k$ space, and suggesting a greater contribution from low coordination number, linear structures, with the Ca$_{CN=5}$ structure contributing, on average, to 65% of the observed EXAFS signal, and the Ca$_{CN=6}$ and Ca$_{CN=7-1}$ structures contributing less than 28% and 7%, respectively.
Figure 30: Simulation of EXAFS from experimental samples with $2 \times 10^{-4}$ M Ca in k space (from 2.3 to 9 Å$^{-1}$) as a linear combination of calculated EXAFS for simulated clusters with a first shell CN = 5, 6 or 7 for the central calcium atom. A: pH 7.5. B: 8.5. In both panels, the red line represents the EXAFS spectrum of the experimental samples, while blue, green and orange curves represent linear combination fits obtained by multiple linear regression of the EXAFS spectra accounting for the simulated clusters with CN = 5 and 6 only, CN = 6 and 7 only, or CN = 5, 6 and 7, respectively. Details of the multiple linear regression are presented in Table 5.
Table 5: Multiple linear regression in k-space of EXAFS from experimental samples containing $2 \times 10^{-4}$ M Ca with calculated EXAFS for simulated clusters with a first shell CN = 5, 6 or 7 for the central calcium atom. Values represent the percent contribution determined for each cluster to the observed EXAFS under each condition. The Pearson product-moment correlation coefficient ($r^2$) for each fit is also provided. Results considering contributions from only the CN = 5 and CN = 6 clusters or from the CN = 5, CN = 6 and CN = 7-1 clusters for each condition are presented.

<table>
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<th>pH 8.5</th>
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</tr>
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<td>CN = 5, CN = 6</td>
<td>CN = 5, CN = 6</td>
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<td></td>
<td>only</td>
<td>only</td>
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<td></td>
</tr>
<tr>
<td>CN = 5 contribution (%)</td>
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<td>-</td>
<td>18.8</td>
<td>41.4</td>
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<tr>
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<td>36.0</td>
<td>28.8</td>
<td>58.6</td>
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<tr>
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<td>64.0</td>
<td>52.4</td>
<td>-</td>
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<tr>
<td>$r^2$</td>
<td>0.45</td>
<td>0.53</td>
<td>0.54</td>
<td>0.61</td>
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</table>
Figure 31: Simulation of EXAFS from experimental samples with $2 \times 10^{-4} \text{ M Ca}$ in $R$ space as a linear combination of calculated EXAFS for simulated clusters with a first shell CN = 5, 6 or 7 for the central calcium atom. **A:** pH 7.5. **B:** 8.5. In both panels the red line represents the EXAFS spectrum of the experimental samples, while the blue and green lines represent the linear combination fits obtained by multiple linear regression of the EXAFS spectra for the simulated clusters with first shell CN = 5 or 6 only, or for the simulated clusters with first shell CN = 5, 6 or 7, respectively. Details of the multiple linear regression are presented in Table 6.
Table 6: Multiple linear regression in R space of EXAFS from experimental samples containing $2 \times 10^{-4}$ M Ca with calculated EXAFS for simulated clusters with a first shell CN = 5, 6 or 7 for the central calcium atom. Values represent the percent contribution determined for each cluster to the observed EXAFS under each condition. The Pearson product-moment correlation coefficient ($r^2$) for each fit is also provided. Results considering contributions from only the CN = 5 and CN = 6 clusters or from the CN = 5, CN = 6 and CN= 7 clusters for each condition are presented.

<table>
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<td>CN = 5, CN= 6 and CN = 7</td>
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<td>CN = 6 contribution (%)</td>
<td>28.8</td>
<td>27.0</td>
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<td>CN = 7 contribution (%)</td>
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<td>$r^2$</td>
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5. Discussion

5.1. Evaluation of experimental approach

In contrast to previous methods used for ACC precipitation (Becker et al, 2003; Michel et al, 2008; Gebauer et al, 2010; Goodwin et al, 2010; Sun et al, 2016), the use of a micro-mixing device and freeze quench apparatus permitted the rapid quenching of nucleation reactions shortly (in less than 20 ms) after having triggering them under homogeneous conditions. Freeze drying of the samples then resulted, as the solvent concentration decreased, in an increase in the concentration of the
calcium species present (e.g. calcium carbonate nanoclusters), enabling acquisition of high quality Ca K-edge EXAFS spectra. The presence of the non-calcium complexing HEPES buffer is additionally expected to have facilitated stabilisation of the calcium species against inter-cluster contact, thus limiting the potential for any potential structural rearrangement through processes like Ostwald ripening (Abdelwahed et al, 2006). Due to the fitting approach, characterisation of first shell around the central calcium atom was relatively accurate and the coordination number was evaluated with an estimated error of 10%. The CN for the second and third shells were evaluated with estimated errors of 15%, while the error in the CN for the fourth shell was estimated to be 20%. It is expected that fitting the of first and second shells was relatively accurate but, in part due to due to the presence of a glitch at around \( k = 9.3 \, \text{Å}^{-1} \), fitting of the third and fourth shells must be interpreted with caution.

EXAFS analysis of the first shell indicates that the freeze dried samples present similar coordination numbers and bond lengths for the first oxygen shell as have been estimated for hydrated calcium, calcite and ACC, all of which possess 6 or 7 oxygen atoms in the first shell (Mazzarella et al, 1967; Leigny et al, 1983; Takagi et al, 1984; Thiele et al, 1984; Faggani et al, 1986; Duhlev et al, 1993; Simonsen et al, 1997; Levi-Kalisman et al, 2000; Jalilehvand et al, 2001; Fulton et al, 2001; Michel et al, 2008; Gebauer et al, 2010). Additionally, while the simulations realised by Demichelis et al (2011) suggest that bidentate bonds between Ca and (bi)carbonate are possible, the bonding between Ca and (bi)carbonate is likely to be predominantly through monodentate linkages in all
samples, since the radial distances for the second (Ca-C) shell ranged from 3.06-3.32 Å, which are similar to the radial distances for monodentate linkages measured in crystalline calcium carbonate minerals, whereas Ca-C radial distances in bidentate linkages in these minerals are typically < 3 Å (Table 7).

Table 7: Atomic bond distances for different types of Ca-C bonds in anhydrous, crystalline calcium carbonate phases.

<table>
<thead>
<tr>
<th>Mineral phase</th>
<th>Monodentate Ca-C bond distances (Å)</th>
<th>Bidentate Ca-C bond distances (Å)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcite</td>
<td>3.21</td>
<td>-</td>
<td>Graf (1961)</td>
</tr>
<tr>
<td>Aragonite</td>
<td>3.24-3.41</td>
<td>2.90-2.93</td>
<td>Dal Negro &amp; Ungaretti (1971)</td>
</tr>
</tbody>
</table>

On this basis, it is possible to estimate the number of oxygen atoms in the first shell that are associated with water by comparison of the CN for the first Ca-O shell with the CN for the Ca-C shell. Assuming that all bonds between Ca and (bi)carbonate are monodentate, the number of O atoms in the first shell associated with Ca-(bi)carbonate bonding will be the same as the number of C atoms in the second shell (which are also associated with Ca-(bi)carbonate bonding). These calculations, which are shown in Table 8, indicate that between 60 and 70% of the oxygen atoms in the first shell are thus likely associated with water molecules.
Table 8: Average number of oxygen atoms in the first shell associated with water molecules (CN O-H$_2$O), calculated from values of coordination number determined for the first and second shells and assuming 100% monodentate bonds between Ca and (bi)carbonate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CN Ca-O</th>
<th>CN Ca-C</th>
<th>CN O-H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.5, [Ca] = 2 × 10$^{-2}$ M</td>
<td>4.9 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>pH 7.5, [Ca] = 2 × 10$^{-3}$ M</td>
<td>5.3 ± 0.5</td>
<td>1.6 ± 0.25</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>pH 7.5, [Ca] = 2 × 10$^{-4}$ M</td>
<td>5.5 ± 0.5</td>
<td>1.6 ± 0.25</td>
<td>3.8 ± 1.1</td>
</tr>
<tr>
<td>pH 8.5, [Ca] = 2 × 10$^{-2}$ M</td>
<td>5.3 ± 0.5</td>
<td>1.6 ± 0.25</td>
<td>3.7 ± 1.0</td>
</tr>
<tr>
<td>pH 8.5, [Ca] = 2 × 10$^{-3}$ M</td>
<td>5.7 ± 0.5</td>
<td>2.3 ± 0.35</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>pH 8.5, [Ca] = 2 × 10$^{-4}$ M</td>
<td>6.4 ± 0.6</td>
<td>2.0 ± 0.3</td>
<td>4.4 ± 1.1</td>
</tr>
</tbody>
</table>

