

Supporting Information

Rapid-Screening of Calcium Carbonate Precipitation in the Presence of Amino Acids: Kinetics, Structure and Composition

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1. Experimental Methods

1.1 Materials

Calcium chloride dihydrate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), anhydrous sodium carbonate, isobutyryl-L-cysteine, *o*-phthalaldehyde, calcein disodium salt, sodium hydroxide, boric acid, potassium hydroxide, L-aspartic acid sodium salt monohydrate, L-glutamic acid sodium salt monohydrate, L-asparagine, L-valine, sodium acetate, ethanol were used as purchased from Sigma Aldrich, UK. Hydrochloric acid (12.1 M), 10-15% sodium hypochlorite, and glacial acetic acid were used as purchased from Fisher, UK. DI water was obtained using a Millipore Milli-Q system.

1.2 Equipment

1.2.1 Well Plates: Vast arrays of crystallisation experiments were performed in 96-well plates in order to miniaturize crystallisation experiments and simultaneously generate large arrays of experiments of different compositions from identical stock solutions. All 96 wells were indexed individually in columns 1-12 and rows A-H (thus 8x12 array of experiments), giving each well a unique identifier allowing for easy referencing. Depending on the requirements of the characterisation methods for each experiment, one of the two following well plates was employed.

For Calcium Carbonate Yield and Quantification of Amino Acid Incorporation. Millipore Solvinert™ 96-well plates with 0.45 µm pore-size hydrophilic membranes (Figure S14) were employed as they can be used to wash and filter the crystal products. They are also compatible with well plate covers, where these were used to reduce evaporation from filled wells and to limit exchange of gas between the solution and air. Further sealing between the well plate cover and well plate was achieved with Parafilm. Solution were filtered through these well plates using a vacuum manifold (comprising a

base plate with adapter for attachment to vacuum pump, a filtrate collection dish to protect vacuum pump and rubberised top plate with a large enough gap to allow removal of liquid from every well and create an airtight seal during filtration (Figure S15)) and vacuum pump at 600 mbar.

For Calcium Carbonate Yield, Raman, SEM, Kinetic and pXRD studies. Greiner μ Clear™ Black 96-well plates (Figure S16) were employed as they have a clear base and are compatible with Corning sealing mats and well plate covers. All of the above characterisation methods could be carried out with these plates, as described in detail below. Calcium concentration determination required the use of two separate well plates. Corning sealing mats (Figure S16) provided both a water-tight seal for each well and a suitable substrate for supporting the precipitation of CaCO_3 when the sealed well plate was inverted; the reacting liquid was therefore in contact with the underside of the sealing mat instead of the well plate bottom. The sealing mats were then easily removed from the well-plate and were gently rinsed and dried with ethanol. Each stud on the sealing mat was corresponded directly to a well on the well plate with known reaction conditions, allowing for easy sample indexing. Precipitation on the sealing mats rather than the well plates was essential for Raman and SEM as both require short working distances. Where it was necessary to shake a well plate, this was carried out using an Eppendorf ThermoMixer C with plate module for 2 min.

1.2.2 Liquid Handling Pipetting Workstation: In order to charge well plates with accurately measured volumes of stock solutions, a Hamilton™ Robotics Microlab^R STAR liquid handling pipetting workstation was used, controlled by Hamilton™ Venus One SP1 software. Known volumes of stock solutions were introduced into specific wells, where this information was provided in the form of a worklist (an array of numbers referring to volumes required in μL of each reagent, listed in columns, and the destination well for each aliquot, listed in rows). Experiment and worklist design took into account the use of different pipette tip sizes (50 μL and 300 μL , Hamilton™ CO-RE conductive non-filtered) and associated accuracy; very small volumes could be accurately transferred using the 50 μL tips while larger volumes could be transferred rapidly with 300 μL tips. Stock solutions were made available to the robot as either bespoke 8 mL Wheaton E-Z EX-Traction, clear, 15-425 barcoded vials capped with PTFE lined screw caps (Wheaton™) or in 120 mL PTFE troughs (Hamilton™). These were targeted by 50 μL and 300 μL tips respectively.

Typically, aqueous solutions of CaCl_2 (200 mM) and amino acid stock solutions (30 and 60 mM) were added from 7 mL vials, while DI water and aqueous Na_2CO_3 solutions (50 mM) were added from 120 mL troughs. 4 identical vials of 200 mL CaCl_2 solution were made available to the workstation in order to increase the efficiency of sample preparation without compromising precision. Amino acids

were made available in 2 different concentrations in order to increase efficiency, but also to reduce the error when very low concentrations of amino acid were required (e.g. $[\text{Ca}^{2+}] = [\text{CO}_3^{2-}] = 3.5 \text{ mM}$, $[\text{amino acid}]/[\text{Ca}^{2+}] = 0.3$) by increasing the volume of amino acid solution required (i.e. taken from the lower concentration solution instead of higher). 50 mM aqueous Na_2CO_3 solution and DI water were added from troughs as larger volumes of these solutions were generally required and, in the case of Raman and SEM sample preparation, to minimise time between reaction initiation and well plate sealing.

1.2.3 Well Plate Reader: Amino acid quantification, calcium concentration determination and kinetic studies were all conducted using a PerkinElmer EnVision 2103 equipped with a liquid dispenser module. Excitation/emission filters and mirrors were selected as appropriate for the responsive fluorescent species under examination (260nm excitation filter, 405nm emission filter and a dichroic mirror with a cut-off wavelength of 315nm for amino acid quantification; 340nm narrow excitation filter, 535 nm emission filter and a dichroic mirror with a cut-off wavelength of 505nm for calcium concentration determination). The measurement height, excitation light intensity and detector gain were optimised before each run, and data acquisition was repeated 8 times to qualify reliability. For turbidimetric kinetic studies, a single 565nm emission filter was used. The associated dispensing module allowed for simultaneous reaction initiation and data acquisition, and the ability to shake the well plate ensured homogeneity in each well. The plates were shaken for 1 min at 600 rpm in a linear configuration after reaction initiation. All kinetic runs were then carried out over 90 min and transmitted intensity readings were recorded at 1 min intervals. Well plate reader control and data acquisition was carried out using PerkinElmer EnVision Manager software.

1.2.4 Scanning Electron Microscopy (SEM): Samples for SEM were prepared in inverted Greiner μ Clear Black 96-well plates capped with Corning sealing mats. Sealing mats with crystals grown on the top of each stud were cut with scissors into 6 x 8 arrays and then attached to a custom-made aluminium sample holder using copper tape (Figure S15). The design of the sample holder was driven by the required compatibility with an FEI Nova Nanoscope 450 FEG-SEM, operated at 2-3 kV in secondary electron mode with ETD detector at a working distance of 5 mm. All samples were coated with 2 nm Pt/Pd, deposited by sputter coating. Microscope control and data acquisition was conducted using software from FEI. Automated screening imaging was facilitated by MAPS software, where low magnification images (HFW = 847 μm) were obtained. Manual navigation of the sample stage for higher magnification (HFW = 254 μm) and high contrast images for analysis (HFW = 847

μm , contrast made such that background was black vs white sample) was facilitated by a NavCam accessory, allowing precise stage movement between samples/studs.

1.2.5 Image Analysis: Circularity data was obtained from high contrast SEM micrographs using the following procedure. Loading image file (tif), convert to 8-bit greyscale, crop to remove scale bar, set threshold to remove background, 'Binary > Close' to form solid structures, 'Analysis Particles', size = 100-infinity; circularity = 0.3-1.0, show Outlines (Figure S16).

1.2.6 Raman Microscopy: Samples for Raman microscopy were prepared in inverted Greiner μClear Black 96-well plates capped with Corning sealing mats. Sealing mats with crystals precipitated on each stud were cut with scissors into 6 x 8 arrays, and were adhered to a standard glass microscope slide with carbon sticky tabs (Agar). This provided compatibility with the microscope's sample stage, allowing efficient sample movement and data acquisition. The crystals were analysed using a Renishaw inVia Raman Microscope (785 nm laser) with a 50x objective using MS20 encoded sample stage control through rollerball XYZ peripheral. Data acquisition was undertaken with Renishaw WiRE 3.4 with a laser intensity of 0.1% under 3 accumulated acquisitions (3 x scan time 30 s) between 1151 to 1051 cm^{-1} . 3 individual crystals were analysed per experiment/stud. The peak centre and full width at half maximum (FWHM) was determined from mathematically calculated peak fits in OriginPro 9.

1.2.7 Powder X-ray Diffraction (pXRD): Samples for pXRD were prepared in μClear Black 96-well plates. Diffraction patterns were obtained directly from each well using PANalytical X'Pert Pro MPD in transmission mode with HTS stage, Cu $\text{K}\alpha$ X-ray source and PIXcel PSD detector. Data was acquired between 27 and 37° 2 θ over 30 min per well. Slow scan rates were required to achieve high signal-to-noise ratios from the low density of crystals in each well. This range was selected as it features significant peaks associated with calcite, aragonite and vaterite. Data was analysed in HighScore Plus, where polymorph identification was undertaken using JCPDS cards for calcite, aragonite and vaterite (86-2339, 76-0606 and 74-1867) as references.

1.3 Preparation of Solutions

1.3.1 Reagents: Aqueous solutions of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (7.3505 g in 250 mL DI water for 200 mM) and Na_2CO_3 (5.2994 g in 250 mL DI water for 200 mM) were prepared in 250 mL volumetric flasks and stored in glass bottles. 100 mM aqueous Na_2CO_3 solution for kinetic studies was prepared by adding 10 mL 200 mM aqueous Na_2CO_3 solution to 10 mL DI water, yielding 20 mL of final solution.

