Does the $\gamma$ Polymorph of Glycine Nucleate Faster?

A Quantitative Study of Nucleation from Aqueous Solution

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Abstract

We quantitatively study the crystallisation of glycine from solution, by following the crystallisation of a plate with 96 wells, each with 0.1 ml of supersaturated solution. Our first aim is to address the difficult problem of obtaining nucleation data that is reproducible. This problem is difficult due to the extreme sensitivity of nucleation times. Nucleation is sensitive to factors that include how the crystallising system is prepared, and even small (1%) variations in the supersaturation. We discuss the appropriate statistical tests needed to show reproducibility. Our second aim is to study the competition between the nucleation of the alpha and gamma polymorphs of glycine. We find that nucleation appears to be heterogeneous: some samples crystallise in minutes, while others do not crystallise after days, which indicates that there is no well defined nucleation rate for the set of samples. Homogeneous nucleation gives a well-defined rate. Those samples that crystallise in minutes mostly yield the metastable alpha polymorph. We speculate that these crystals may be the result of seed crystals formed by transient local increases in supersaturation in pipette tips during sample preparation. However, those that crystallise in hours are largely in the equilibrium gamma polymorph. This is perhaps surprising as typically the alpha polymorph is obtained from crystallisation from aqueous solutions near neutral pH. We speculate that the nucleation rate of the gamma polymorph may be higher than that of the alpha form, but that in earlier work with larger solution volumes (increasing the probability of seeding) and with stirring, the alpha polymorph dominates because of its much faster growth rate.

Introduction

Crystallisation begins with nucleation: the formation of a small stable crystal nucleus, after which crystal growth occurs. As nucleation is the first step in crystallisation, nucleation determines whether crystals appear at once, after hours or days, or not at all. It also determines whether we get many or a few crystals, and if there are competing polymorphs, which ones can form. Thus nucleation is important in a diverse range of fields, from the manufacture of pharmaceuticals, to modelling the contribution of clouds to climate change.
Here we study the nucleation of crystals of glycine from aqueous solution. Glycine is a small amino acid. It is an ideal model system that has been studied extensively in earlier work.\textsuperscript{4–19} Glycine is an easy-to-work-with molecule, which forms three polymorphs: $\alpha$, $\gamma$ and $\beta$. The $\gamma$ polymorph is the most stable of the glycine polymorphs.\textsuperscript{20} Now, while the $\gamma$ polymorph can be obtained,\textsuperscript{4–7,11,12,14} the $\alpha$ polymorph is generally found on crystallisation from aqueous solution at neutral pH.\textsuperscript{4–17} Glycine’s solubility is very sensitive to temperature,\textsuperscript{21} making it easy experimentally to vary the supersaturation. The $\alpha$ polymorph has a very fast growth rate (radial growth rates on the order of 1 mm/s have been observed\textsuperscript{17}). Han \textit{et al.}\textsuperscript{18} estimated the growth rate of $\gamma$ glycine to be about half that of the $\alpha$ polymorph; they found slower growth rates than Toldy \textit{et al.}\textsuperscript{17}

The aim of our work is to advance the state of the art in quantitative studies of isothermal nucleation from solution. Ultimately we want to understand the microscopic mechanism of nucleation, and to be able to control nucleation. We are very far from these objectives at the moment, but we believe that reproducible, quantitative experimental data on isothermal nucleation from solution will take us toward this objective.\textsuperscript{1} We provide and analyse reproducible and quantitative data here.

To achieve our aim, we developed a technique of studying almost one hundred crystallising samples in parallel. Each sample is of small (0.1 ml) volume, which has two advantages: only one or a few crystals nucleated, and growth is typically much faster than nucleation. Many samples are needed to get statistics on the random process of nucleation. Previous researchers have used microfluidics\textsuperscript{22,23} or other techniques\textsuperscript{24–28} to achieve the same goal. Quantitative analysis of these data requires statistical tools. We apply Kolmogorov-Smirnov statistical tests\textsuperscript{29,30} to nucleation data. As far as we are aware, this is the first time this has been done in this way, although Kuhs \textit{et al.}\textsuperscript{27} have used this test on nucleation data. These statistical tests are used to test for reproducibility. As nucleation is a very sensitive process, reproducible data are difficult to obtain, and so it is important to prove that data are reproducible.

Part of this sensitivity is due to nucleation almost always being heterogeneous, i.e., the crys-
tal nucleus forms at an interface, or is (possibly accidentally) seeded. Another reason for this sensitivity is due to the fact that nucleation rates vary very rapidly with supersaturation. This is true in general, and in our system an increase in supersaturation of 1% is enough to increase the nucleation rate by about 10%. This means that supersaturation variations during an experiment have to be kept significantly below 1% in size to avoid them affecting the measured rate.

While undertaking our experiments, we were surprised to find that, except for the fast nucleating samples, the majority of our crystals were of the equilibrium $\gamma$ polymorph. Most previous work reports that for crystallisation from aqueous solution near neutral pH, it is the metastable $\alpha$ polymorph that dominates. Apart from varying pH, $\gamma$ glycine has been obtained by applying an electric field, adding salts or in emulsion systems using the anionic surfactant AOT. It is unusual to observe $\gamma$ glycine without using one of these methods specifically to induce $\gamma$ nucleation. Our findings may be useful; the pharmaceutical industry often wants the equilibrium polymorph, and we found it without varying the pH, adding salt, etc.. The equilibrium polymorph is desired because it is guaranteed not to transform to a more stable form (it is already in the most stable form). Different polymorphs can have very different stability, solubility and bioavailability.

The structure of the paper is as follows. In the next section we describe the statistical tests needed to test for reproducibility, and to test if there is a well defined nucleation rate. We then describe our experimental method, and follow that with our results on nucleation rates and studies of polymorph formation. We end with a conclusion.