These results are consistent with the molecular dynamics simulations performed by Demichelis et al (2011), in which PNC structures with 5 or 6 oxygen atoms in their first shell were typically bonded with two (bi)carbonate molecules. This result differs when the CN of Ca with oxygen increases to 7, as more complex branched structures are seen to develop on simulated clusters, implying that if these structures are present in the samples considered in this study, they are in the minority. These calculations therefore imply that freeze drying did not remove water bound in the first coordination shell of Ca, suggesting that the local coordination environment was likely to have been preserved. They also suggest that the decrease in CN for the first oxygen shell with increasing calcium concentration is due to a decrease in the amount of water bound in the first shell.

Contrary to other EXAFS studies examining calcium carbonate phases formed soon after nucleation, the favourable signal/noise ratio of the data presented here allowed accurate fitting of second and, to a lesser extent, third and fourth shells.
While characterisation of these shells has previously been possible in samples of calcium carbonate biominerals (Levi-Kalisman et al, 2000; Neumann & Epple, 2007; Gower, 2008), studies on abiotic ACC have usually failed to accurately describe the furthest shells (Ca-C, Ca-O₂, Ca-Ca) (Gebauer et al, 2010; Michel et al, 2011). The ability to fit these shells in samples resulting from homogenous nucleation in this work is most likely linked to use of the micro-mixing device. The use of rapid mixing and quenching techniques may facilitate the differentiation of these shells by limiting the development of different structures possessing different short range order within the samples. More specifically, all structures present in the samples used in this work formed within 18 ms of initiating the reaction, in contrast to the precipitation methods used by Gebauer et al (2010) or Michel et al (2011), which is expected to have limited the formation of secondary phases that can potentially induce peak broadening and/or destructive interferences.

Due to the absence of a well-defined structural model for such systems, accounting for multiple scattering in the samples is difficult. This problem was approached by examining the potential contribution of multiple scattering pathways to the theoretical EXAFS signal in several simulated PNCs. This analysis seems to suggest that oxygen atoms in triangular carbonate structures are the main contributors to multiple scattering. Non-consideration of these pathways can lead to an underestimation or overestimation of the experimental EXAFS amplitudes, especially for highly coordinated calcium ions. Nonetheless, correcting for these effects in experimentally determined EXAFS is difficult to do accurately due to the lack of reference structures and the small number of simulated structures considered.
in this study. However, linear combination fits of the simulated EXAFS (accounting for multiple scattering) from various combinations of the hypothetical clusters with different first shell CN values to the experimental EXAFS data for the samples containing $2 \times 10^{-4}$ M Ca suggests that these samples contain a combination of mainly low CN structures. Attempts to perform similar fitting of the EXAFS data for samples containing higher calcium concentrations were unsuccessful, however, reinforcing the likelihood that these sample are highly heterogeneous. The main discrepancies between the experimental and simulated EXAFS spectra were associated with the Ca-C and Ca-O$_2$ shells, as seen in the linear combination fitting. This can be attributed to a range of factors including the difficulty to fit scattering from light elements like carbon, the fact that structural water molecules were removed from simulated clusters, the likely presence of several calcium carbonate structures within samples, non-consideration of effects of pH on the simulated structures, and the relatively small number of simulated structures considered in this study.

5.2. Impact of calcium concentration and pH on the local calcium coordination environment

5.2.1. Saturation state

At both pH 7.5 and 8.5, increasing the Ca concentration resulted in changes in the local calcium coordination environment. The CN for the first (Ca-O$_1$) and fourth (Ca-Ca) shells both decreased with increasing calcium concentration, suggesting dehydration and increasing heterogeneity of the structures. A similar
trend was visible at pH 7.5 for the CN of the third (Ca-O$_2$) shell, which has previously been attributed in ACC to oxygen present in structural water (i.e. the hydrated layer surrounding ACC) (Gebauer et al., 2010) but which is more likely attributable here to oxygen in (bi)carbonate bound through monodentate bonds. This is supported by the fact that values of the CN for carbon present in the second shell and for oxygen present in the third shell are relatively similar (except for the lowest calcium concentration at pH 7.5), suggesting that most of the oxygen in the third shell is likely associated with carbonate groups.

Incorporation of water in the structure of small amorphous calcium carbonate clusters has been shown to increase their stability (Raiteri & Gale, 2010). Under certain conditions, growth of the cluster can be faster than diffusion of water, preventing the core of the cluster from equilibrating with the surrounding solution. These processes occur on millisecond time scales and, at particular levels of supersaturation, can lead to the development of a core/hydrated shell structure with a negative gradient of hydration from the outer shell to the core of the cluster (Raiteri & Gale 2010). These results are once again consistent with results from the small angle scattering experiment presented in Chapter V. It should be noted, however, that a similar decrease in hydration could also be caused by the formation of secondary calcium carbonate mineral phases within the samples.

5.2.2. pH

Previous studies on the impact of pH in calcium carbonate systems have indicated formation of proto-mineral phases possessing short range order similar to calcite at lower pH or vaterite at higher pH (Gebauer et al, 2008 and Gebauer et al,
It might be expected that the structure of PNCs would change significantly between a system dominated by carbonate ions (pH > 10) and a bicarbonate ion dominated system (pH < 9). However, the change in pH from 8.5 to 7.5 in the system studied here also resulted in changes in the short range structure of calcium carbonate clusters, despite both conditions being dominated by bicarbonate ions. This suggests a critical role for carbonate ions, which are 10-fold more abundant at pH 8.5 than pH 7.5, potentially due to different reaction kinetics resulting from competition between carbonate and bicarbonate ions at the different pH values. The changes in short range structure observed here seems related to dehydration coupled with a progressive increase in complexity as the calcium concentration increases. Such changes in short range order may be associated with the formation of core/shell structures, as has previously been observed and simulated (Gebauer et al, 2008; Goodwin et al, 2010; Demichelis et al, 2011). Even so, it remains unclear which parameters, other than pH, control development of such short range order.

5.3. Implications to Non-Classical Nucleation Theory

The samples containing $2 \times 10^{-4}$ M Ca at both pH 7.5 and 8.5 are expected to most likely represent the structure of PNCs due to significant undersaturation with respect to various calcium carbonate minerals phases, including ACC. Furthermore, the presence of scattering structures consistent with PNCs was shown to occur under these conditions in Chapter V. While the radial distances for the first and second shells in these samples were similar to those for calcite, the CN values for the second, third and fourth shells were lower than those for any of the crystalline or proto-crystalline standards previously considered (Figure 32). Furthermore, the
mean coordination number and atomic radial distances acquired via EXAFS for these samples are similar to those predicted using molecular dynamics simulations of PNCs by Demichelis et al (2011), where simulated chain-like structures consisted of calcium and carbonate ions possessing coordination numbers less than 3 for both calcium-carbon and calcium-calcium pairs. Therefore, the results of EXAFS analysis in this study are broadly consistent with previous findings under similar pH conditions and beyond (7 to 11.5) in relation to the fundamental nature of calcium carbonate PNCs involved in NCNT.