Aqueous solutions amino acids L-aspartic acid sodium salt monohydrate (1.0386 g in 50 mL DI water), L-glutamic acid sodium salt monohydrate (1.1228 g in 50 mL DI water), L-asparagine (0.7927 g in 50 mL DI water) and L-valine (0.7029 g in 50 mL DI water) were prepared in separate 50 mL volumetric flasks, yielding 120 mM aqueous solutions of each solution.. The pH of each solution was checked and adjusted to 7 where required with HCl solution (62.5 mL 37% concentrated HCl into 187.5 mL DI water (total 250 mL) for 3 M) or NaOH solution (29.9978 g in 250 mL DI water for 3 M). 60 mM, 30 mM and 1 mM aqueous solutions of each amino acid were prepared by dilution of stock (10 mL DI water into 10 mL stock, 15 mL DI water into 5 mL stock and 19.333 mL DI water with 667 μ L 30 mM amino acid solution yield 20 mL of 60, 30 and 1 mM aqueous solutions).

1.3.2 Buffers: An aqueous solution of ethylenediaminetetracetic acid disodium salt dihydrate (EDTA.2Na.2H₂O; 9.306 g in 250 mL DI water for 100 mM) was prepared in a 250 mL volumetric flask and stored in a glass bottle. Aqueous solutions of NaOAc (2.0509 g in 250 mL DI water for 100 mM) and AcOH (1.43 mL glacial AcOH into 248.57 mL DI water (total 250 mL) for 100 mM) were prepared in 250 mL volumetric flasks and stored in separate glass bottles. In order to obtain 250 mL acetate buffer at pH 4, 211.75 mL AcOH solution and 38.25 mL NaOAc solution were mixed in a separate glass bottle. The pH was checked and adjusted with either NaOAc or AcOH solutions as appropriate. An aqueous solution of potassium borate (pH 10.4) buffer (1 M) was prepared in a 500 mL glass beaker under constant stirring. The correct mass of boric acid (30.915 g for 500 mL 1 M solution) was added to approximately 300 mL DI water. Under constant monitoring of the pH, potassium hydroxide pellets were carefully added until all potassium hydroxide and boric acid had dissolved and the pH was 10.4. The solution was then decanted into a 500 mL volumetric flask and the volume made up to 500 mL with DI water before being stored in a clean glass bottle.

1.3.3 Derivatisation and Fluorimetric solutions: Amino acid derivitisation solution was generated by dissolving 0.1 g isobutyryl-L-cysteine (IBLC) and 0.045 g o-phthaldialdehyde (OPA) in 20 mL 1 M pH 10.4 potassium borate buffer to yield a solution with 26 mM IBLC and 17 mM OPA. This hereafter is referred to as 'IBLC/OPA derivitisation solution'. Calcium concentration determination solution was generated by dissolving 0.01 g calcein disodium salt in a mixture of 6.66 mL DI water and 13.34 mL 3 M NaOH solution to yield 20 mL 0.75 mM calcein solution in 2 M NaOH. This, hereafter, is referred to as 'calcein solution'. 2 mM aqueous CaCl₂.2H₂O solution in 100 mM acetate buffer (pH 4) was generated by dissolving 0.0058 g CaCl₂.2H₂O in 20 mL 100 mM acetate buffer (pH 4). **Note that these solutions must be prepared immediately before use, and cannot be satisfactorily stored as stock solutions.**

1.3.4 Other: Approx. 7% sodium hypochlorite solution was prepared by mixing 100 mL DI water into 100 mL 10-15% sodium hypochlorite solution in a glass beaker, and used immediately.

1.4 Preparation of Samples

Sample preparation for all characterisation methods is summarised in a schematic (Figure S1), where similarities and differences between the sample preparation methods are detailed.

1.4.1 Samples for Kinetic Studies: In these studies, the total final volume in each well was 200 μL . 180 μL of solution was added using a liquid handling pipetting workstation, which charged each well with DI water, calcium chloride and amino acids. The final 20 μL of reaction volume was added as the sodium carbonate solution, which was added using the dispensing module on the well plate reader.

Greiner μClear Black 96-well plates were charged with appropriate volumes of DI water (from 120 mL trough using 300 μL tips), followed by aqueous CaCl_2 and amino acid solutions (from 7 mL sample vials using 50 μL tips) using the liquid handling pipetting workstation. The volumes added to each well ensured that a final $[\text{Ca}] = 10 \text{ mM}$ and $[\text{amino acid}]/[\text{Ca}^{2+}] = 0, 0.3, 0.6, 0.9$ and 1.5 would be present for each amino acid (totalling 5 different conditions per amino acid), and repeated 5 times per well plate (Figure 2a). After addition of DI water and aqueous CaCl_2 and amino acid solutions, the volume of liquid in each well was 180 μL , leaving 20 μL for addition of 20 μL aqueous 100 mM Na_2CO_3 solution in order to initiate the reaction. Reactions were initiated and analysed in a PerkinElmer EnVision 2103 well plate reader. The final 20 μL of 100 mM Na_2CO_3 was added to each well using the associated dispensing module followed by shaking in linear configuration for 1 min at 600 rpm, and a kinetic run for 90 min at 1 min intervals. Because the final 20 μL was added within the plate reader, whole experimental arrays on a single well plate can be initiated and analysed simultaneously.

1.4.2 Samples for SEM and Raman Analysis: Crystallisation reactions for Raman analysis and SEM shape analysis were conducted in inverted Greiner μClear Black 96-well plates capped with Corning sealing mats. Well plates were charged with DI water (from 120 mL trough using 300 μL tips), followed by aqueous CaCl_2 and amino acid solutions (from 7 mL sample vials using 50 μL tips), and finally with aqueous Na_2CO_3 solutions (from a 120 mL trough using 300 μL tips) with the liquid handling pipetting workstation. The appropriate volumes of each reagent were added such that

conditions $[\text{Ca}^{2+}] = [\text{CO}_3^{2-}] = 3.5, 5, 7.5$ and 10 mM and $[\text{amino acid}]/[\text{Ca}^{2+}] = 0, 0.3, 0.6, 0.9$ and 1.5 were present for each amino acid (totalling 20 different conditions per amino acid) to a final volume in each well of $200 \mu\text{L}$ (Figure 2b). Immediately after addition of aqueous Na_2CO_3 solution, well plates were sealed with Corning sealing mats, inverted and shaken for 1 min to ensure that the solution was in contact with the sealing mat. Importantly, the indexed and labelled studs on the sealing mat corresponded to the wells of the same identifier on the well plate (i.e. A1 corresponds to A1, E6 to E6 etc.). Aqueous Na_2CO_3 solution was added last from 120 mL troughs to initiate the crystallisation reaction and to ensure that all experiments on the well plate were initiated at the same time. It was also necessary to rapidly initiate, seal and invert all of the reactions to ensure that crystal growth occurred on the sealing mat rather than on the well plate bottom. The well plates were left to stand for 2 days to allow completion of nucleation and growth of calcite in the presence of additives. After this time, the sealing mat was carefully peeled away from the well plate and gently rinsed with ethanol to remove water, additives and salts, and was dried in air for 1 day before analysis.

1.4.3 Samples for pXRD Analysis: Crystallisation reactions for pXRD phase identification were conducted in Greiner μClear Black 96-well plates covered with well plate covers and sealed with Parafilm. Well plates were charged with DI water (from 120 mL trough using $300 \mu\text{L}$ tips), followed by aqueous CaCl_2 and amino acid solutions (from 7 mL sample vials using $50 \mu\text{L}$ tips); and finally with aqueous Na_2CO_3 solutions (from a 120 mL trough using $300 \mu\text{L}$ tips) with the liquid handling pipetting workstation. The appropriate volumes of each reagent were added such that conditions $[\text{Ca}^{2+}] = [\text{CO}_3^{2-}] = 3.5, 5, 7.5$ and 10 mM; and $[\text{amino acid}]/[\text{Ca}^{2+}] = 0, 0.3, 0.6, 0.9$ and 1.5 were present for each amino acid (totalling 20 different conditions per amino acid) to a final volume in each well of $300 \mu\text{L}$ (Figure 2b). A larger volume was used in these experiments to increase the number density of crystals, and thus the signal-to-noise ratio of the pXRD data.