**Methods for analysis of nucleation data**

As experiments and computer simulations have found, and as the classical theory predicts, nucleation is a random process: in two apparently identical samples, nucleation will occur at different times. Thus, to quantitatively study and understand nucleation, we need the statistical tools and models appropriate to a random process.
The simplest model follows from the assumptions that nucleation is a one-step process that occurs at a constant rate that is the same in all samples. Then the probability that a sample has not yet crystallised at time \( t \), \( P(t) \), is a simple exponential function of time\(^1 \)

\[
P(t) = \exp \left[ -kt \right]
\]

(1)

where \( k \) is the nucleation rate. This is the simplest case, and is what we have called Class I nucleation.\(^1 \)

Nucleation is almost always heterogeneous; it can occur on impurities in the solution.\(^1,31,32,39 \)
Then different samples, even if prepared in identical ways, will have different impurities in them, and so different nucleation rates, \( k \).\(^1 \) These rates could also be changing with time, but here we assume these rates are not changing with time. If in each of our wells, nucleation is still random but with varying values of \( k \), then Proschan\(^40 \) has shown that the effective nucleation rate of a set of wells always decreases with time. This is what we term Class II nucleation. A simple model of this behaviour is the Weibull model for \( P(t) \):

\[
P(t) = \exp \left[ -\left( \frac{t}{\tau} \right)^\beta \right]
\]

(2)

with \( \beta < 1 \). This is also known as a stretched exponential.\(^41 \) Here \( \beta \) is a measure of how widely varying the nucleation rate is from sample to sample.\(^42 \) The smaller its value, the larger the spread. This has been used previously in nucleation studies.\(^1,37 \) It can be derived from survival data analysis,\(^43,44 \) as discussed in the review of Sear.\(^1 \)

**Testing models of \( P(t) \)**

To estimate \( P(t) \) we take \( n \) nominally identical samples and record the fraction that have not yet crystallised as a function of time, \( f(t) \). For large \( n \), \( f(t) \) is a good approximation to \( P(t) \). We also work with datasets where we have extracted out the samples that crystallised after the first hour, and before hour 48, and dropped the rest. Then we denote the fraction that have not crystallised
by $f^*(t)$, which for large $n$ is a good approximation to the $P(t)$ for the subset of samples that crystallised after 1 hour and before 48 hours.

As $n$ is finite, $f(t)$ is not exactly equal to $P(t)$, because there will be statistical fluctuations about the true $P(t)$. When we fit $P(t)$ models to our data we need to be able to determine whether the differences between $f(t)$ or $f^*(t)$, and the model $P(t)$ (e.g., exponential $P(t)$), are due to the data being inconsistent with the model, or whether they are sufficiently small that the differences may be just statistical noise due to small $n$.

We use the well-known Kolmogorov-Smirnov (KS) test,\textsuperscript{29,30} to test if $f(t)$ for a dataset is, or is not, consistent with a model $P(t)$. To perform this test we evaluate the supremum $D$ between $P(t)$ and $f(t)$: $D$ is the maximum absolute difference $|f(t) - P(t)|$ for any value of $t$. It measures the difference between our measured $f(t)$ and the model $P(t)$. Then we conclude that this difference is large enough that our model $P(t)$ is unlikely to be correct if

$$Dn^{\frac{1}{2}} \geq C \quad (3)$$

for $C = 1.36$. If the difference is smaller we conclude that the model $P(t)$ is consistent with the measurements.

For brevity, we refer to the left hand side of eq. (3) as the KS number. Equation (3) is related to the central limit theorem of statistics. It is saying that the size of the fluctuations in $f(t)$ around the true $P(t)$ should be no more than approximately $1/n^{\frac{1}{2}}$. If they are larger, we can conclude that our assumed $P(t)$ is likely to be incorrect. If our assumed model $P(t)$ is indeed the true distribution, then for 95 out of 100 measured $f(t)$s, the KS number should be less than 1.36.

**Testing for reproducibility**

Nucleation is very sensitive to many parameters, and so achieving reproducibility can be difficult. So, we also need a test to distinguish between two types of variation in $f(t)$ from one experiment to another one performed under identical conditions. The first type of variation between the $f(t)$s
of the two experiments is just due to statistical noise, and occurs even if the experiments are done truly reproducibly, i.e., when both \( f(t) \)s are samples from the same underlying \( P(t) \). The second type occurs when the experiments are not done reproducibly, i.e., when the two \( f(t) \)s are samples from different underlying \( P(t) \)s because there is a real difference in the crystallisation behaviour.

To perform this check on experiments \( A \) and \( B \), we use the Kolmogorov-Smirnov two-sample test. To do this for two experiments \( A \) and \( B \) we calculate the supremum of the pair of \( f(t) \)s of experiments \( A \) and \( B \), \( D_{AB} \). Then if

\[
\left( \frac{n_An_B}{n_A+n_B} \right)^{1/2} D_{AB} \geq C
\]  

(4)

we conclude that experiments \( A \) and \( B \) are not reproducible. Here, \( n_A \) and \( n_B \) are the numbers of samples in datasets \( A \) and \( B \). When the inequality of eq. (4) is satisfied we conclude that the two \( f(t) \)s are unlikely to be samples from the same true \( P(t) \), and so the experiments are not reproducible. We conclude this because if the two \( f(t) \)s are samples from the same \( P(t) \), then the inequality of eq. (4), with \( C = 1.36 \), is only satisfied 5% of the time. The same test is also useful to check that when we intentionally change a parameter, that this change has significantly changed the nucleation rate.

**Experimental Methods**

Glycine solutions were made by adding solid glycine (≥ 99% HPLC from Sigma, cat. no. G7126) to de-ionised water (Milli-Q, 18.2 MΩ cm) in a glass vial. This solid glycine consisted of mainly the \( \gamma \) polymorph, but with a small amount of the \( \alpha \) polymorph; the XRD pattern is shown in Figure S6. We fully dissolved the glycine during preparation of the solution. Experiments were carried out at concentrations, \( c \), in the range of 306.66 mg/ml to 353.33 mg/ml. The solution was heated to 70°C in a sealed vial and stirred at 1200 rpm for 1 h using a hotplate and magnetic stirrer bar. The pH of a solution with \( c = 333.33 \) mg/ml was 6.2 ± 0.1 obtained using a digital pH meter.