**Figure 32**: Coordination numbers (symbols) and radial distances (bars) for various coordination shells in calcite standard (red bars and circles), the sample at pH 7.5 with $\text{[Ca]} = 2 \times 10^{-4} \text{M}$ (blue bars and diamonds), and the sample at pH 8.5 with $\text{[Ca]} = 2 \times 10^{-4} \text{M}$ (orange bars and triangles).

As recently pointed out by Sun et al (2016), it remains unclear from where the short range order in ACC arises. Hypothetical hydrated chain-like structures, similar to what have been observed in this system by SAXS (Chapter V), could be present in ACC. A second hypothesis developed by Rez et al (2014) suggested that the presence of randomly oriented nanocrystals in the nanometre size range separated by water molecules could generate coherent diffraction profiles similar to
those exhibited by ACC. Such nanostructures have been evoked by Sun et al (2016) with the use of amphipathic ligand binding nanoparticles forming during the early stage of nucleation and creating a hydrated complex, \((\text{CaCO}_3)\text{H}_2\text{O}(\text{PCDA})_3\), possessing a calcium carbonate core size comparable with that of PNCs in aqueous solutions. With both the shape and number of CaCO\(_3\) units being quite close to those for the primitive rhombohedral unit cell of calcite, the short range order of such complexes was found to possess Ca-O bond close to calcite.

6. Conclusion

Although potential limitations must be carefully considered, EXAFS has proven to be an effective method to characterise the coordination environment of early stage precursors of calcium carbonate nucleation. By granting access to a detailed picture of the coordination environment, EXAFS can be used to investigate the formation and evolution of short range order in putative PNCs and their possible role in the formation of ACC.

As shown, multiple scattering can have a critical impact on the EXAFS signal, and failing to account for multiple scattering in EXAFS modelling may cause underestimation or overestimation of the true EXAFS amplitude. Coupling computer simulations and XAFS experiments at lower pH may be the best way to further characterise and define a structural model for the coordination environment of putative PNCs. Total scattering analysis may also help to provide an even more detailed definition of the short and medium range structure of putative calcium carbonate PNCs and ACC during the process of nucleation.
As this study has been conducted under different conditions to previous studies, it remains unclear, in the context of homogeneous nucleation, exactly which parameters can encode information in putative PNCs relating to the post-nucleation structure of resulting calcium carbonate minerals. However, a similar approach could also be used to characterise the transformation of ACC as well as the interaction of similar nanoscale objects with organic and/or inorganic ligands, potentially providing further information on this issue.
Chapter VII

Characterisation of liquid-liquid like phase separation in the CaCO$_3$ nucleation process using Small Angle Neutron Scattering
1. Introduction

Non-classical nucleation theory has increasingly been invoked in studies of mineral nucleation over the past decade. Characterisation of PNCs, which are an important feature of NCNT, has also been progressed via different methods (Quigley et al, 2008; Gebauer et al, 2008; Pouget et al, 2009; Liu et al, 2010; Nudelman et al, 2010; Demichelis et al, 2011; Sear 2012; Gebaeur et al, 2014) but their exact role in the formation of crystalline mineral phases remains poorly understood. In particular, the role of liquid-liquid like phase separation (i.e. the formation of two liquids possessing different physical density) as either a precursor to PNC formation, or a consequence of PNC aggregation, has become a central issue in relation to calcium carbonate nucleation via NCNT.

This alternative mineralisation pathway, proposed by Gower et al (2008) and extended by Wolf et al (2011), describes how a liquid-liquid like phase separation could lead to the aggregation of pre-critical hydrated complexes. Such pathways were simulated for PNCs, investigating cluster stability, structure and nucleation pathways. The outcome of these studies suggested the formation of a second, ion-rich liquid phase possessing higher calcium ion activity that facilitated formation of favourable nucleation sites able to host hydrated PNCs (Wallace et al, 2013). The free energy profile of such processes presents a monotonic decrease characteristic of the existence of a stable state within the dense liquid phase. In this second ion-rich phase, the coordination number of calcium with carbonate ions evolves smoothly (from CN = 2 to CN = 3 when the cluster size reaches 26 ions), revealing
that the formation of a dense liquid doesn’t induce an abrupt change in cluster characteristics (Wallace et al., 2013).

However, the processes leading to the formation of such a dense liquid phase remain experimentally unproven (Nielsen et al., 2014). Indeed, there are still key questions as to whether the accumulation of hydrated PNCs may induce the phase separation, or if the higher ionic activity in the second phase will trigger local supersaturation, favouring formation of PNCs (Gebaeur et al., 2014). In both cases, the change in local saturation levels implies that a different population of PNCs could be present in the ion-rich phase. While not providing direct evidence of phase separation, a recent SAXS experiment on the calcium carbonate system highlighted formation of a polydisperse population of PNCs from 0.5 to 3 nm in size during the early stage of nucleation (Chao et al., 2014). This study demonstrated formation of a sharp interface between relatively large scattering objects (presenting as a power law regime with \( I \propto q^{-4} \) at low \( q \)), similar to the data presented in Chapter V, that could be associated with liquid-liquid like phase separation or the presence of large nanodroplets. However, the potential relationship between these features and liquid-liquid like phase separation remains inconclusive.

In this chapter, I present an attempt to further characterise PNCs, and seek evidence for liquid-liquid like separation or otherwise, using the contrast variation technique with Small Angle Neutron Scattering (SANS). As neutrons scatter due to interactions with the core of the atoms, different isotopes of the same element possess different neutron scattering length densities. With the contrast variation technique, the contrast between different materials and an aqueous solvent can be
changed by varying the H$_2$O/D$_2$O ratio. Certain critical values of this ratio can be reached such that the scattering length density of a part of the material matches with the solvent, rendering it effectively “invisible” to neutrons. Under these conditions, particular features of the material under study can be highlighted due to contrast with the remaining “invisible” parts of the material. This technique is particularly effective with biological samples rich in hydrogen atoms, however contrast matching can also be used to characterise, amongst other things, hydrated crystalline phases (Carlile & Willis, 2009). This property of neutron scattering also makes it particularly useful to investigate changes in the hydration state of materials, as would be expected to occur due to liquid-liquid like phase separation that possibly occurs during the early stages of calcium carbonate nucleation (Wallace et al, 2013; Nielsen et al, 2014).

2. Research objectives

The objective of this experiment was to use the contrast matching technique with Small Angle Neutron Scattering (SANS) to:

i. seek evidence of liquid-liquid like phase separation, or otherwise, during the nucleation of calcium carbonate;

ii. further characterise the hydrated diffuse interface of nanoparticles that form in the early stages of calcium carbonate nucleation.

3. Experimental section

3.1. Chemical preparation
CaCl$_2$.2H$_2$O (assay $\geq$ 99.0%) was purchased from Ajax Finechem. Na$_2$CO$_3$ and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (assay $\geq$ 99.5%) were purchased from Sigma-Aldrich. All solutions were prepared in varying ratios of ultrapure 18 M$\Omega$.cm resistivity milliQ water (hereafter referred to as MQ) obtained from a Millipore Academic water purification system and D$_2$O available at the SANS beamline. Samples were prepared by mixing $4 \times 10^{-3}$ M calcium chloride solution with $2 \times 10^{-2}$ M sodium carbonate plus $3 \times 10^{-2}$ M HEPES buffer solution in a 1:1 volume ratio, resulting in final concentrations of 2 mM Ca$^{2+}$ and 10 mM total carbonate. HEPES was used as a non-calcium complexing pH buffer, and was additionally favoured due to its fast response time and ability to maintain a quasi-steady-state pH after about 10 ms (Schulz et al, 2006). HEPES buffer was also used in this study due to its lack of impact on the scattering signal (Shukla et al, 2008).