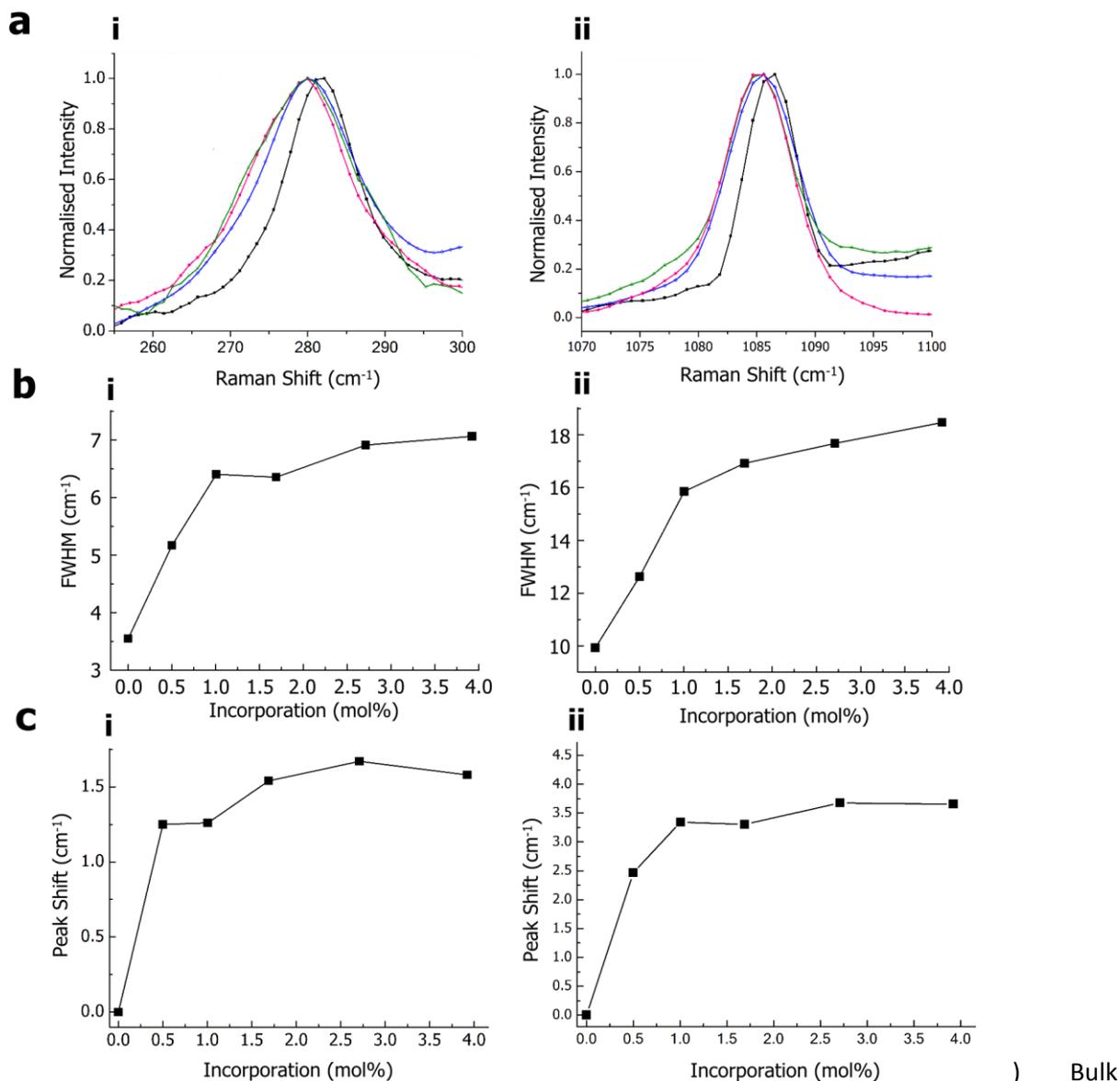
Immediately after addition of aqueous Na_2CO_3 solution, the well plates were covered with well plate covers and sealed with Parafilm, shaken for 1 min, and left to stand for 2 days. After this time, the Parafilm and well plate covers were removed, the solutions were discarded and the well plates were rinsed. The plates were allowed to dry in air for at least 1 day before analysis. Minimal loss of calcite from the well plate bottom was observed due to strong adhesion of the crystals to the polypropylene surface. It is noted that filter plates, such as Millipore SolvintTM 96-well plates, would have ensured higher yield of calcite in each well, but the bases were not sufficiently X-ray transparent to yield good diffractograms.

1.5 Quantification of Amino acid Incorporation

1.5.1 Sample Preparation: Crystallisation reactions for the quantification of amino acid incorporation were conducted in Millipore Solvinert™ 0.45 µm hydrophilic 96-well filter plates covered with well plate covers and sealed with Parafilm. For a single well plate, 2 different amino acids were investigated to allow for repeats. Thus, for assessment of all 4 amino acids (Asp, Glu, Asn and Val), 2 separate well plates were used. Well plates were charged with DI water (from 120 mL trough using 300 µL tips), followed by aqueous CaCl₂ and amino acid solutions (from 7 mL sample vials using 50 µL tips; and finally with aqueous Na₂CO₃ solutions (from a 120 mL trough using 300 µL tips) with the liquid handling pipetting workstation. The appropriate volumes of each reagent were added such that the conditions $[Ca^{2+}] = [CO_3^{2-}] = 3.5, 5, 7.5$ and 10 mM and $[amino\ acid]/[Ca^{2+}] = 0.3, 0.6, 0.9$ and 1.5 were present for each amino acid (totalling 16 different conditions per amino acid with repeat for each) to a final volume in each well of 200 µL. Columns 1, 6, 7 and 12 were left unoccupied for future calibration curve preparation steps (Figure S2). Immediately after addition of the aqueous Na₂CO₃ solution, the well plates were covered with well plate covers and sealed with Parafilm, shaken for 1 min and left to stand for 2 days.

After this time, the Parafilm and well plate covers were removed, and the well plate placed upon a vacuum manifold. The crystallisation liquor was removed through the filter in the well plate bottom by applying a vacuum (pressure in manifold 600 mbar). The emptied well plate was then placed on absorbent paper to remove excess water from its underside. Sodium hypochlorite solution (approx. 7% by volume) was carefully added to each well using a disposable plastic pipette to completely fill the wells and the plate was allowed to stand for 20 min to remove trace organic additives on the surfaces of the crystals. After this time, the sodium hypochlorite solution was removed by vacuum filtration as described previously. With the vacuum still in place, each well was then rinsed with DI water and then ethanol. The well plate was then removed from the manifold; the underside was dabbed with absorbent paper to remove excess ethanol, and was allowed to dry for 1 day in air.

1.5.2 Amino Acid Quantification: A fluorescence-based assay was used to provide accurate measurements of the extent of amino acid incorporation in calcite single crystals. (1



samples were solubilised with EDTA to release the incorporated amino acid into solution. (2) The amino acid was derivitised to yield a fluorescent reporter. (3) The fluorescence intensity of each well was then measured and contrasted against a calibration curve of known amino acid concentration to determine the number of moles of amino acid molecules in a given volume. (4) This value was then set against a determined value for the final number of moles of calcium (and therefore calcium carbonate) present in each corresponding well in a separate experiment, to provide an extent of incorporation given in mol%. The latter step is described in detail below.

Dried, clean well plates containing amino acid/calcite crystals were transferred back to the liquid handling pipetting workstation for the preparation of the calibration curve of known amino acid, and for the dissolution of amino acid/calcite composites with 100 mM EDTA solution. Well plates were charged with 100 mM aqueous EDTA solution (from a 120 mL trough using 300 μL tips into all wells),

followed by amino acid solutions (1 mM) (from 7 mL sample vials using 50 μ L tips) into the designated calibration curve columns 1, 6, 7 and 12 with the liquid handling pipetting workstation (Figure S2). The final volume in every well was 100 μ L. The calibration curves were generated from known concentrations 0, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM in rows A-H respectively. Since 2 different amino acids were analysed per plate, a calibration curve plus repeat was required for each amino acid. Therefore, a single amino acid calibration curve was allocated columns 1 and 7, where the other was allocated columns 6 and 12.

The well plate was then covered with a well plate cover and was shaken for 1 h at 600 rpm in a radial configuration to ensure dissolution of all CaCO_3 . Meanwhile, IBLC/OPA derivitisation solution in 1 M potassium borate buffer was prepared as described above. 100 μ L of IBLC/OPA derivitisation solution was added to each well using an Eppendorf Multipipette M4 (both calibration curve columns and dissolved composite solutions) to yield a final volume of 200 μ L in each well. Well plates were then covered and shaken for 5 min before analysis on a well plate reader (PerkinElmer EnVision 2103) using the procedure described above. By comparing the fluorescence intensity values from wells which contained amino acid/calcite crystals to the calibration curves, it is possible to identify to quantity of amino acids present in each well.

1.5.3 Analysis of the Yield of Calcium Carbonate: In order to determine the mol% of amino acid in the crystals it is essential to determine the yield of CaCO_3 in each well. It was not possible to do this using gravimetric methods so a calcium-sensitive fluorescent reporter dye, calcein in 2 M aqueous NaOH (calcein solution), was used. The fluorescence intensity of calcein is heavily quenched at such high pH values. However, upon chelation with calcium, there is a recovery of fluorescence intensity yielding a reliable, linear relationship between calcium concentration and fluorescence intensity between approximately $[\text{Ca}^{2+}] = 0.2$ and 1.2 mM.

Samples for determination of the CaCO_3 yield were prepared using the procedure described in Section 1.5.2, with the exception of the dissolution and analysis steps, which were carried out as follows. To all columns except 1, 6, 7 and 12, 200 μ L 100 mM acetate buffer (pH 4) was added using an Eppendorf Multipipette M4 (acetate buffer was used here instead of EDTA because of the preferential chelation between EDTA and calcium interfering with calcein). Well plates were then covered with well plate covers and shaken for 1 h at 600 rpm in a radial configuration to completely dissolve all CaCO_3 . Meanwhile, calcein solution was prepared as described above. 100 mM acetate buffer (pH 4) was then pipetted into a separate, new Greiner μ Clear Black 96-well plate using an

Eppendorf Research plus 8 channel autopipette. The volumes pipetted were as follows: 71.4 μL into all wells in columns 2 and 8, 80 μL into all wells in columns 3 and 9, 86.7 μL into all wells in columns 4 and 10 and 90 μL into columns 5 and 11. In columns 1 and 12, 100, 90, 80, 70, 60, 50 and 40 μL of the 100 mM acetate buffer (pH 4) were added by autopipette into rows A-G respectively. 2 mM aqueous $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 mM acetate buffer (pH 4) was added by autopipette into columns 1 and 12 in the following volumes: 0, 10, 20, 30, 40, 50 and 60 μL in rows A-G respectively. All occupied wells in columns 1 and 12 then contained total volumes of 100 μL , and constitute the calcium concentration calibration ($[\text{Ca}^{2+}] = 0, 0.2, 0.4, 0.6, 0.8, 1.0$ and 1.2 mM in rows A-G respectively).

From the Millipore Solvinert well plate containing solubilised amino acid/calcite composites in 100 mM acetate buffer (pH 4), the following volumes were then transferred to the corresponding wells in the new Greiner μClear Black well plate using an Eppendorf Research Plus 8 channel autopipette: 28.6 μL in columns 2 and 8, 20 μL in columns 3 and 9, 13.3 μL in columns 4 and 10; and 10 μL in columns 5 and 11. Each of the wells in the new Greiner μClear Black well plate then contained 100 μL total volume. These dilutions were essential to reduce the calcium concentration to within the functional range of calcein (≈ 1 mM). Prior to analysis with the plate reader using the protocol described above, 100 μL of prepared calcein solution was added to each well with an Eppendorf Multipipette M4 to yield final volumes of 200 μL , followed by shaking for 2 min at 600 rpm in a radial configuration.