Each experiment used a microplate (Nunclon Delta Surface) with 96 wells (arranged as eight
Figure 1: (a) A schematic illustrating the experimental setup. The light comes down vertically from the ceiling. A translucent sheet of yellow tracing paper is fixed in position above the microplate. It is used to improve the contrast between the crystals and rest of the well. (b) A camera image recorded 48 hours after the solution was injected into the wells. Crystallisation has occurred in many but not all wells.

rows of 12 wells). For each run a new microplate was used. Each well initially contained 100 µl of tridecane (≥ 99% from Sigma Aldrich). We chose tridecane because of its very low vapour pressure (0.056 mm Hg at 25°C) and very low miscibility with water. At 25°C, the mol fraction solubility of water in tridecane is $6.1 \times 10^{-4}$, whereas the mol fraction solubility of the slightly smaller oil, dodecane in water is $8.9 \times 10^{-10}$. Tridecane will be less soluble in water than dodecane. Then 100 µl of the heated glycine solution was transferred simultaneously into each successive row of wells using a 12-channel multi-pipette (Scipette). The same set of pipette tips were used row to row within each run, but a fresh set of pipette tips were used for each new run. It was found that injecting glycine solution under the oil resulted in trapping air bubbles at the interface of the two liquids in many of the samples. For this reason, the glycine solution was deposited on top of the tridecane after which it would immediately sink below the oil due to its
Figure 2: (a) Images of the same well at six different times ranging from 5 min to 48 h. In this well a crystal (circled in red) nucleates at 4 h and proceeds to grow. (b) Images of another well at five different times from 5 min to 48 h. Several crystals, circled in red, can be observed forming simultaneously from 10 min onwards.

higher density. The plate and samples cool to room temperature within the first hour of the experiment, as can be seen in Figure S1 (in the Supporting Information). The experiments are then isothermal after the first hour.

Our use of oil to cover multiple wells was inspired by a technique pioneered for protein crystallisation by Chayen and coworkers.\textsuperscript{46,47} Their technique is called microbatch crystallisation under oil. It is called microbatch, because it is ‘micro’ as the droplets are small, and ‘batch’ as many droplets are studied in parallel in a single plate. We copy Chayen and coworkers’ use of oil to cover the droplets and prevent evaporation. There are however differences between our approach and theirs: Our droplets are 100\,$\mu l$, whereas in microbatch protein crystallisation they are typically around 1\,$\mu l$, and in microbatch crystallisation often the conditions in different wells will be different — a single run is used to rapidly try (screen) many different crystallisation conditions.

The experiments were conducted in a temperature controlled room with a setpoint temperature of $T = 21^\circ\text{C}$. Images of samples were recorded with a Logitech HD Pro Webcam C920 placed underneath the microplate. Background light travelling at an angle from the side towards the microplate was blocked with a wooden frame to reduce any glare. The setup is illustrated in Figure 1(a).
Captured images were recorded at a resolution of 1080 × 1920 pixels in PNG format. An image was recorded every 10 minutes for the first three hours, then every 30 minutes for the following six hours, then at every hour for the remainder of the experiment. The images were then analysed by eye to determine the time at which each sample crystallised. Crystallisation events within individual wells can be seen in Figure 2, and the crystallisation events for an entire microplate can be seen in Figure 1b.

**Estimating crystal growth rates**

We are assuming that crystals grow rapidly with respect to the nucleation rate, and hence the time at which we first see the crystal is a good approximation to the nucleation time. We can justify this by quantifying the growth rates of the crystals. By measuring the largest distance across the crystals in the images recorded in 10 minute intervals for an hour after each crystal is formed, an approximation of the growth rate (within the plane of view) can be obtained. With this we can calculate the time it took for the crystals to grow to an observable size. A histogram of the crystal growth rates for all the crystals that nucleated after the first hour is plotted in Figure 3. This is from a single run.

![Histogram of growth rates](image)

Figure 3: A histogram of the growth rates of crystals from the 57 wells where nucleation occurred after the first hour in a single microplate. This is at 21°C and a supersaturation $S = 1.81 \ (c = 333.33 \ mg/ml)$. 

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Our distribution of rates is very broad but most crystals have growth rates around 0.02 mm/min, or 0.3 µm/s. This is smaller than the growth rates measured by Han et al.\textsuperscript{18} at the lower supersaturation of $S = 1.43$, and at the slightly higher temperature $T = 23^\circ$C. They find (at our neutral pH) growth rates of approximately $1.4 \pm 0.3$ µm/s for the $\alpha$ polymorph and $0.8 \pm 0.2$ µm/s for the $\gamma$ polymorph. As we work at higher supersaturations, we would expect our rates to be higher not lower than those of Han et al., but there are differences between their and our system. For example they study larger crystals (theirs are approximately 5 mm and larger) and study specific crystal faces.

We estimate that crystals are observable at a size of about 0.11 mm. Our wells are $6.8 \pm 0.2$ mm in diameter which is 90 pixels in our images, so one pixel is approximately 0.076 mm. The smallest crystal we observe is then two pixels across diagonally, or a centre-to-centre distance of $2^{1/2} \times 0.076 = 0.11$ mm. We assume that the time to grow large enough (0.11 mm) to be observed = (minimum observable size)/(growth rate). Then, 98% of crystals are observable within 20 minutes and 77% are observable within 10 minutes of nucleation. This means there is a small uncertainty in our early nucleation time measurements but the uncertainty is smaller for longer nucleation times.

**Temperature Variation**

Temperature was found to vary slightly throughout the duration of an experiment. To clarify whether temperature variation was significant, the room temperature was recorded over a one week time period. The mean temperature as a function of time of day can be seen in Figure 4. On average the temperature ranged from a minimum of 20.7°C at 04:00, to a maximum of 21.4°C at 12:00.