3.2. Sample preparation

As the number of scattering events is much lower with SANS than with SAXS (principally due to the lower flux of the collimated beam), use of the mixing chip in continuous flow, as described in Chapter V, was not possible in this case. Instead, the reaction was initiated by mixing the calcium solution and the carbonate plus HEPES solution (set at pH 8.5) in a polyethylene centrifuge tube. Samples with increasing percentage of D$_2$O (0%, 10%, 20%, 25, 30%, or 40%) were prepared in the same way. Sample pH was adjusted to 8.5 after mixing the two solutions by adding small amounts of HCl or NaOH. The pH was measured using a standard glass pH electrode calibrated using a three-point curve with pH 4, 7 and 10 buffer solutions in H$_2$O (pH 4 solution from Australian Scientific and pH 7 and 10 solutions
from LabServ). Due to the difference in activity of hydrogen and deuterium species, pH was adjusted following the equation defined by Mikkelsen & Nielsen (1960):

\[ pD = pH + 0.42 \]

*Equation 29*

This resulted in target values of measured pH using the calibrated pH electrode as shown in Table 9.

**Table 9**: Target values of measured pH using the calibrated pH electrode to obtain an equivalent total \( H^+/D^+ \) activity of \( p(H,D) = 8.5 \) in solutions containing varying proportions of \( D_2O \) and \( H_2O \) (based on Mikkelsen & Nielsen, 1960).

<table>
<thead>
<tr>
<th>( D_2O% )</th>
<th>0%</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>( pH ) targeted</td>
<td>8.5</td>
<td>8.544</td>
<td>8.588</td>
<td>8.632</td>
<td>8.676</td>
<td>8.72</td>
</tr>
</tbody>
</table>

The mixed solutions were then quickly transferred into either a 2 mm path length quartz cell (for samples containing < 30% \( D_2O \)) or a 4 mm path length quartz cell (for samples containing at least 30% \( D_2O \)) and the cell installed on the beamline.

### 3.3. SANS data acquisition

Quartz cells were placed in a rotating sample holder at the Quokka Small Angle Neutron Scattering beamline of the Bragg Institute, Australia (Gilbert *et al*, 2006). Data were acquired using an Ordela 21000N High Count-Rate Two-Dimensional neutron counter detector. The detector was sequentially positioned at distances of 1.3 m, 12 m and 20 m from the sample with counting times of 2 min for the 1.3 m sample/detector distance, 8 min for the 12 m sample/detector distance and 20 min for the 20 m sample/detector distance, resulting in a full scan time of approximately 40 min per sample. Subsequently, data were reduced from the raw
counts on the two-dimensional detector to a radially averaged absolute intensity as a function of the scattering vector, \( q \), defined as:

\[
q = \frac{4\pi}{\lambda} \sin \frac{\theta}{2}
\]

\textit{Equation 30}

with \( \lambda \) the neutron beam wavelength (set at 5 \( \pm \) 0.5 Å) and \( \theta \) the scattering angle.

Data from the three detector positions were combined into a single scattering curve spanning a \( q \)-range from 0.00049 to 0.64 Å\(^{-1}\). Data were background corrected by the subtraction of empty beam scattering and H\(_2\)O/D\(_2\)O scattering intensity used as a standard, then the signal was normalised at each detector pixel by beamstop intensity (a flat isotropic cadmium scatterer) to yield scattering intensity as an absolute value.

4. SANS data analysis

Due to the low signal/noise ratio of the data set and the absence of a distinct power law regime, application of the Unified Model, as described in Chapter V, was impossible. Instead, the absolute intensity from SANS at selected \( q \) values was compared with the absolute intensity from SAXS in an attempt to relate the change in scattering length density to the hydration state of the sample. A first comparison was performed at \( q \) values of 0.001 and 0.01Å\(^{-1}\), which were chosen as the error around the mean was the minimum for all samples.

As the scattering intensity is proportional to the square of the contrast, i.e. the difference between the scattering length density (SLD) of the solvent and the sample, the principle behind contrast matching is to change the contrast value
between the solvent and a scattering object to better define a part or the whole of a sample. Contrary to X-ray scattering where the SLD increases with the atomic number, neutrons interact with the nucleus of an atom and the SLD of a given element varies depending its isotopic composition. For instance, D$_2$O and H$_2$O have significantly different SLD values of $6.33 \times 10^{-6}$ and $-5.6 \times 10^{-7}$ Å$^{-2}$, respectively. Thus, in the case where there is no exchange of H$_2$O/D$_2$O or H$^+$/D$^+$ between the sample and the solvent, the contrast, and thus the scattering intensity, will reach a maximum (or minimum) at a certain H$_2$O/D$_2$O ratio, enabling determination of the SLD of the sample.

However, when exchange of H$_2$O/D$_2$O or H$^+$/D$^+$ between the sample and solvent is possible, the sample SLD becomes a function of the percentage of H$_2$O/D$_2$O or H$^+$/D$^+$ incorporated in the sample, since the SLD of a particular material is given by:

$$
\text{SLD} = \frac{\sum_{i} b_i}{V} = \frac{N_A \sum_{i} c_i M_i b_i}{\sum_{i} c_i M_i} = \frac{d N_A \sum_{i=1}^{N} c_i b_i}{\sum_{i=1}^{N} c_i M_i}
$$

Equation 31

where $b_i$ is the SLD of atom $i$ in the material, $V$ is the volume of a “unit cell” of the material, $N_A$ = Avogadro’s number, $M_i$ is the atomic mass of atom $i$, $c_i$ is the stoichiometric number of atom $i$ in the chemical formula for the material, and $d$ is the bulk physical density of the material of interest (sample or solvent). This relationship has been used as the basis for further analysis of the hydration state of the sample and is developed in more detail in the next section.

4.1. Estimation of the hydration level
As noted above, a change in absolute scattering intensity with changing proportion of D$_2$O in solvent reflects a change in contrast between the solvent and the scattering object. To quantitatively relate the change in absolute scattering intensity, and hence contrast, to the hydration state of the samples investigated in this work, the following method was used:

i. both neutron and X-ray contrast were calculated as a function of the percentage of D$_2$O in the solvent, assuming no isotopic fractionation during the formation of hydrated phases (i.e. any water in the hydrated samples has the same isotopic composition as the solvent);

ii. the ratio of absolute scattering from X-rays to that from neutrons was calculated as a function of ACC composition and its physical density.

In order to calculate neutron and X-ray contrast, the SLD of ACC was expressed as a function of the water content of the ACC (i.e. expressed as H$_2$O/D$_2$O : Ca ratio in mol/mol), the molar ratio of bicarbonate to total dissolved carbonate (denoted as HCO$_3$/TDIC in mol/mol), and the molar ratio of carbonate to calcium atoms in the ACC (expressed as C/Ca ratio in mol/mol). The third parameter was constrained at 1.02 mol/mol using the EXAFS data, presented in Chapter VI, for this specific calcium concentration. The physical density was initially estimated at 1.62 g.cm$^{-3}$, which is the physical density of ACC as given in Liu et al (2008). The neutron scattering contrast was then calculated from Equation 31 for varying hydration levels (H$_2$O/D$_2$O : Ca ratio) of the scattering object to determine which hydration level best simulated the trends in absolute neutron scattering intensity as a function of the proportion of D$_2$O in the solvent, and the experimentally calculated
X-ray/neutron scattering ratios. Sensitivity analysis was performed by varying values of the physical density of the ACC to determine the density at which calculated X-ray/neutron scattering ratios were unable to simulate the experimental values when the remaining model parameters were varied over a reasonable range.

A second set of simulations was also performed to test the impact of density and C/Ca ratio over a wider range of values (density from 1.1 to 1.9 g/cm$^3$ and C/Ca ratio from 1 to 5, with 5 being the ratio present in the bulk solution), corresponding to the conditions present at the lower $q$-range.