By comparing the intensity of fluorescence from wells containing the amino acid/calcite crystals to those of the corresponding calibration curves, it is possible to identify how many moles of calcium are present in each well, and therefore how many moles of calcium carbonate were present in the original well plate. These values were then used in the mol% amino acid calculations.

2. Supporting Figures

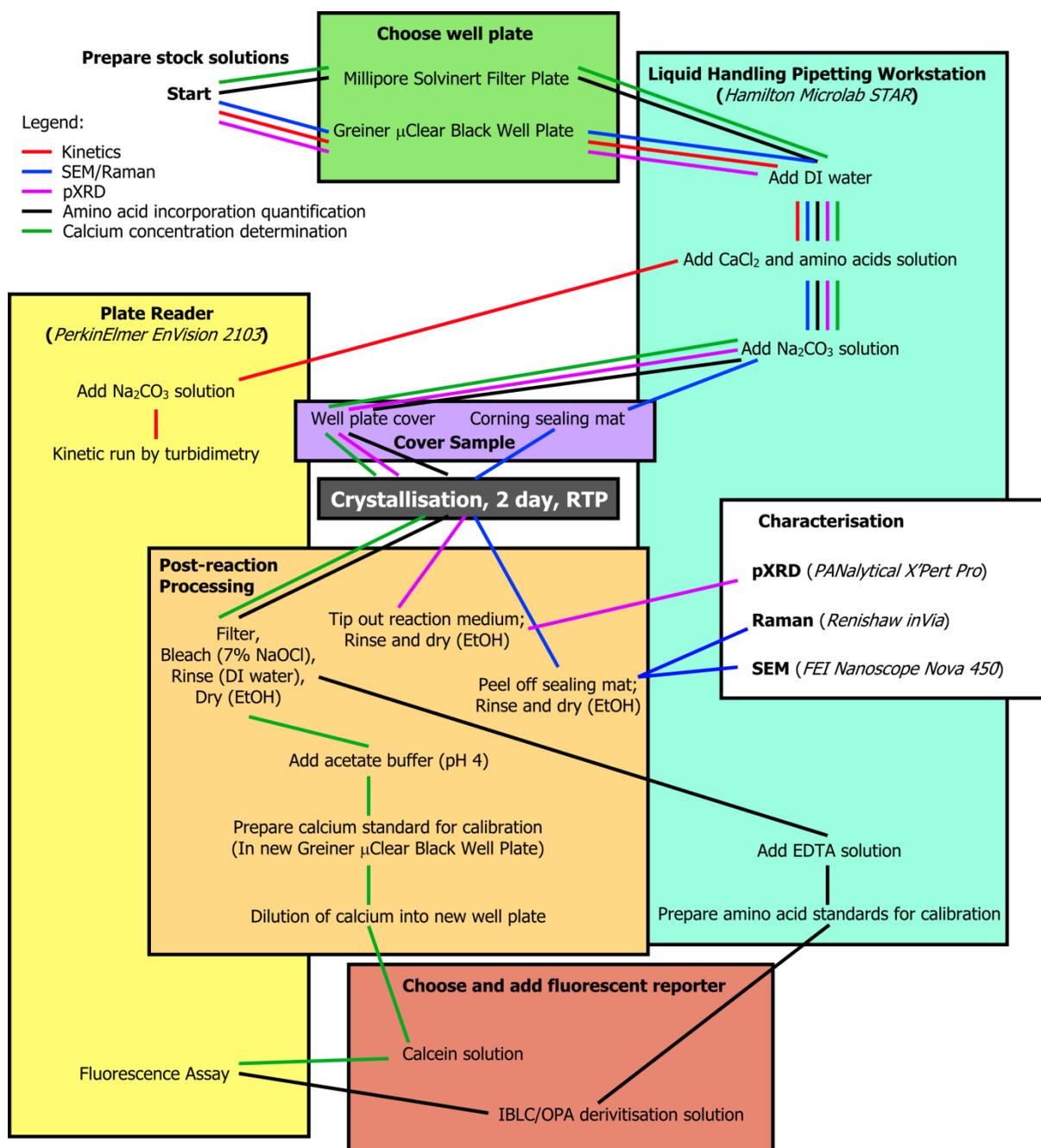


Figure S1: A detailed schematic of the complete high-throughput protocol described examining shape, structure, phase and composition of calcite/amino acid composites.

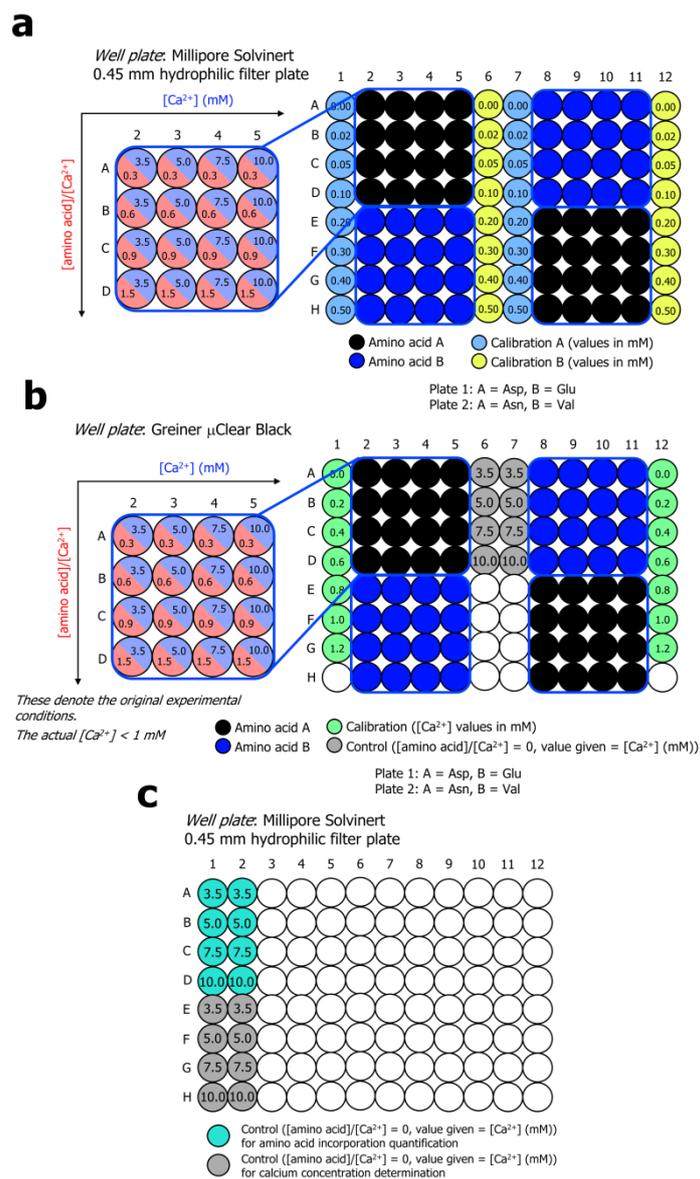


Figure S2: (a) Well plate ‘map’ showing the experimental design of the plates used for quantification of amino acid incorporation and the $CaCO_3$ yield. Wells are denoted with different colours depending on the amino acid present in solution (amino acid A = Black (Asp or Asn), amino acid B = Blue (Glu or Val)). Each 4 x 4 array per amino acid contains the same $[Ca^{2+}]$ and $[amino\ acid]/[Ca^{2+}]$ values as shown in the detailed diagram on the left, where numbers with a red back ground correspond to $[amino\ acid]/[Ca^{2+}]$ number with a blue background correspond to $[Ca^{2+}]$. Calibrations, represented with light blue (amino acid A) or yellow (amino acid B), are denoted with a number corresponding to the known amino acid concentration in each well. (b) Plate layout after dilution for calcium concentration determination experiments, with calibrations (light green) and controls (no amino acid; grey). (c) Well plate layout for control samples for both amino acid quantification (turquoise) and calcium concentration determination (grey).

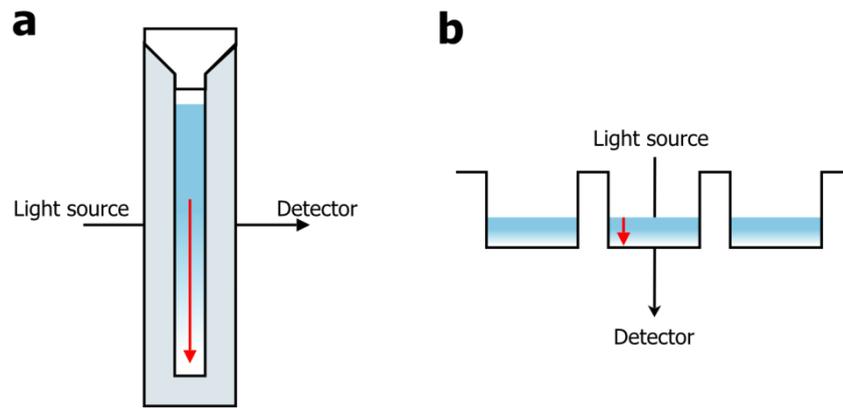


Figure S3: Schematic diagram showing the differences between the configuration of turbidimetric studies conducted in (a) a quartz cuvette/standard UV-Vis spectrometer and (a) the well plate/plate reader. The direction of light through the sample chamber is perpendicular or parallel to the direction of sedimentation respectively, as denoted by a red arrow.