The solubility of glycine is very sensitive to temperature, which means that supersaturation is varying during the experiments. Using published solubility data,\textsuperscript{21} it can be estimated that the temperature difference between 21.4°C (solubility 186 mg/ml) and 20.7°C (183 mg/ml) corresponds to a supersaturation change of around 1.7%.
X-ray diffraction

X-ray diffraction (XRD) was carried out on crystals from some experiments to determine which of glycine’s three polymorphs formed. Immediately after completing an experiment, all the crystals in the microplate were removed with tweezers from their wells and placed onto filter paper. This procedure removed any water still on the crystals and stopped any crystals transforming from one polymorph to another between the end of the experiment and the XRD analysis. The crystals were ground up to a fine powder before analysis. Powder XRD was carried out using a PANalytical X’Pert Pro diffractometer across a 2θ range of 10° to 70° using copper Kα radiation.

Results

We list all our datasets in Table 1, each is identified by a letter. Here we define a run as the nucleation times recorded for one 96-well microplate. We define a dataset as all runs carried out at the same concentration and time of day. We performed experiments at a number of concentrations, c. The solubility at 21 °C was calculated to be $c_s = 184$ mg/ml by interpolating solubility data from Yang et al,21 so the supersaturations $S = c/184$ are around 1.8.

For each dataset we give the total number of wells, $N$, in the dataset. For datasets A through D
(only), we also controlled the time, \( t_{\text{held}} \), the solution was held in the pipette, before being injected into the wells. For each dataset, we give \( F_{\leq 1} \), \( F^* \), and \( F_{>48} \), these are the fraction of samples that nucleated within the first hour, the fraction that nucleated after the first hour, and the fraction that did not nucleate within 48 hours, respectively. For some datasets we have fitted an exponential to the extracted data where the extracted data are all nucleation events that fall into the \( F^* \) region. For these we give the best fit values of \( k \), with error bars calculated from the standard deviation of \( k \) values fitted to each component run, divided by the square root of the number of runs. The numbers in the rightmost column are the KS numbers comparing pairs of \( f^*(t) \)'s for runs in that line’s dataset. These KS numbers should be less than 1.36 when the runs are reproducible.

Table 1: A table of our datasets; each is identified by a letter in column one.

<table>
<thead>
<tr>
<th>Runs</th>
<th>( N )</th>
<th>( c/\text{g/l} )</th>
<th>( t_{\text{start}} )</th>
<th>( t_{\text{held}} / \text{s} )</th>
<th>( F_{\leq 1} )</th>
<th>( F^* )</th>
<th>( F_{&gt;48} )</th>
<th>( k / \text{h}^{-1} )</th>
<th>KS numbers</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>1.25</td>
<td>120</td>
<td>333.33</td>
<td>16:00</td>
<td>5</td>
<td>0.24</td>
<td>0.31</td>
<td>0.45</td>
<td>0.16</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>96</td>
<td>333.33</td>
<td>16:00</td>
<td>20</td>
<td>0.23</td>
<td>0.22</td>
<td>0.55</td>
<td>0.14</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>96</td>
<td>333.33</td>
<td>16:00</td>
<td>25</td>
<td>0.59</td>
<td>0.21</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>D</td>
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<td>120</td>
<td>333.33</td>
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<td>30</td>
<td>0.92</td>
<td>0.08</td>
<td>0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>192</td>
<td>353.33</td>
<td>16:00</td>
<td>-</td>
<td>0.77</td>
<td>0.09</td>
<td>0.14</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>288</td>
<td>341.13</td>
<td>16:00</td>
<td>-</td>
<td>0.50</td>
<td>0.43</td>
<td>0.07</td>
<td>0.234 ± 0.007</td>
</tr>
<tr>
<td>G</td>
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<td>480</td>
<td>333.33</td>
<td>16:00</td>
<td>-</td>
<td>0.30</td>
<td>0.55</td>
<td>0.14</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>H</td>
<td>3</td>
<td>288</td>
<td>325.53</td>
<td>16:00</td>
<td>-</td>
<td>0.20</td>
<td>0.53</td>
<td>0.27</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>192</td>
<td>320.00</td>
<td>16:00</td>
<td>-</td>
<td>0.22</td>
<td>0.39</td>
<td>0.39</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>J</td>
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<td>192</td>
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<td>16:00</td>
<td>-</td>
<td>0.04</td>
<td>0.04</td>
<td>0.93</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
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<td>333.33</td>
<td>13:00</td>
<td>-</td>
<td>0.25</td>
<td>0.24</td>
<td>0.51</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>288</td>
<td>333.33</td>
<td>10:00</td>
<td>-</td>
<td>0.51</td>
<td>0.28</td>
<td>0.21</td>
<td>0.096 ± 0.005</td>
</tr>
</tbody>
</table>

In some wells more than one crystal was found to form. We studied this phenomenon by counting the number of crystals in all five of the runs that made up dataset G (146 wells that crystallised in the first hour and 266 that crystallised in hours 2 to 48). For the samples where the first nucleation event was within the first hour of starting the experiment, a mean of 2.21 crystals per well formed. In the three plates, for the fast-nucleating wells, 38% of wells had 1 crystal, 27% had 2 crystals, 11% had 3 crystals and 24% had 4 or more crystals. For the samples where the first nucleation event was after the first hour of starting the experiment, a mean average of 1.61 crystals
per well formed. For these slower-nucleating wells, 49% of the wells had 1 crystal, 42% had 2
crystals, 9% had 3 crystals, and 1% had 4 or more crystals.

When multiple nucleation events occurred in the same well, in some cases the two crystals
would then co-exist. In a few cases the growth of the second crystal would cause the first crystal
to shrink and eventually dissolve back into the solution. We also found that in some cases two
or more crystals would form within the same time interval. This occurred particularly often for
crystals nucleating within the first hour of the experiment.

**Reproducibility**

We first need to determine if our experiments are reproducible. Hence, we consider dataset $L$,
which consists of three runs: $L_1$, $L_2$ and $L_3$. In Figure 5(a) we plot the fraction not crystallised,
$f(t)$, for these three runs, which are all at the same conditions (concentration, and time of start of
experiment). We see that there are large differences between the $f(t)$ curves. The corresponding
KS numbers are presented in Table 2. The KS numbers in the second row of the table are for
the three possible pairwise comparisons between the runs. Two are greater than 1.36, the value
that is expected to be crossed only 5% of the time if data are reproducible. We conclude that our
experiments are not reproducible.