5. Results

5.1. SANS absolute scattering intensity

The absolute scattering intensity from SANS for the six samples prepared in mixtures containing different proportions of H$_2$O and D$_2$O is presented in Figure 33. SAXS results (see Chapter V) for the sample prepared with the same Ca and total carbonate concentrations at pH 8.5 are also shown for comparison. SANS curves were similar to those obtained from SAXS in the low $q$ region, which was characterised by an extensive Porod region with a slope close to -4. This Porod region extended as far as the lower $q$ limit of $4.9 \times 10^{-4}$ Å$^{-1}$, with no indication of a transition to a Guinier regime evident. The high $q$ feature present in the SAXS data was not observable in the SANS curves due to the much weaker scattering intensity of SANS and the high background scattering in this region, which was due to the use of pure H$_2$O/D$_2$O for the background solvent (compared to use of carbonate + pH buffer solution with SAXS). However, in contrast to the SAXS data, a small
hump was present in the SANS data between about 0.005 Å\(^{-1}\) and 0.02 Å\(^{-1}\) for the sample containing 25% D\(_2\)O.

**Figure 33:** Absolute neutron scattering intensity of samples possessing between 0% and 40% D\(_2\)O and containing [Ca] = 2 \times 10^{-3} M. The purple line represents X-ray scattering for the sample containing [Ca] = 2 \times 10^{-3} M as presented in Chapter V. The black line illustrates a line with slope of –4 for comparison with the low q data.

Closer analysis of the data at \(q = 0.001\) Å\(^{-1}\) and \(q = 0.01\)Å\(^{-1}\) (q values where the relative error was the minimum for all samples) indicates a non-linear evolution of the absolute SANS intensity with increasing D\(_2\)O concentration, reaching a maximum at 25% D\(_2\)O for both q values (**Figure 34**).

The ratio of the absolute SAXS intensity compared to the absolute SANS intensity, hereafter denoted as \(I_{\text{X-ray}}/I_{\text{neutron}}\), was relatively independent of the D\(_2\)O proportion in the solvent in the region between 0.03 Å\(^{-1}\) < \(q\) < 0.05 Å\(^{-1}\),
corresponding to the high $q$ structural level characterised in more detail by SAXS (see Chapter V), exhibiting a median value of 27.6 in this range. However, when considering the entire $q$ range, $I_{\text{X-ray}}/I_{\text{neutron}}$ gradually decreased with increasing $q$ from $\sim$200-700 for a few points at the lowest $q$-values to $< 1$ at the highest $q$ value. Too few points were available for samples containing 100% H$_2$O to determine an accurate average of the $I_{\text{X-ray}}/I_{\text{neutron}}$ ratio over entire the $q$ range investigated.

**Figure 34:** Absolute neutron scattering intensity at $q = 0.001 \, \text{Å}^{-1}$ and $q = 0.01 \, \text{Å}^{-1}$ with increasing D$_2$O concentration in the solvent. The scattering intensity was maximum at 25% D$_2$O.

**5.2. Estimation of hydration level**

Simulation of neutron scattering contrast and $I_{\text{X-ray}}/I_{\text{neutron}}$ assuming a physical density for calcium carbonate clusters of 1.62 g/cm$^3$ (as defined in Liu et al, 2010) and C/Ca ratio set at 1.02 mol/mol (from EXAFS results in Chapter VI), and with an optimal value of H$_2$O/D$_2$O : Ca ratio = 8.5 mol/mol, is presented in Figure 35.
While the simulation matched the experimental data reasonably well, the sharp rise in neutron scattering intensity with 25% D₂O in the solvent as presented in Figure 34 was not reproduced in this simulation. However, such sharp rises in scattering intensity are not typical of contrast matching experiments, as further addressed in the discussion.

Theoretical values calculated for the SANS contrast between the solvent and ACC and $I_{\text{X-ray}}/I_{\text{neutron}}$ that best simulated the experimental data as a function of the H₂O/D₂O ratio in the solvent, H₂O/D₂O : Ca molar ratio in the scattering objects, and physical density of the scattering objects are presented in Table 10 and Table 11. As expected, a decrease in physical density for a constant C/Ca ratio corresponded to an increase in the hydration level of the ACC.
Figure 35: Simulated values of neutron scattering contrast ($\Delta \rho$)² (black circles) and ratio of X-ray/neutron absolute scattering intensity (red squares) as a function of the proportion of D₂O in the solvent. The experimentally determined ratio of X-ray scattering intensity / neutron scattering intensity between $0.03 \text{ Å}^{-1} < q < 0.05 \text{ Å}^{-1}$ is also shown (black triangles). The physical density of the calcium carbonate clusters was set at 1.62 g/cm³, the $\text{HCO}_3^-$/TDIC ratio was set at 0.98, the C/Ca ratio was set at 1.02 mol/mol, and the $\text{H}_2\text{O}/\text{D}_2\text{O}$: Ca ratio was optimised to produce the best fit of the simulation to the experimental data, yielding a value of 8.5 mol/mol.
Table 10: Variation in the ratio of X-ray/neutron absolute scattering intensity with varying physical density of the ACC. The HCO$_3$/TDIC ratio was set at 0.98, the C/Ca ratio was set at 1.02 mol/mol, and the H$_2$O/D$_2$O: Ca ratio was optimised to produce the best fit of the simulation to the experimental data for each value of ACC physical density. Values of the ratio of X-ray/neutron absolute scattering intensity represent the average over the range 0-40% D$_2$O in the solvent. The X-ray contrast value was calculated in 100% water solvent.

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<tr>
<th></th>
<th>Reference value</th>
<th>Sensitivity analysis</th>
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<td><strong>C/Ca ratio (mol/mol)</strong></td>
<td>1.02</td>
<td>1.02 1.02 1.02</td>
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<tr>
<td>Physical density of ACC</td>
<td>1.62 1.7 1.5 1.4</td>
<td></td>
</tr>
<tr>
<td>(g/cm$^3$)</td>
<td></td>
<td></td>
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<tr>
<td><strong>HCO$_3$/TDIC (mol/mol)</strong></td>
<td>0.98 0.98 0.98 0.98</td>
<td></td>
</tr>
<tr>
<td>Simulated H$_2$O/D$_2$O : Ca molar ratio (mol/mol)</td>
<td>8.5 7.4 10.7 13.8</td>
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<tr>
<td>Average X-ray/neutron ratio for 0% &lt; %D$_2$O &lt; 40%</td>
<td>27.4 27.1 27.9 28.9</td>
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Table 11: Simulated neutron scattering contrast ($\Delta p)^2(\text{Å}^{-4})$ calculated under an extended range of conditions.

<table>
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<th>Increasing Density →</th>
<th>C/Ca ratio (mol/mol)</th>
<th>HCO$_3$/TDIC (mol/mol)</th>
<th>Simulated H$_2$O/D$_2$O : Ca molar ratio (mol/mol)</th>
<th>Physical density of ACC (g/cm$^3$)</th>
<th>Average X-ray/neutron ratio for 0%&lt;%D$_2$O&lt;40%</th>
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</tr>
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6. Discussion

6.1. Evaluation of experimental approach

Results published after data collection seem to suggest that solvent entropy is the key driver of ion association. As the structuring of H\textsubscript{2}O and D\textsubscript{2}O is distinct, it is most likely that the release of water upon ion binding will occur at different rates. While it is now almost certain that use of deuterated vs hydrogenated solvent will cause differences in PNC formation, the exact nature of these differences is still unknown.