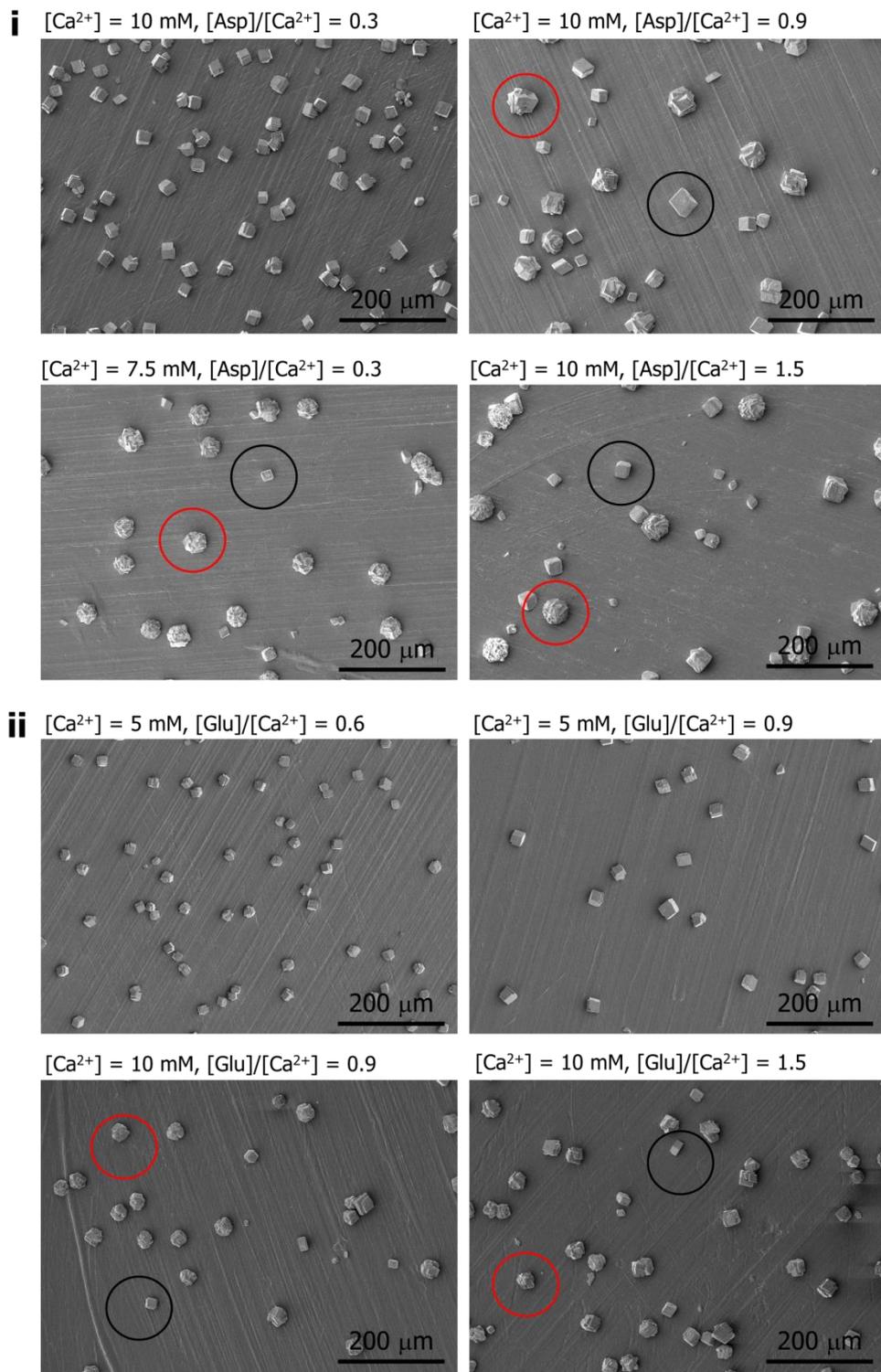


Figure S4: Low magnification SEM micrographs of samples prepared under the given conditions, to demonstrate the presence of polycrystals (red circle) amongst a population of single crystals (black circle) in calcite grown in the presence of Asp (i) and Glu (ii).

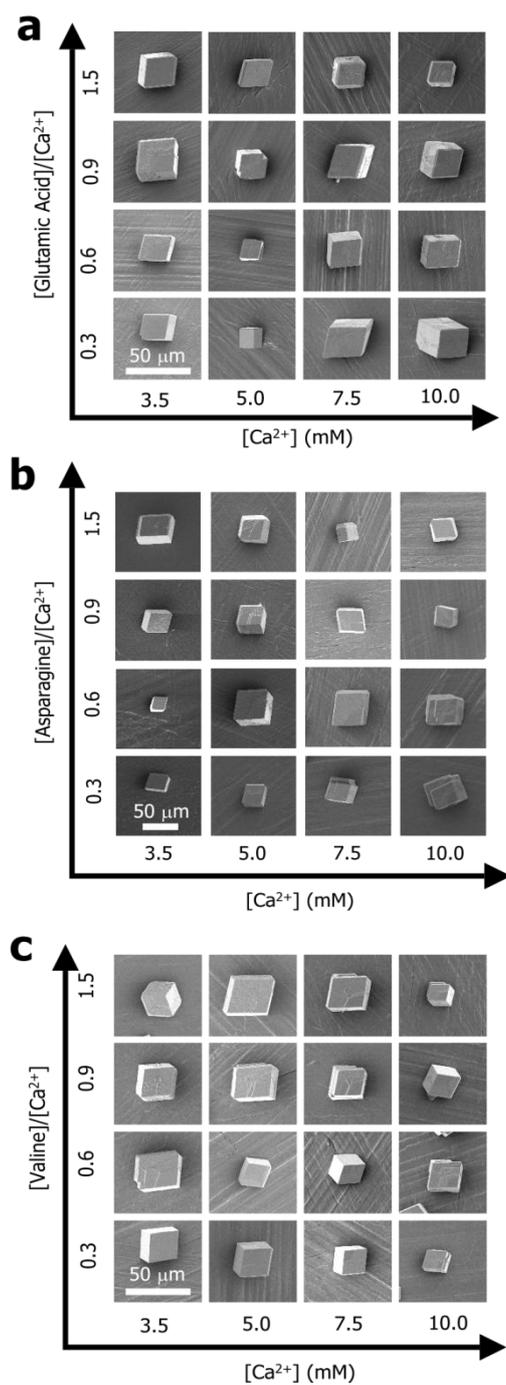


Figure S5: Micrographs of calcite single crystals observed using high-throughput sample preparation methods grown in the presence of glutamic acid (a), asparagine (b) and valine (c). Micrographs are arranged with increasing $[Ca^{2+}]$ and $[amino\ acid]/[Ca^{2+}]$.

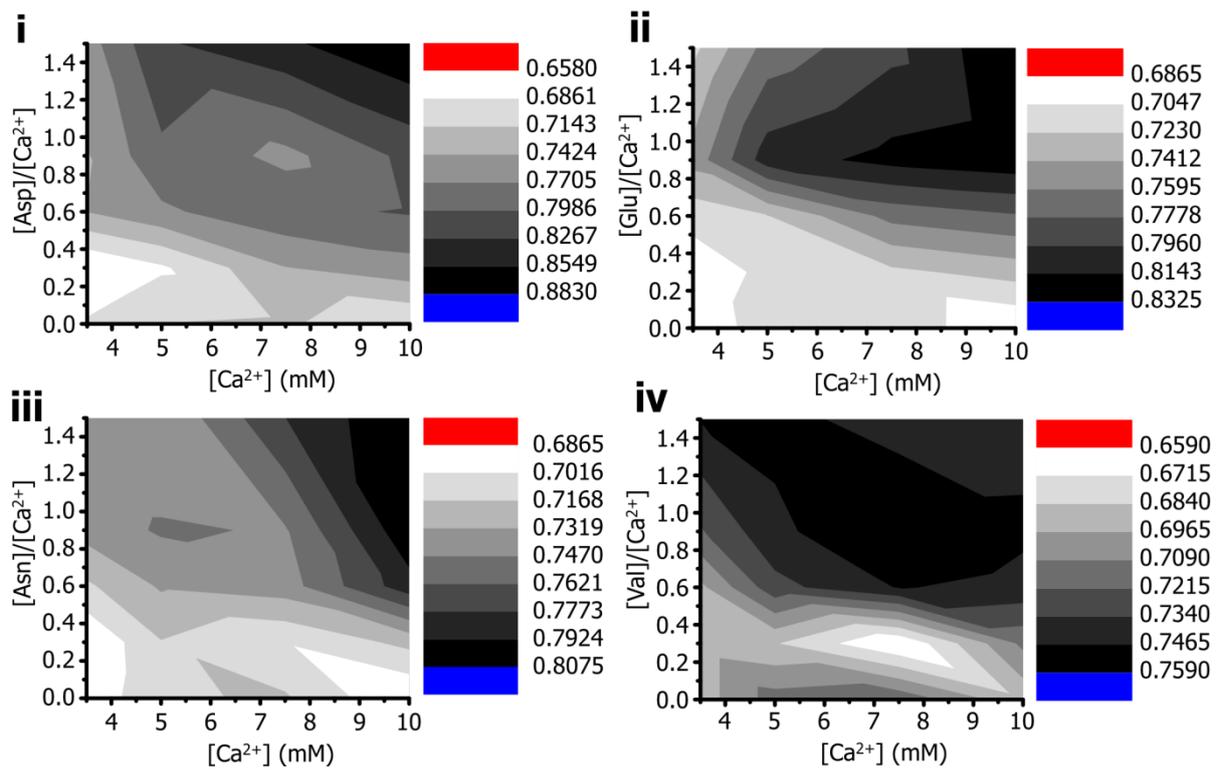


Figure S6: Contour plots (XYZ: X = $[Ca^{2+}]$, Y = $[amino\ acid]/[Ca^{2+}]$ and Z = circularity, white to black = lowest to highest) describing the trends in circularity taken from SEM micrographs of calcite grown in the presence of **(i)** Asp, **(ii)** Glu, **(iii)** Asn and **(iv)** Val.