We can see in Figure 5(a) that the fractions that crystallise in the first hour vary between runs. It
appears that some factor causing nucleation that immediately, or almost immediately, is changing
from one run to another. We note that it takes 30 minutes from the start of the experiment for all
wells to cool to within 1°C of room temperature, and hence nucleation in the first hour is not at
constant supersaturation. The temperature in the wells as a function of time is plotted in Figure
S1 in the Supporting Information. However, the cooling curves are reproducible. Although the
cooling will affect nucleation in the first hour, variability in cooling rate is not the cause of the
variability in the amount of nucleation that we observe.

In the next section, we consider a candidate for the cause of the lack of reproducibility for
nucleation in the first hour. But here we consider data in which we remove all samples that crys-
tallised in the first hour, and shift the $t = 0$ point to one hour after the start. We are not the first to use this approach, in this we follow the work of Carvalho and Dalnoki-Veress.\textsuperscript{25,48} Removing all samples that crystallised in the first hour will affect the fraction that do not crystallise during the 48 hours of our experiment, so we also remove from the data all samples that never crystallise during our experiment. We replot the remaining samples in Figure 5(b). We use the notation $f^*(t)$ to indicate that we have extracted out the samples that crystallised after the first hour, and before hour 48.

The three $f^*(t)$ curves are very similar and the differences between them are consistent with statistical fluctuations, as indicated by the KS numbers in the bottom row of Table 2. The numbers in the rightmost column of Table 1 are the KS numbers for $f^*(t)$s for runs in that line’s dataset. Most are below 1.36. We conclude that our method generates mostly reproducible data for nucleation events occurring between 1 and 48 hours after starting the experiment, but that nucleation in the first hour is not reproducible.

![Figure 5](image-url)

**Figure 5:** Plots of the fraction of samples where nucleation has not occurred, as a function of time, for the three runs $L_1$, $L_2$ and $L_3$ that make up dataset L. All runs are carried out under identical conditions. In (a) we plot $f(t)$, the fraction not crystallised, taken from the complete set of 288 samples. In (b) we plot $f^*(t)$, the fraction where nucleation has not occurred, taken only from the subset of 81 of these samples that crystallised between 1 h and 48 h.
Table 2: The KS numbers comparing the three datasets in Figure 5(a) and (b). The middle and bottom rows are for the \( f(t) \) and \( f^*(t) \) functions, respectively.

<table>
<thead>
<tr>
<th>Datasets</th>
<th>( L_1 ) and ( L_2 )</th>
<th>( L_1 ) and ( L_3 )</th>
<th>( L_2 ) and ( L_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f(t) )</td>
<td>2.17</td>
<td>1.35</td>
<td>0.99</td>
</tr>
<tr>
<td>( f^*(t) )</td>
<td>0.86</td>
<td>0.55</td>
<td>0.51</td>
</tr>
</tbody>
</table>

For nucleation in the first hour, homogeneous nucleation is ruled out as the nucleation mechanism by the lack of reproducibility of the \( f(t) \) curves. Each run is at the same supersaturation where the homogeneous nucleation rate will be the same. The \( f^*(t) \) plots in Figure 5(b) are close to exponentials, and this functional form is consistent with homogeneous nucleation.\(^1\) However, in Figure 5(a) we see that the \( f(t) \)'s have plateaus; these are not consistent with homogeneous nucleation. The pronounced plateaus imply that some samples have a smaller (possibly much smaller) nucleation rate than those that nucleate after the first hour. Assuming impurities can only increase nucleation rates, i.e., that homogeneous nucleation rate is a lower bound to the total nucleation rate, then nucleation after the first hour cannot be homogeneous as a number of samples have lower rates. As expected, nucleation is heterogeneous.

**Pipette times**

To understand the lack of reproducibility in the fast crystallising samples, we performed further experiments in which we measured and varied the time that the solution was held in the pipette, \( t_{\text{held}} \). In the earlier experiments of Figure 5 we did not record this time. When we did not measure this time, there is a dash in the \( t_{\text{held}} \) column in Table 1. In Figure 6 we plot both \( f(t) \) and \( f^*(t) \) for experiments in which we varied \( t_{\text{held}} \) while keeping all other parameters the same. In Figure 6(a) and Table 1 we see that holding the solution in the pipette for 30 s results in over 80% of the samples nucleating in the first hour, whereas if the pipette is held for 5 s, only 25% crystallise in the first hour. Clearly \( t_{\text{held}} \) affects nucleation in the first hour.

In Figure 6(b) we can see the effect of \( t_{\text{held}} \) on the extracted \( (f^*(t)) \) datasets. The KS numbers comparing the datasets in Figure 6(b) can be seen in Table 3. The largest KS number is below the
1.36 threshold. The differences between the extracted datasets for each $t_{held}$ are sufficiently small that they are consistent with statistical noise, and so we see no effect of $t_{held}$ on nucleation in the extracted data. We conclude that the extracted data are not affected by $t_{held}$. The nucleation rates of the extracted data displayed in Table 1 show no clear relationship with $t_{held}$. This is consistent with our finding of reproducible $f^*(t)$ curves for runs in which we did not control the pipette holding time (Figure 5).

Table 3: KS numbers comparing $f^*(t)$s for the datasets A to D, for which $t_{held}$ is varied.