While SANS data were successfully collected for a range of D\textsubscript{2}O/H\textsubscript{2}O ratios, analysis and interpretation of the data suffered from several problems. First, the integration time of 40 min poses problems in distinguishing PNCs from other forms of calcium carbonate, potentially including crystalline phases. Therefore, the scattering curves presented here do not represent scattering from a static system, but the time-averaged neutron scattering intensity resulting from any clusters or particles forming and/or dissolving during the 40 min of data acquisition. The large error bars associated with most of the samples, except for 25% D\textsubscript{2}O, may thus reflect the non-equilibrium aspect of the system and the evolution of hydrated structures during data acquisition.

Despite the differences in acquisition time between the SANS and SAXS experiments (40 min for SANS vs < 10 ms for SAXS), both scattering curves present similarities including the presence of a strong interface at low $q$, which was attributed to possible liquid-liquid like phase separation in the SAXS study (Chapter
V), suggesting a potential likeness in the processes occurring under each set of experimental conditions. This is, however, hard to justify when the level of saturation is taken into account. At this level of saturation \(\Omega_{\text{ACCII}} = 7.0\) and \(\Omega_{\text{ACCI}} = 8.5\), formation of gel-like or hydrated ACC inside and outside the analytical window is most likely and could potentially generate similar scattering patterns, making the differentiation between PNCs and more developed ACC phases difficult, if not impossible (Liu et al, 2008; Gebauer et al, 2008; Liu et al, 2010). Other approaches were attempted during beamtime to address this issue (i.e. summing results from scans with much shorter integration times by considering the neutrons’ time-of-flight, and on-line reagent mixing using stopped-flow equipment in front of the neutron beam), but did not prove useful.

6.2. Interpretation of neutron scattering curves

Despite the issues identified above, it remains that scattering intensity for the sample containing 25% D\(_2\)O seems fairly constant with small error bars. Studies like that conducted by Pouget et al (2009) have been able to identify PNCs in both undersaturated and oversaturated conditions. If PNCs are not directly involved in the nucleation process as suggested by Zahn et al (2015), it should theoretically be possible to distinguish them from secondary ACC phases forming. In this case, the hump in scattering intensity at high \(q\)-values (around 0.01 Å\(^{-1}\)) could be associated with PNCs persisting in oversaturated conditions.

Due to their hydrated character, it is expected that PNCs should have a scattering length density closer to that of the solvent than that of crystalline phases (Raiteri & Gale 2010; Demichelis et al, 2011; Wallace et al, 2013). Even if the
evolution of the scattering intensity seems quite abrupt for the sample containing 25% D$_2$O, such an evolution could be caused by reaching a critical H$_2$O/D$_2$O ratio corresponding to the contrast matching point of the hydrated layers. Under this special circumstance, the neutron scattering length density of the particles will become measurably different from that of the solvent ($\Delta\rho_{80\% \text{H}_2\text{O}} \ll \Delta\rho_{75\% \text{H}_2\text{O}}$), resulting in a substantial increase in scattering intensity. Again, the presence of scattering objects possessing multiple hydrated domains, each of them responding differently to changes in D$_2$O/H$_2$O, could potentially explain this phenomenon.

Alternatively, this abrupt change could result from an error of background removal. However, the errors in the measured scattering intensity for this sample were the smallest of all the conditions and the high scattering intensity is consistent over the entire $q$ range investigated, strongly arguing against this possibility. This hypothesis was further tested by subtracting 25% of the scattering intensity of pure H$_2$O standard and comparing the scattering curves. Both background removal protocols yielded very similar results, suggesting that such an error could not result in such a substantial change in scattering intensity.

The similarity in scattering intensity profiles at 0.001 and 0.01Å$^{-1}$ over all samples suggests a possible link between the two regions and the processes yielding their formation. Based on SAXS data demonstrating the development of hydrated layers around nanoparticles formed under similar pH and reactant concentrations (Chapter V), aggregation of ion-dense nanodroplets containing PNCs could generate scattering patterns similar to liquid-liquid like phase separation (Gower et al, 2008; Wolf et al, 2011; Demichelis et al, 2011; Wallace et al, 2013). Changing the
H$_2$O/D$_2$O ratio of the solvent could therefore potentially emphasise the influence of the physical density of the nanodroplets containing PNCs while suppressing the influence of the physical density of the solvent, thus resulting in a scattering pattern close to that recorded.

### 6.3. Estimation of the hydration state of scattering objects

Although the general trends in scattering intensity with varying H$_2$O/D$_2$O ratio were successfully reproduced and scattering objects possessing similar hydrated phases to those described in the literature were simulated (Raiteri & Gale, 2010), these calculations failed to describe the abrupt change in contrast observed for the sample prepared in 25% D$_2$O.

Simulations for scattering objects possessing physical density values similar to those reported in the literature for ACC (Liu et al, 2010) yielded a calculated H$_2$O/D$_2$O : Ca molar ratio of 8.5 mol/mol, emphasising that the scattering structures possess a relatively high hydration level. By assuming PNCs have a composition similar to other crystalline calcium carbonate forms, it is possible to compare this ratio to values given in the literature. In this case, the ratio evolves from 1.46 to 2.76 as physical density decreases from 1.7 to 1.4 g/cm$^3$. These ratios are much higher than those reported by Michel et al (2008) of 1.19 to 1.38 water molecules per formula unit, or by Raiteri & Gale (2010) where 1.4 to ~2 water molecules per formula unit were found for ACC.

Assuming that the H$_2$O : Ca content provides a rough indication of the size of ACC particles, as expressed by Raiteri & Gale (2010), the scattering objects simulated should have a size ~ 20 to 25 Å, which is consistent with SAXS results in
Chapter V. The finding that a decrease in physical density for simulated calcium carbonate clusters must correspond to a significant increase in hydration level is coherent with knowledge of the initial processes involved in ACC formation, in which an increase in the hydration level of the first mineral phases was quantified (Michel et al, 2008; Raiteri & Gale 2010).

The simulated ratio of X-ray/neutron scattering intensity for a total C : Ca molar ratio of 1.02 as given by the EXAFS analysis, is slightly higher than the experimental values. This could possibly be driven by the relatively low C/Ca ratio estimated via EXAFS, which was not corrected for multiple scattering. Higher values for this ratio, associated with a higher level of solvation, generate X-ray/neutron scattering intensity ratios closer to the experimental observations.

Extended results from simulated neutron contrast of a theoretical ACC particle show that similar $I_{\text{X-ray}}/I_{\text{neutron}}$ ratios can also be generated by other combinations of input parameters over realistic conditions relevant to ACC and, to a further extent, an ionic liquid phase. However, the experimental SANS trends can only occur if the simulated scattering objects contain a substantial amount of water. Therefore, these findings provide experimental support for what is known about the composition of PNCs and early stage forms of ACC from simulation (Raiteri & Gale, 2010; Demichelis et al, 2011; Wallace et al, 2013).
7. Conclusion

SANS analysis proved to be more complex than SAXS due to weak scattering intensity and the resulting requirement for long integration times. Although precise characterisation of the diffuse interface identified by SAXS was not possible using SANS, the presence of hydrated nanoparticles containing around 25% water as well as a strong interface possibly associated with a liquid-liquid like phase separation were identified. Comparison of SAXS and SANS measurements coupled with a simulation of the scattering contrast expected to result from hydrated calcium carbonate nanoparticles also seems to confirm the presence of highly hydrated compounds, either being PNCs or early stage forms of ACC.