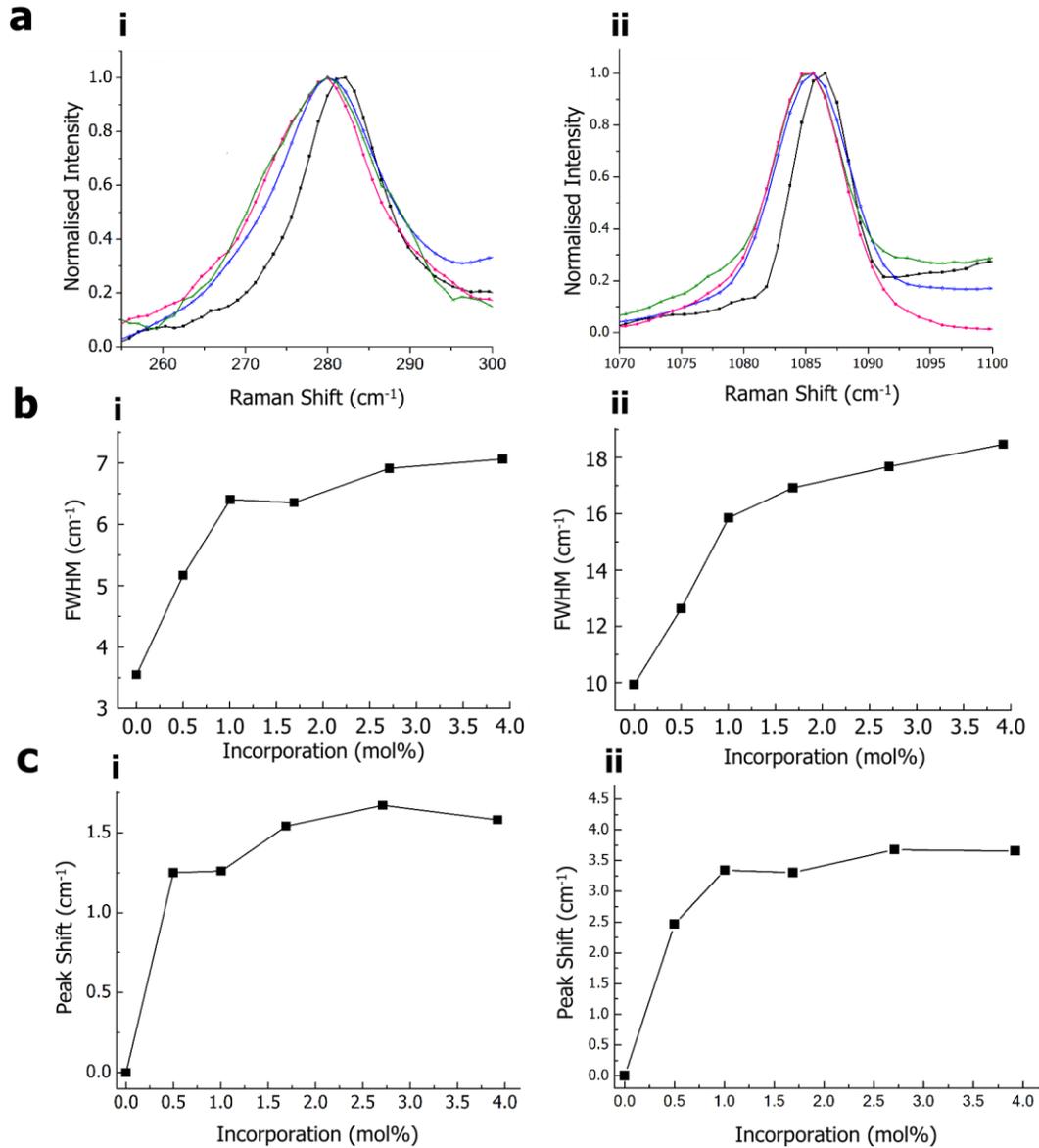


Figure S7: **a)** Raman spectra obtained from Asp/calcite composites focused on peaks attributed to 283 cm^{-1} lattice mode (**i**), and 1085 cm^{-1} ν_1 vibration mode (**ii**) demonstrating peak shift and broadening from samples grown under different conditions (initial [Asp] = 0 mM (black), 10 mM (blue), 20 mM (pink) or 50 mM (green)). **b)** Line broadening (FWHM) vs. values for incorporated Asp for lattice mode (**i**) and ν_1 vibration mode (**ii**) demonstrating the link between incorporated Asp and inhomogeneities within composites. **c)** Peak shift (deviation of peak centre from samples incorporating no Asp) vs. values for incorporated Asp for lattice mode (**i**) and ν_1 vibration mode (**ii**) demonstrating the link between incorporated Asp and structural distortion.

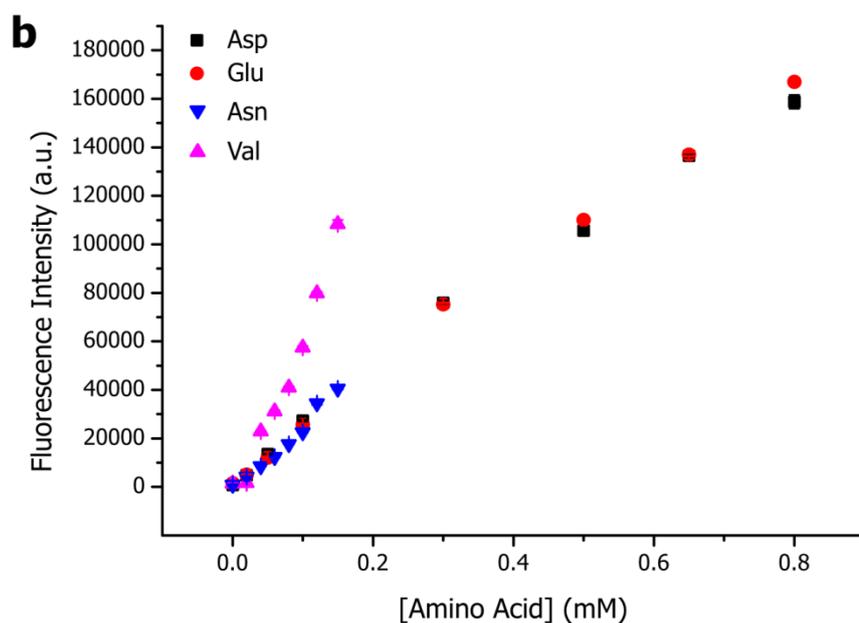
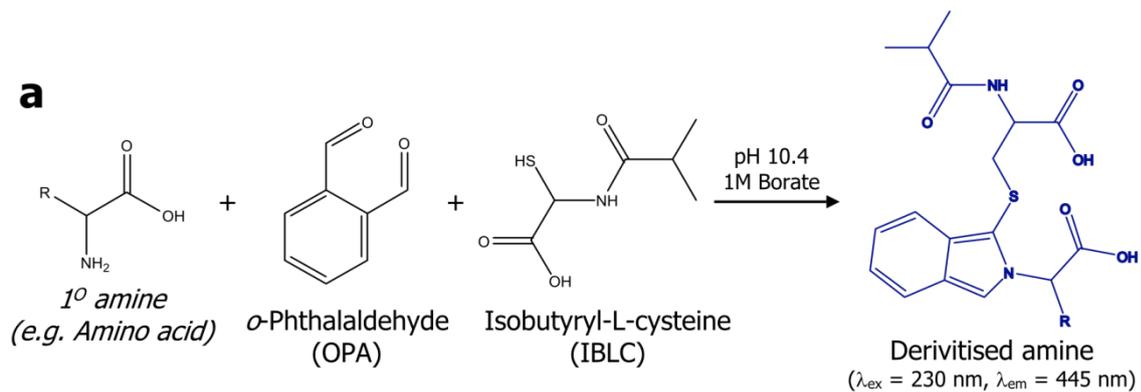


Figure S8: (a) Diagram of the reaction scheme leading to the fluorescent derivative of amino acids used for quantification experiments. (b) [amino acid]/ $[\text{Ca}^{2+}]$ vs fluorescence intensity calibration plots demonstrating the linear relationship between amino acid concentration and detectable fluorescence intensity.