<table>
<thead>
<tr>
<th>Datasets</th>
<th>KS number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A and B</td>
<td>0.93</td>
</tr>
<tr>
<td>A and C</td>
<td>0.57</td>
</tr>
<tr>
<td>A and D</td>
<td>0.80</td>
</tr>
<tr>
<td>B and C</td>
<td>1.03</td>
</tr>
<tr>
<td>B and D</td>
<td>0.79</td>
</tr>
<tr>
<td>C and D</td>
<td>0.51</td>
</tr>
</tbody>
</table>

We have no direct evidence on why holding the solution in the pipette tips for 30 s results in many samples crystallising immediately or almost immediately. It may be that small amounts of evaporation in the pipette tips locally raises the supersaturation enough to drive rapid nucleation, creating seed crystals that are then pipetted into some wells. We note that a sub-micrometre volume of solution is more than enough to allow nucleation, so only a microscopic volume of the solution in the pipette needs to reach higher supersaturations to trigger nucleation. This is a speculative explanation. Whether or not it is indeed the mechanism, future experiments using this methodology should control $t_{held}$ to ensure reproducibility.
Concentration

Next we consider the effect of concentration on nucleation rate. We acquired data at concentrations of 353.33, 341.13, 333.33, 325.53, 320.00 and 306.66 mg/ml. The $f^*(t)$s in the range from 320.00 to 341.13 mg/ml are plotted in Figure 7. As expected, we find that nucleation is faster at higher supersaturations.

Figure 7: Plot of the fraction of samples where nucleation has not occurred, for concentrations between 320.00 to 341.13 mg/ml. Only data from those samples that crystallised between 1 and 48 h are shown here. The solid curves are fits of exponential functions to the data; the best fit values of $k$ are given in Table 1. At the highest supersaturation, dataset F, the exponential function is a poor fit. For this dataset (only) we also fit a Weibull to the data. This is shown as the dashed blue curve; the fit parameters are $\beta = 0.67 \pm 0.06$ and $\tau = 3.6 \pm 0.4$. 

Figure 6: Plots of the fraction of samples where nucleation has not occurred, for runs with different pipette holding times. In (a) and (b), we plot $f(t)$ and $f^*(t)$, respectively. All runs are at $c = 333.33$ mg/ml.
In Figure 7 we show exponential fits to the $f^*$ plots. See Supporting Information for our fitting procedure. We use KS numbers to assess the quality of the fits. These numbers are in Table 4. The KS numbers are consistently relatively high. It seems unlikely that the true $f^*(t)$s are simple exponentials, which suggests that nucleation is heterogeneous, and that there is sample-to-sample variability in the surfaces on which nucleation is occurring on. It is known that variability makes $f^*(t)$ non-exponential.\(^1\) We note that non-exponential $f^*(t)$s are common,\(^1\) so this finding is not a surprise.

Table 4: The KS numbers comparing each of the datasets in Figure 7 to their respective exponential fits.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>$F$</th>
<th>$G$</th>
<th>$H$</th>
<th>$I$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS number</td>
<td>1.75</td>
<td>1.68</td>
<td>0.97</td>
<td>0.98</td>
</tr>
</tbody>
</table>

We have also fit the data with Weibull functions. The highest concentration of 341 mg/ml is best fit by a Weibull function with $\beta = 0.67$ and $\tau = 3.6$ h. The $\beta < 1$ puts it into Class II.\(^1\) The Weibull fits for the datasets $G$, $H$ and $I$ gave $\beta$ values of 1.00, 0.93 and 1.05, respectively. As for these fits $\beta \approx 1$, the Weibull fits are not a significant improvement on an exponential fit.

![Figure 8](image_url)  

Figure 8: The fractions of samples that: (1) nucleate within the first hour (blue circles), (2) nucleate between 1 and 48 hours (red diamonds) and (3) do not nucleate within the 48 hours (green triangles) of the experiment. This is from datasets E to J, and the numbers are in Table 1.

We find that some samples do not nucleate during the 48 h we monitor them. This fraction decreases as the supersaturation increases, as shown in Figure 8. The fraction that crystallises in
the first hour (blue circles) increases with increasing supersaturation. This suggests that the process that induces nucleation in the first hour, and that depends on the time the solution is held in the tips, becomes more effective at high supersaturations. The nucleation rate in the period after the first hour, also increases with supersaturation. Both factors decrease the fraction of samples that do not crystallise during our experiments.

With our experiments we can accurately measure times in the 1 to 48 h range. Most droplets nucleate within this time range for concentrations between 320mg/ml to 340mg/ml, and so this is the range of concentrations we can study quantitatively.

Figure 9: The logarithm of the median nucleation time, t_{1/2}, of each extracted dataset from F to I, plotted as a function of 1/ln(S)^2, for S the supersaturation. We fit a dashed straight line to the data. The fit parameters are a gradient of m = 2.1, and a y-intercept of -4.9. Error bars are the standard errors of t_{1/2}, which have been calculated from the runs making up the respective dataset.

Comparison with classical nucleation theory

Classical nucleation theory predicts that the logarithm of the rate varies as \(-1/(\Delta \mu)^2\), for \(\Delta \mu\) the difference between the chemical potential of the solution and that at saturation. If the solution is ideal then \(\Delta \mu/kT = \ln S\), for supersaturation \(S = c/c_S\). Here \(c_S\) is the saturation concentration or solubility. The solubility at 21 °C was calculated to be \(c_S = 184 \text{ mg/ml}\) by interpolating solubility data from Yang et al.\(^{21}\)

As the datasets in Figure 7 are not all well fitted by an exponential \(P(t)\), we do not look at how the fitted rate parameter \(k\) varies with supersaturation. Instead we look at how the median
nucleation time, $t_{1/2}$, of each dataset, varies with $\ln S$. This is plotted in Figure 9. Note that this is calculated from the extracted data, i.e., the median time is defined by $f^*(t_{1/2}) = 1/2$.

We see that our data are consistent with the natural log of $t_{1/2}$ varying with $1/\ln S$. However, we note that the range of $S$ values is not large, and also that as our concentrations are over 300 mg/ml, the solution will not be ideal, and hence deviations of $\Delta \mu$ from $kT \ln S$ are likely to be significant.

**Time of Day**

Our samples are subject to a small temperature drop at night, see Figure 4. The temperature in our temperature-controlled room varied from 20.7 to 21.4°C. Using the solubility data of Yang et al., this corresponds to a solubility that varies from 183 to 186 mg/ml, and hence a supersaturation $S$ that varies from 1.82 to 1.79. This is a variation of 1.7% in the supersaturation between night and day.