Although it was not possible to either confirm or refute the hypothesis of liquid-liquid like phase separation during the nucleation of calcium carbonate, the samples studied appeared to possess a similar hydration level as those predicted by computer simulations of non-classical nucleation of calcium carbonate (Raiteri & Gale, 2010). To rigorously test this hypothesis, it is essential to improve the sample environment and adapt the micro-mixing device for use with SANS in order to perform this experiment using a continuous flow approach. With such apparatus operational, a more detailed examination of the early stages of calcium carbonate nucleation via contrast matching and analysis of partial scattering should be achievable (Endo et al, 2003). Working with newer neutron sources (e.g. spallation sources), which provide much greater neutron fluxes and more scattering events per time unit, could also give a better definition of the processes occurring during the early stages of calcium carbonate nucleation.
Chapter VIII

Summary and concluding remarks
1. Characteristics of calcium carbonate PNCs near neutral pH

1.1. Impact of solubility limit

This thesis focused on better understanding the formation of PNCs and their implications to subsequent processes during the early stages of calcium carbonate mineral formation. In it, I have presented a novel way to trigger homogeneous nucleation using a micro-mixing device and to acquire high-resolution data at short timescales required to observe nanoparticle formation during the early stages of nucleation. By coupling this device with synchrotron-based techniques, it has been possible to obtain new information regarding the short range order of PNCs (Chapter VI) and their long range order (Chapter V). In particular, I examined the impact of pH and saturation state on the physical and chemical characteristics of these nanoclusters and the calcium coordination environment within them (Chapter IV and Chapter VII). I have also presented an attempt to characterise the hydration state of PNCs, including explicitly examining the possibility of liquid-liquid like phase separation, using X-ray and neutron scattering (Chapter V and Chapter VII).

Small Angle X-ray Scattering (SAXS) and X-ray Absorption Fine Structure (XAFS) analysis realised on very short timescales allowed the definition of PNCs over a range of pH and ion concentrations comparable to conditions in many natural surface water environments, such as seawater. These experiments distinguished hydrated nanoparticles exhibiting pH-dependent behaviour and possessing planar-like shapes, low calcium coordination number and similar sizes to what was previously described in the literature for PNCs. The results are consistent with non-
classical nucleation theory, which predicts the pH-dependent formation of nanoscale objects by the aggregation of hydrated calcium carbonate cluster with various shapes (Gebauer et al, 2008; Pouget et al, 2009; Raiteri & Gale 2010; Chao et al, 2014; Wallace et al, 2013; Gebauer et al, 2014). Additionally, the results suggest that PNCs might possess a well-defined short range order. EXAFS analysis revealed that the first coordination shell in PNCs possesses similar bond distances and coordination numbers as calcite. As has already been shown for ACC, the establishment of short range order similar to that in crystalline calcium carbonate phases appears to be mainly controlled by pH. However, it remains unclear why such a well-defined short range order can be identifiable in the early stage of ACC formation and at what stage of the nucleation process this is being developed.

Hypothetical hydrated chain-like structures similar to those defined via computer simulation, and experimentally observed in this work using SAXS, could be present in ACC. A second hypothesis developed by Rez et al (2014) suggested that the presence of randomly oriented nanocrystals in the nanometer size range separated by water molecules could generate coherent diffraction profiles similar to those of ACC.

Such results have been observed for ACC and in silicate and silicate-stabilised calcium carbonate systems (Kellermeier et al, 2012). In the latter, aggregates of hydrated PNCs possessing a growing hydrated interface were stabilised into nanodroplets possessing similar features to those observed through SAXS in this study (Kellermeier et al, 2012). It is most likely that the calcium carbonate system examined in this study possess the same features, however these
are difficult to characterise precisely due to a low contrast in physical density and/or X-ray scattering length density with the solvent.

Even though the SANS results are inconclusive due to the limitations of the experiment performed, the SAXS results demonstrate that formation of PNCs is closely linked to the appearance of hydrated structures. The aggregation of these structures, and partial dehydration of their core, was seen respectively in the SAXS and XAFS data. While the presence of features consistent with liquid-liquid like phase separation were observed, it remains unclear if spinodal or binodal demixing will generate local supersaturation and trigger the formation of PNCs or if the coalescence of hydrated ion-rich droplets could generate patterns consistent with liquid-liquid like phase separation.

1.2. Impact of pH

In the early stage of calcium carbonate nucleation, pH is expected to directly impact the kinetics of the reactions involved. It is generally accepted that PNCs form by the random collision of calcium ions with carbonate and bicarbonate ions. However, due to lower binding strength with calcium ions, bicarbonate ions will limit the growth of the cluster by acting as a chain terminator. As pH is the main parameter controlling the relative concentrations of carbonate and bicarbonate, it will thus directly affect the kinetics of PNC formation and growth, with higher pH favouring creation of larger clusters due to the greater proportion of carbonate relative to bicarbonate.

The different experimental results presented in this thesis appear consistent with this hypothesis. SAXS analysis shows that at higher pH the nanoparticles
present characteristics different to those expected for PNCs, and closer to those expected for ACC with the formation of a hydrated layer, a lower number of particles per volume unit and higher mean particle volume. The relationships observed between particle volume and zero $q$ scattering intensity under these conditions were not consistent with either of the conventional particle growth processes considered (monomer addition and particle-particle addition), suggesting the occurrence of more complex aggregation processes (e.g. several aggregation processes occurring at the same time).

Analysis of short range order via EXAFS further confirmed that pH will have an impact on the kinetics of the reaction, given that dehydration in the first shell of calcium appears to occur to a greater extent at higher pH.

2. PNCs and nucleation processes

As raised by Pouget et al (2009), Nudelman et al (2010) and Sear (2012), PNCs are expected to be present both before and after nucleation. In order to participate in the formation of crystalline phases, PNCs must overcome two barriers to nucleation: a first barrier between the solvent and the formation of the PNCs (or larger particles possessing the same physical and chemicals characteristic), and a second more significant barrier corresponding to an internal structural rearrangement leading to formation of the macroscopic structure.

As explained by Zahn (2015), this second cluster-nucleus barrier could be, under certain conditions and depending on the size distribution of clusters, larger than the barrier to nucleation from bulk solution (i.e. the kinetic barrier to form an ordered nucleus from ions in solution is much less than the one to order and
desolvate the cluster). However, kinetic stabilisation to such a large extent seems unlikely, and it is plausible that thermodynamics should play an important role. Similar results have been obtained via computer simulation by Andersson et al (2016) where calcite growth at a high level of supersaturation was observed to follow classical nucleation theory, driven by the attachment of paired ions and polynuclear complexes. Once again, at this level of saturation, such processes are expected as the kinetic barrier would be much lower than the thermodynamic one.

With these prerequisites, nucleation will occur within the bulk ionic solution without affecting the previously formed prenucleated clusters via processes described in the classical nucleation theory (Zahn, 2015). PNCs would then persist thanks to enhanced stability resulting from their hydrated characteristics until later stages of crystal growth. Such results have been presented by Nielsen et al (2014), where both classical and non-classical nucleation processes were identified during the early stage of ACC formation, over a similar pH range and at similar saturation levels to the conditions explored in this thesis. Pre-existing clusters might still be outperformed thermodynamically once the nuclei reach the mature stage of crystal growth (Zahn, 2015). Thus, PNCs can either collide and merge with a forming nucleus or dissociate into solution to compensate for the solvent depletion in ions’ concentration arising from crystal precipitation (Zahn, 2015).

Both pathways lead to a fundamentally different understanding of the behavior of PNCs: as described by De Yoreo (2013), Hu et al (2014) and Anderson et al (2016), either they are formed by ion-by-ion addition process, playing no direct role in the nucleation process at low concentration and should be considered as a
buffer to the concentration of ions in solution rather than precursors to crystal nucleation; or they are candidate building blocks to crystal growth but not nucleation (Zahn, 2015). The results from this thesis indicate that the calcium concentration doesn't seem to have a direct impact on the concentration of PNCs at pH 7.5, but they increase in size and volume with increasing calcium concentration. These observations, combined with the concordance of aggregation processes with the NCNT, the possible presence of liquid-liquid interface, and their presence in undersaturated and supersaturated conditions, suggest that the first pathway may be more likely. If so, the concentration of PNCs would be expected to decrease with time under supersaturated conditions. Such substantial changes in concentration were unable to be observed in this work due to the examination of short times only and under relatively low levels of supersaturation, but could certainly be explored by extending this work using similar techniques.