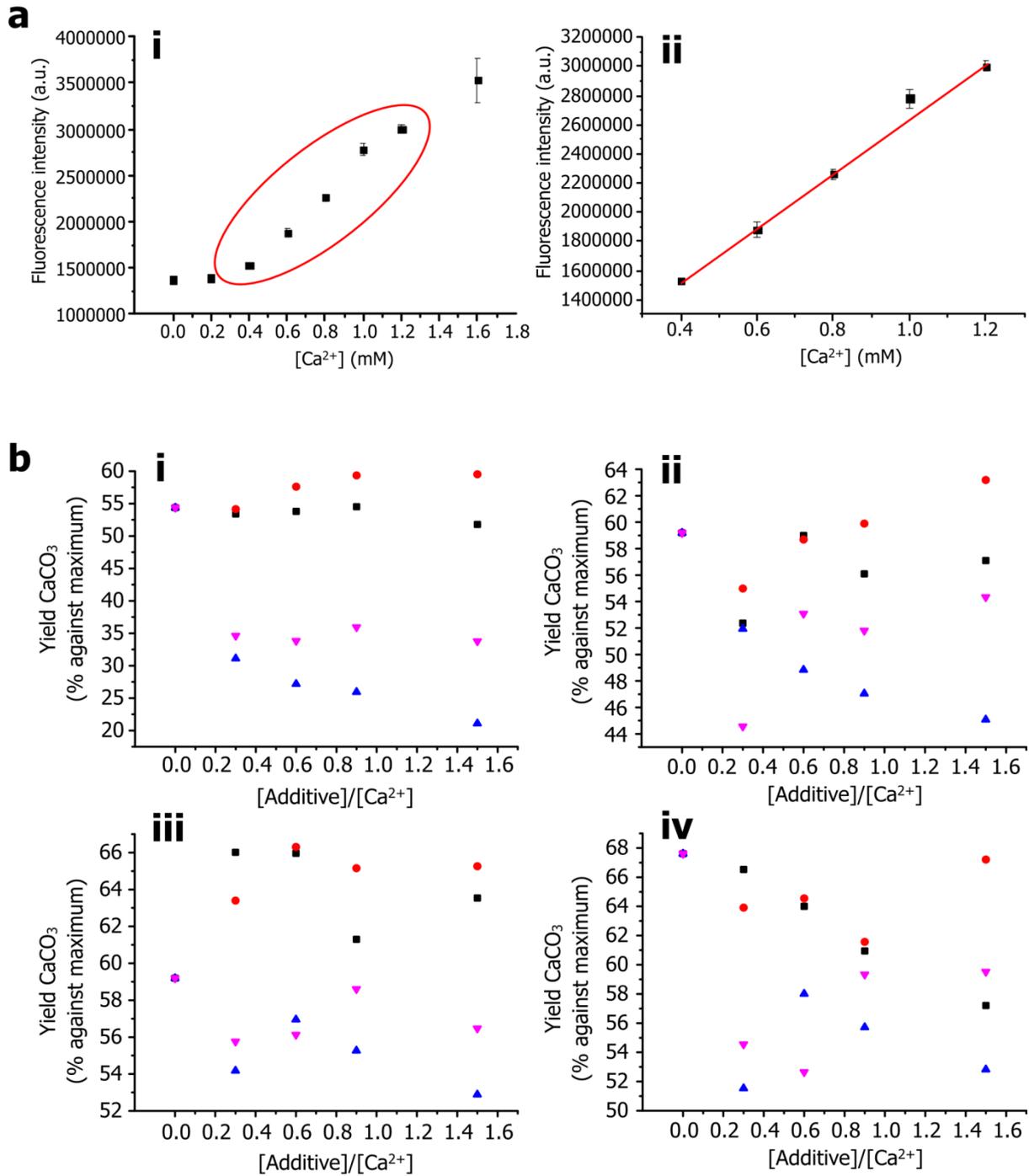


Figure S9: (a) Calibration curves across (i) a wider range, showing a sigmoidal relationship between recovered fluorescence intensity and [Ca²⁺], and (ii) a narrow range, showing a functional calibration curve with a linear trend. (b) Non-gravimetric calcite mass determination, shown as a percentage against the maximum theoretical mass of calcite prepared in the presence of Asp (black square), Glu (red circle), Asn (Blue triangle) and Val (pink triangle) from experiments where [Ca²⁺] = (i) 3.5 mM, (ii) 5 mM, (iii) 7.5 mM and (iv) 10 mM. These values were used in amino acid incorporation quantification calculations.

Sample	[Ca]	[Asp] (mM)	Known Value (mol%)	High-throughput value (mol%)
390D	10	75	1.94	1.95
390B	10	20	0.99	1.05

Figure S10: Mol% values for Asp incorporation from previous studies (Known Value from REF; analysed by HPLC) and values obtained using the analysis method described in the current research (High-throughput Value).

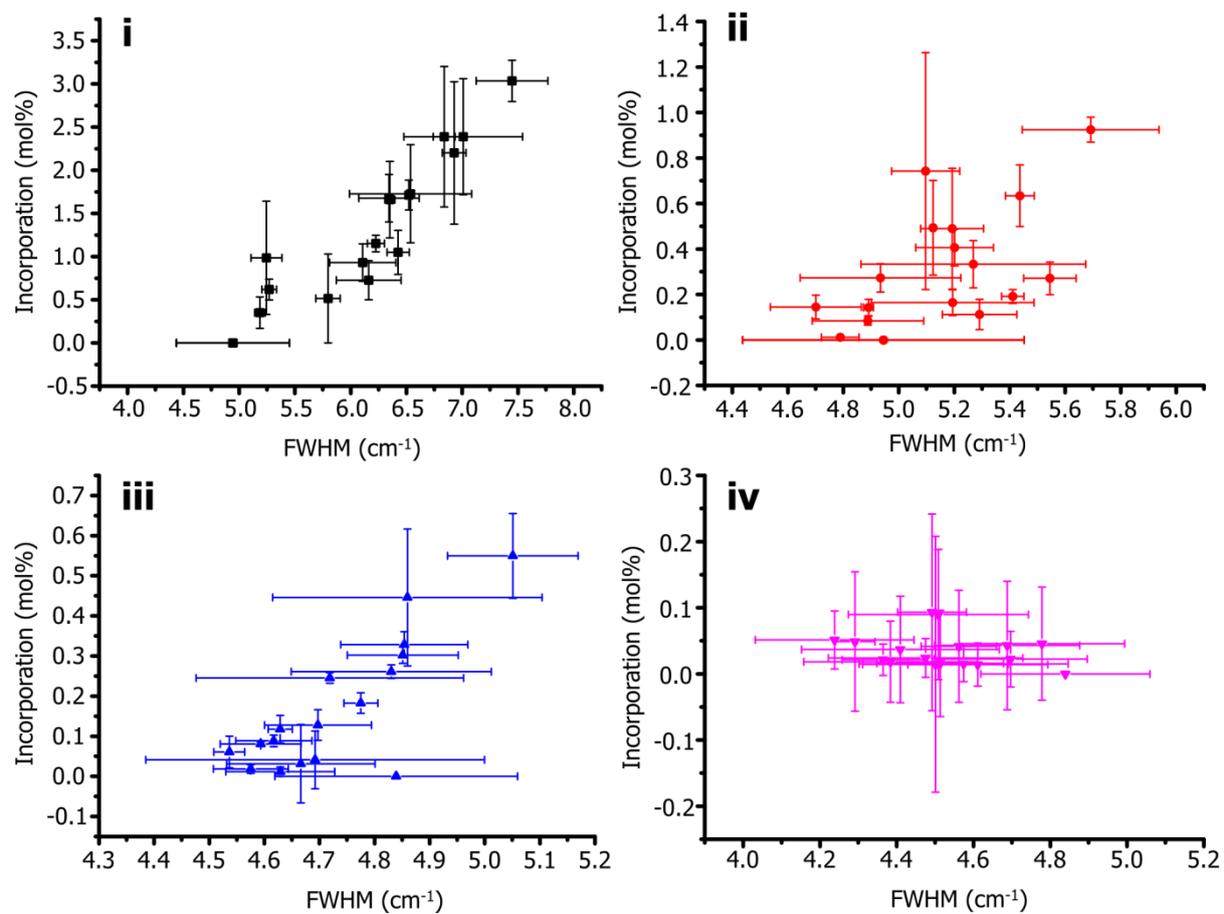


Figure S11: Values of incorporated amino acid (Asp (i), Glu (ii), Asn (iii) and Val (iv)) vs. FWHM of C-O symmetrical stretch Raman peak at 1085 cm⁻¹.

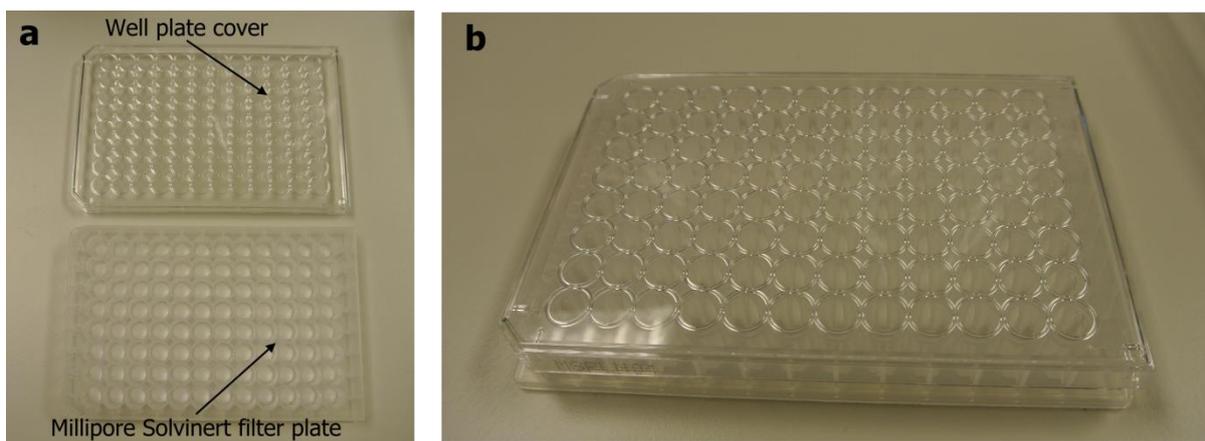


Figure S12: (a) A Solvinert 96-well hydrophilic Filter Plate (Millipore) was used for amino acid quantification and is composed of 2 parts: the well plate cover and the filter plate itself. (b) The well plate cover fits exactly over the top of the well plate, and can be sealed with Parafilm to yield a controlled environment on the interior.

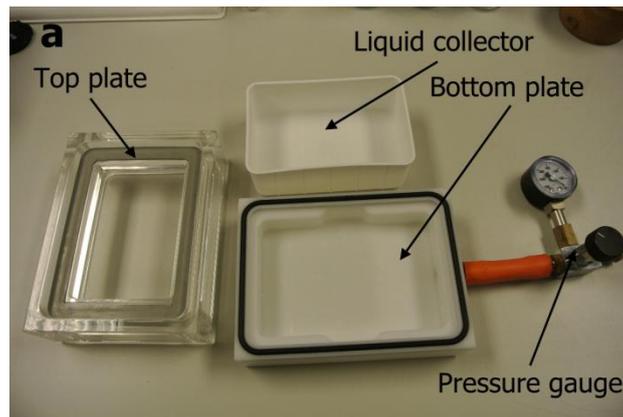


Figure S13: (a) The vacuum manifold used for filtration through filter plates is composed of 3 parts: a bottom plate with outlet to pressure gauge and vacuum pump, a liquid collector dish and a top plate which holds the well plate in place. (b) The liquid collector fits within the recess of the bottom plate and the top plate can then be placed on top, forming a tight seal with the O-ring shown in black on the bottom plate. (c) The complete vacuum manifold has a gap on top upon which the filter plate sits, forming a tight seal with the grey, rubberised section on the top plate.