To estimate the effect of this change in $S$, we can use the fit in Figure 9 and write down an expression for the ratio of median nucleation times at supersaturations $S$ and $S + \Delta S$, for small $\Delta S$: 

$$
t_{1/2}(S + \Delta S)/t_{1/2}(S) = \exp[-2m\Delta S/(S(\ln S)^3)].
$$

For a supersaturation $S = 1.81$, this is 

$$
t_{1/2}(1.81 + \Delta S)/t_{1/2}(1.81) = \exp[-11.1\Delta S],
$$

which for a 1.7% increase in supersaturation gives a 17% decrease in median nucleation time. Thus even a 1.7% change in supersaturation changes the rate by over 10%.

To test the effect of this variation in $S$, we conducted experiments with different start times. Changing the start times shifts the temperature variation with respect to the time since the start of the experiment. For example, the temperature minimum at 04:00 (Figure 4) occurs 11 h (note that 1 h is subtracted off for $f^*$ plots) after the $f^*$ start time for an experiment starting at 16:00, but it occurs at 17 h after the start of an experiment at 10:00.

The $f^*(t)$ plots are shown in Figure 10. The results of the KS test comparing $G$, $K$ and $L$ can be seen in Table 5. As we can see in Table 5 there is a significant difference between dataset $G$ (started at 16:00) and $L$ (started at 10:00). The variation in the $f^*(t)$ plots is consistent with
Figure 10: A plot of $f^*(t)$, the fraction of samples that have not yet crystallised, as a function of time, and including only those samples that crystallise within the time window 1 to 48 h.

There is a slightly higher supersaturation at night accelerating nucleation. Note that the $f^*(t)$ for the late (16:00) starting experiment has the fastest initial rate of decrease. Also, the best fit values of the rate $k$ decrease significantly as start time is made earlier, see Table 1. The periodic variation of the temperature, and hence the supersaturation, is having a measurable effect. In particular, different start times give best-fit $k$ values from $0.096 \pm 0.005$ to $0.17 \pm 0.02$ h$^{-1}$. So our best guess for the true best-fit rate, $k$, for nucleation at a truly fixed $21^\circ$C (and $c = 333$ mg/ml) is approximately $0.14 \pm 0.05$ h$^{-1}$. With our data we cannot be more accurate than that. Reducing this uncertainty would require better temperature control, as well as better statistics.

Table 5: The KS numbers comparing datasets $G$, $K$ and $L$.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>$G$ and $K$</th>
<th>$K$ and $L$</th>
<th>$G$ and $L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS number</td>
<td>1.30</td>
<td>1.08</td>
<td>2.45</td>
</tr>
</tbody>
</table>

**Polymorphism**

In this section we examine which of glycine’s three polymorphs ($\alpha$, $\beta$ and $\gamma$) were formed in our experiment. XRD samples were prepared using the same conditions as dataset $G$ ($c = 333$ mg/ml, and experiments started at 16:00). We obtained XRD patterns separately from those wells where nucleation was rapid, blue curve in Figure 11, and where nucleation occurred between 1 and 48...
hours, red curve in Figure 11. The blue curve is obtained using XRD on all crystals that nucleated in the first hour, while the red curve is for all crystals that nucleated later. We needed to collect crystals from a number of wells in order to obtain enough crystals for the XRD experiment.

For those wells where nucleation was effectively instantaneous, we see almost 100% $\alpha$ glycine. There is a peak at $2\theta = 25.1^\circ$, which is characteristic of the equilibrium $\gamma$ polymorph, but this peak is very small in comparison to the $\alpha$ peaks. This result is expected. For crystallisation at neutral pH for aqueous solution; the $\alpha$ polymorph is the expected polymorph.\textsuperscript{4–17} The $\alpha$ polymorph has been found in many experiments.\textsuperscript{4–17} These experiments were done with a range of volumes, supersaturations etc, thus the preference for the $\alpha$ polymorph (at neutral pH) is a robust result.

However, if we look at the XRD pattern (red curve) of the crystals that nucleated after 1 hour, then we see that they are mostly the $\gamma$ polymorph, although the $\alpha$ polymorph is present. Also, a few of the crystals that nucleated after the first hour have the classic distinctive morphology of the $\alpha$ polymorph; we show one in Figure 11(d). The presence of the $\gamma$ polymorph is surprising.\textsuperscript{4–17} Although the ratio of the peak heights of the main $\gamma$ and $\alpha$ peaks does not directly correspond to the relative proportion of the two polymorphs, we can use it as a rough approximation. The ratio of the height of the main $\gamma$ peak ($25.1^\circ$) to the $\alpha$ peak ($29.8^\circ$), is 0.02 : 1 for the wells that nucleated in the first hour, and 1 : 0.25 for the wells where crystallisation occurred later. A duplicate run yielded very similar results, the patterns for this run are shown in the Supporting Information.
Figure 11: (a) X-ray diffraction patterns of the crystals generated from one 96-well plate. The blue line (top) is the pattern for the samples that nucleated in the first hour, and the red line (bottom) is the pattern for the samples that nucleated in the following 47 hours. The symbols identify diffraction peaks for the $\alpha$ and $\gamma$ polymorphs. The crystals were prepared using the same conditions as dataset G. See Supporting Information for the XRD pattern of a duplicate run. (b), (c), (d) and (e) are images of single wells with crystals in them, obtained from a run at $c = 333$ mg/ml. (b) and (c) are wells where the crystals formed in the first hour, while (d) and (e) are wells where the crystals nucleated after the first hour of the experiment. Note that in (b) two crystals have formed while in (c) many crystals have formed; for nucleation in the first hour we frequently observe multiple crystals. There is only one crystal in (d) and one in (e); (d) has the classic morphology of the $\alpha$ polymorph,\textsuperscript{18} while (e) may be the $\gamma$ polymorph. Each well is 6.8 mm across.