Based on the results obtained in this thesis, the model presented by Gebauer et al (2013) for calcium carbonate mineral formation via the PNC pathway can be validated and extended (Figure 36). However, the fundamental question remains as to whether PNCs are formed by liquid-liquid like phase separation or whether the aggregation of hydrated nanoclusters creates spinodal/binodal demixing.
Figure 36: Schematic representation of calcium carbonate mineral formation process, with the prenucleation cluster pathway represented in the shaded box. Despite the advances made in this thesis, many of the processes involved remain poorly understood. However, the work reported in this thesis has helped to better define the prenucleation cluster pathway as well as the definition of transition phases toward polycrystalline phase.
3. Outlook

Despite advances made in recent work, including this thesis, the link between classical and non-classical nucleation theory and the exact role of PNCs in the nucleation process remains a major conundrum. Additional information to help resolve remaining knowledge gaps could be obtained by the use of similar apparatus to that employed in this work by reducing the mixing time, thus allowing better definition of the early stages of nucleation. Additionally, comparison of similar saturation states at several pH values could allow better definition of the processes leading to formation of ACC polymorphs with various short range orders.

Reducing the kinetics of reactions by decreasing the calcium concentration could also provide new insights into the very early stage of nucleation and give a better definition of the (potential) relationship between liquid-liquid like phase separation and formation of the first PNCs. These results would need to be coupled with computer simulations such as those performed by Wallace et al (2013) or Andersson et al (2016) at a low saturation state and over a wider pH range.
4. General conclusion

The concept of the solubility limit ($\Omega$) is based on classical thermodynamics and defined as the concentration of ions in solution beyond which a solid phase is spontaneously formed. However, this definition does not consider the formation of PNCs, which modify the activity of the ions in solution (Kellermeier et al, 2014) and may be stabilised by thermodynamic and kinetic phenomena. Moreover, PNC formation involves not just one equilibrium process, but a sequence of consecutive equilibria. Because of this combination of stabilising parameters and the intrinsic nature of PNCs in forming under conditions that would thermodynamically be impossible, we can consider that PNCs are acting as calcium carbonate mineral nanoparticles.

Classical nucleation theory neglects the role of kinetic barriers to particle or cluster formation, instead expressing the nucleation rate in terms of overcoming a thermodynamic barrier. Formation of PNCs contradicts this assumption and instead highlights the importance of kinetic processes at low ion concentrations (Figure 37). If we want to understand the importance of these mineral nanoparticles, and the way they will form and react with their environment, the fundamental concept of solubility limit can no longer be seen as an abrupt and absolute limit, but must instead be considered as a conceptual boundary defined by mechanistic and kinetic processes, including those developed through classical nucleation theory almost 150 years ago, but also accounting for new concepts such as the diversity of nucleation pathways, the importance of precursor phases and the behaviour of PNCs. The notion of $\Omega'$ as per Figure 37 is therefore difficult to define accurately due to the
presence of a range of different species at low ionic concentrations, such as the early stages of PNCs, ion pairs and/or free ions in solution.

**Figure 37:** Schematic relationship between PNCs and the concept of the solubility limit as per classical nucleation theory. The concept of the solubility limit is based on thermodynamics and does not consider kinetic processes. The solubility limit \( \Omega \) is defined based on the concentration of ions in solution over which a mineral phase spontaneously forms. While the thermodynamic barrier \(-\Delta G_{ex}/k_BT\) mainly controls the nucleation rate at \( \Omega > 0 \), the kinetic barrier \(-E_A/k_BT\) plays an important role in stabilising prenucleation clusters at \( \Omega < 0 \).

It is evident that experimental measurements and computer simulations are complementary methods when it comes to investigating mineral formation reactions and the evolution of such systems. In the present study, it was shown that, despite a substantial difference between the timescales accessible through each method (nanoseconds for computer simulation vs milliseconds for experimental measurements), results were transferable and consistent with one another. Moreover, this suggests that over the range of saturation states investigated in this thesis, while ionic ordering associated with liquid-liquid like phase separation linked to the formation of PNCs occurs on the nanosecond scale, these artefacts of
nucleation persist without formation of more ordered phases (e.g. ACC) on the millisecond time scale accessible in this thesis.

It is hoped that the experimental tools developed during this study will open the door to further work focusing on characterisation of the very early stages of homogenous nucleation processes and their kinetics in various aquatic systems and under various experimental conditions.
References


Appendix I

Calculation of the solubility limit for calcium carbonate mineral phases
The solubility limit for a substance in aqueous solution is defined as the threshold at which additional solute will no longer dissolve at a given temperature and salinity as a fundamental threshold. The saturation state, $\Omega$, which is a function of the product of activities of the relevant solute ions, reflects the thermodynamic favourability of mineral formation (mineral formation is favoured when $\Omega > 0$, whereas mineral dissolution is favoured when $\Omega < 0$. As one of the aims of this thesis is to investigate the influence of saturation state on the early stage of calcium carbonate nucleation, accurate calculation of the solubility limit for potential calcium carbonate mineral phases is essential.

Saturation state values for various mineral phases ($\Omega_{\text{mineral}}$) have been calculated as the following:

$$\Omega_{\text{mineral}} = \ln \left( \frac{[\text{Ca}^{2+}][\text{CO}_3^{2-}]}{K_{\text{sp(mineral)}}} \right)$$

*Equation 32*

where $[\text{Ca}^{2+}]$ and $[\text{CO}_3^{2-}]$ represent the concentrations of calcium and carbonate ions, respectively, and $K_{\text{sp(mineral)}}$ is the solubility product for that particular mineral phase.

$K_{\text{sp}}$ values for calcite ($K_{\text{sp(Cal)}}$), aragonite ($K_{\text{sp(Ara)}}$) and vaterite ($K_{\text{sp(Vat)}}$) were calculated (see Table 12) using the equations given by Mucci *et al* (1983) for calcite and aragonite and Plummer and Buseberg (1981) for vaterite below:

$$\log K_{\text{sp(Cal)}} = -171.9065 - 0.077993T + \frac{2839.319}{T} + 71.595 \log T$$
$$+ \left(-0.77712 + 0.0028426T + \frac{178.34}{T}\right)S + 0.0041249S^{1.5}$$

*Equation 33*
\[
\log K_{sp}^{*}(\text{Ara}) = -171.945 - 0.077993 T + \frac{2903.293}{T} + 17.595 \log T \\
+ \left( -0.068393 + 0.0017276 T + \frac{88.135}{T} \right) S^2 - 0.10018 S \\
+ 0.0059415 S^{1.5}
\]

*Equation 34*

\[
\log K_{sp}^{*}(\text{Vat}) = -172.1295 - 0.077993 T + \frac{3074.688}{T} + 17.595 \log T
\]

*Equation 35*

where \( T \) is the temperature and \( S \) the salinity; values of \( T = 294.15 \) K and \( S = 0 \) were used for the calculations in this work.

\( \Omega \) values for two amorphous calcium carbonate phases (\( \Omega_{\text{ACC-I}} \) and \( \Omega_{\text{ACC-II}} \)) identified by Gebauer et al (2008) have also been calculated using the \( K_{sp} \) values given by Gebauer et al (2008) (see Table 12).

The concentration of carbonate ions under the experimental conditions used in this work was calculated using “CO₂Sys” software from Lewis & Wallace (2006) to be 12.62 µM and 133.04 µM at pH 7.5 and 8.5 with 10 mM total carbonate.

**Table 12: \( K_{sp} \) values for calcite (Mucci et al, 1983), aragonite (Mucci et al, 1983), vaterite (Plummer and Buseberg 1981), and amorphous calcium carbonate (ACC) I and II (Gebauer et al, 2008) at \( T = 294.15 \) K and \( S = 0 \).**

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