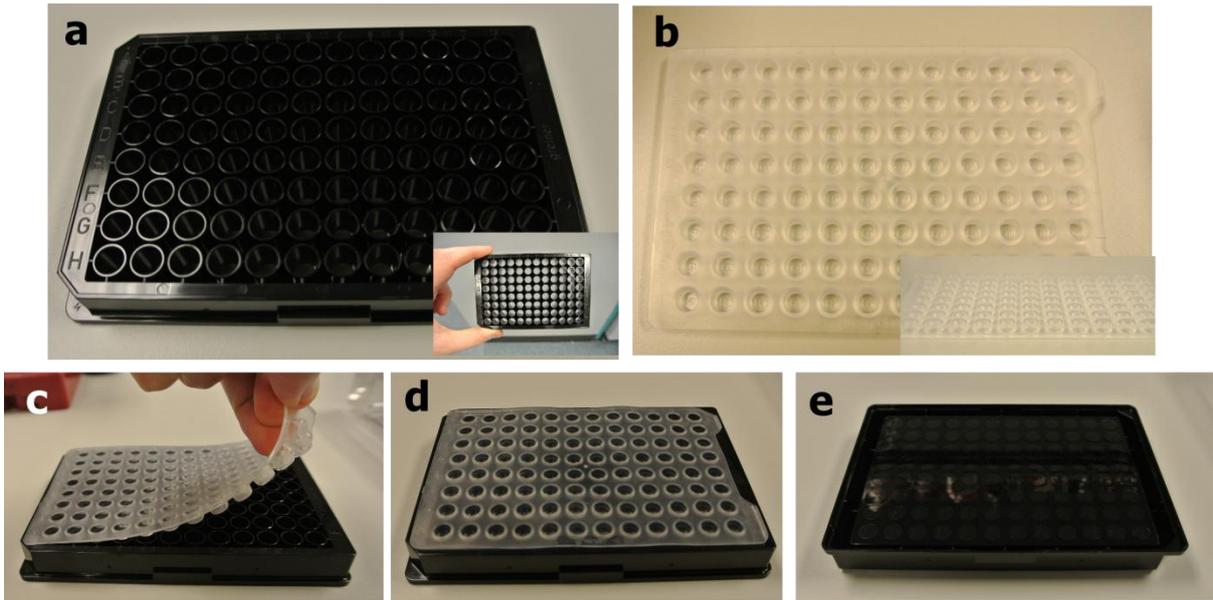


Figure S14: (a) Greiner μ Clear 96-well plate used for pXRD, kinetics; and SEM/Raman sample preparation. Inset: the clear bottom is ideal for turbidimetry studies. (b) The Corning sealing mat comprises 96 studs which are designed to fit into a 96-well plate, creating a water and air tight seal on each well. (c) The sealing mat is placed on the top of a well plate. (d) By applying a small amount of pressure, the studs on the sealing mat sink into each individual well. (e) In order to grow calcite on the surface of the studs of the sealing mat, the sealed well plate as shown in (d) is inverted, such that the sealing mat is on the bottom.

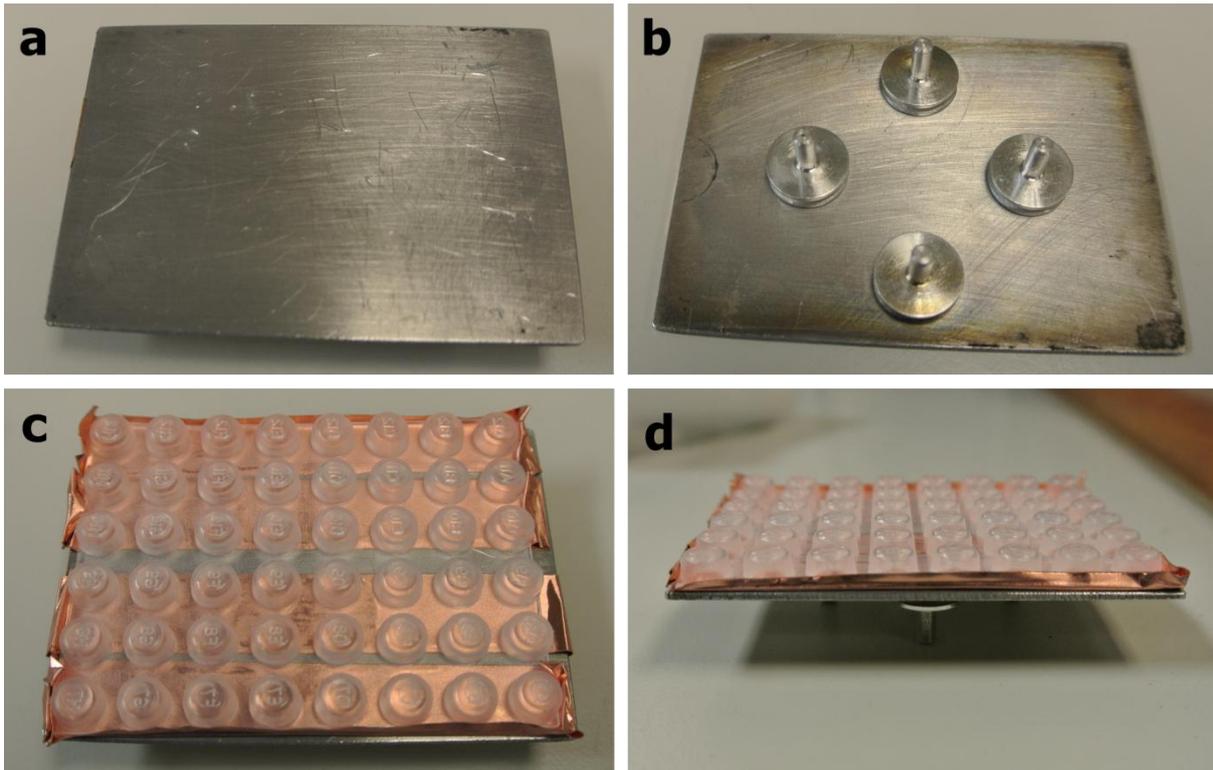


Figure S15: (a) A customised SEM sample platform was formed from a flat aluminium plate. (b) Aluminium stubs were placed in the correct position so that the aluminium plate could sit firmly in the sample stage of the microscope. (c) A 6 x 8 section of the stud mat that had been used as a substrate for growing calcite was adhered to the top surface of the aluminium plate using conductive, double sided copper tape. (d) The substrate holding 48 unique samples is flat, and with no obstruction above, enables viewing at optimal working distances.

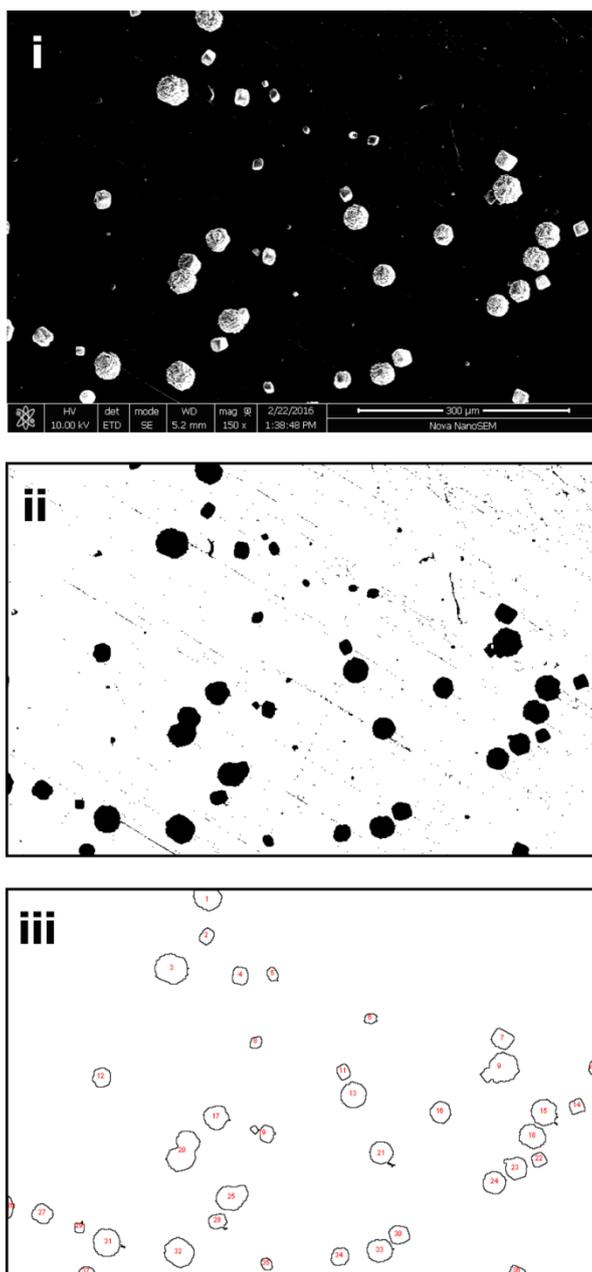


Figure S16: Image analysis (ImageJ) procedure conducted on a high-contrast SEM micrograph. (i) Initially, images were cropped, threshold values set such that background is reduced at much as possible, and remaining objects (i.e. crystals) are made solid through a Binary > Close function. (ii) Finally, circularity values were taken from analysed particles, detected automatically as shown by the 'Outline' image (iii).