When we estimated the growth rates of crystals that nucleated after the first hour (see Figure 3) we found that a few crystals appeared to grow roughly 10 times faster than the most common growth rate, which is near 0.02 mm/min. As we expect most of the growing crystals to be the
γ polymorph, with a few of the α polymorph, it is possible that the crystals near the peak of the growth rate histogram of Figure 3 are of the γ polymorph while those in the fast-growth-rate tail are of the α polymorph. In other words although there is variability in growth rate between crystals of the same polymorph, that some of the spread in growth rates seen in Figure 3 is due to us following the growth of crystals of both polymorphs. We plotted the growth rates as a function of nucleation time, to look for a correlation, but the data did not show a significant correlation.

### Nucleation of the second crystal in samples where a crystal is already present

![Figure 12: A plot to compare nucleation of the first crystal, to nucleation of the second crystal in the same well, from the five runs of dataset G. The blue circles are \( f_{23}^\#(t) \), the fraction of samples that have not yet crystallised, as a function of time, including only those samples that crystallise within the time window 1 to 23 h. The red diamonds are \( f_{23}^{(2)}(t) \), the fraction of samples in which one crystal has appeared but in which a second crystal has not yet appeared. This is as a function of the time since the first crystal nucleated, and it is calculated using only those samples that crystallise within the time window 1 to 23 h. In that time window, 256 samples formed at least one crystal, of which 119 formed a second crystal. The green curve is a fit of a Weibull function to \( f_{23}^{(2)} \); the fit parameters are \( \beta = 0.25 \) and \( \tau = 165 \) h.

In this work we focus on the formation of the first crystal in a solution that does not contain pre-existing crystals. However, we do in fact have data on the times for the formation of additional crystals in samples where there is already a crystal. This is plotted in Figure 12. To compare formation of the first and second crystal on an equal footing, we compare nucleation data over
a period of 23 hours. We only have 48 h of data and so can only study both first and second nucleation over 23 hours each. So in Figure 12 we have plotted $f_{23}^*(t)$, the fraction of samples that have not yet crystallised, including only those samples that crystallise within the time window 1 to 23 h. We compare this to $f_{23}^{(2)}(t)$, the fraction of the samples in which a second crystal has not nucleated, as a function of the time since the first crystal nucleated. The fraction $f_{23}^{(2)}$ is calculated using the same samples as used for $f_{23}^*$.

We see that the effective nucleation rate for a second crystal starts off fast but rapidly slows over a few hours. The observation that the rate decreases with time is not surprising, because the supersaturation is decreasing with time as the first crystal grows. Note that as we saw in Figure 3, there is substantial variability in the growth rates and so some crystals do take a few hours to grow. Thus, we expect that at least in some cases the supersaturation takes a few hours to drop down to the saturation concentration for the polymorph of the first crystal. This decreasing supersaturation is an obvious explanation for the decreasing nucleation rate over the first few hours.

Secondary nucleation is, by definition, nucleation caused by a pre-existing crystal. For example, if early in crystal growth a nanocrystallite broke off from an existing crystal and started growing, we would see two crystals appear simultaneously or nearly simultaneously. It is possible that the initial fast rate (steep decrease of $f_{23}^{(2)}$) is due to secondary nucleation. But as we cannot observe nucleation directly, we do not know whether or not the second crystals we observe form via secondary nucleation.

**Conclusion**

Quantitatively studying the nucleation of crystals is difficult. The rate is extremely sensitive both to impurities and to the conditions in which samples are prepared. It also varies very rapidly with supersaturation; in the case of our glycine experiments, even a 1% change in supersaturation can introduce a 10% variation in the rate. We have mostly overcome these challenges to produce data that we show is reproducible, over a range of supersaturations. For example we found a best-fit
rate, \( k \approx 0.14 \pm 0.05 \text{ h}^{-1} \), at a concentration \( c = 333 \text{ mg/ml} \ (S = 1.81) \), and a nominal \( T = 21^\circ \text{C} \). Reducing the uncertainty in \( k \) would require temperature control to within 0.1°C, as well as more data to reduce the statistical uncertainty.

We believe that our approach, with its application of statistical tests for reproducibility, will be useful in future work. For example, it could be used to test for the effects of impurities or materials deliberately added to induce nucleation. It is likely that our crystals are nucleating heterogeneously on impurities, but we do not know what these impurities are or where they come from. By systematically varying the purity of the glycine, water and oil and applying our statistical tests, it should be possible to infer if impurities are indeed causing nucleation, and if so from where they come. Nucleation may also be occurring at the oil/solution interface, in which case it may depend on which oil is used. In many systems where the solubility varies rapidly with temperature, it may also be good to test for the effect of variations in temperature, even if they are only 1°C or less.

Finally, we were surprised to find that although those crystals that nucleated immediately or almost immediately were of the expected \( \alpha \) polymorph,\(^4-17\) those that nucleated later were of the \( \gamma \) polymorph. It is possible that, if seeding is excluded, the nucleation rate of the \( \gamma \) polymorph is higher than that of the \( \alpha \) polymorph. If so, we may need to rethink our understanding of why the \( \alpha \) polymorph is usually obtained.

**Acknowledgements**

It is a pleasure to thank Violeta Doukova (Surrey) for technical assistance, Dan Driscoll (University of Surrey) for performing the X-ray diffraction, and Sharon Cooper (University of Durham) and Baron Peters (University of California Santa Barbara) for helpful discussions. We acknowledge EPSRC for funding a PhD studentship for LJL.
Data Availability

The authors confirm that data underlying the findings are available without restriction. Details of the data and how to request access are available from the University of Surrey publications repository: [http://epubs.surrey.ac.uk].

Supporting Information

This contains: a description of our fitting procedure; a plot of temperature as a function of time during cooling to room temperature; details of how we estimate growth rates; a temperature profile over one week, and an additional XRD pattern. The Supporting Information is available free of charge on the ACS Publications website at DOI: TBA.

References


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Does the $\gamma$ Polymorph of Glycine Nucleate Faster?

A Quantitative Study of Nucleation from Aqueous Solution

Little, Sear and Keddie

We advance the quantitative study of nucleation by combining the study of hundreds of samples, with statistical tests for reproducibility, and for well defined nucleation rates. Unlike in most previous work, we find that in the samples that nucleate after the first hour of our experiment, the equilibrium glycine polymorph predominates over the alpha form.