

Optical spectroscopy of crystal nucleation one nucleus at a time

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Abstract

While crystallization is a ubiquitous and an important process, the microscopic picture of crystal nucleation is yet to be established in spite of decades of dedicated research.^{1,2} Recent studies agree that the nucleation process is more complex than the view offered by the classical nucleation theory.³⁻⁸ Although optical spectroscopy has the potential to capture the details of nucleation dynamics, its application has been hampered by the stochastic and heterogeneous nature of crystal nucleation. Here, we implement an innovative Single Nucleus Spectroscopy (SNS) by combining Raman microspectroscopy and optical trapping induced crystallization (OTIC) to investigate crystallization one nucleus at a time. Raman spectral evolution during a single glycine crystal nucleation measured by SNS and analyzed by a non-supervised spectral decomposition technique uncovered the Raman spectrum of pre-nucleation aggregates as well as its critical role as an intermediate species in the dynamics. The agreement between the Raman spectrum of pre-nucleation aggregates and our simulated spectrum from glycine solutions further suggested that loosely-bound linear chains of glycine are the likely precursors of crystallization. These results highlight the power of performing optical spectroscopy at one nucleus at a time and could change how optical spectroscopy tackles the crystal nucleation problem.

Crystallization is an important process in a wide range of disciplines from fundamental science to industrial application.^{1,9,10} Despite the importance of controlling the crystallization and its morphology (*e.g.* polymorphism), the lack of microscopic description of crystal nucleation often limits the rational approach to its engineering and control.⁹ A major challenge in establishing the molecular level understanding of crystal nucleation is its stochastic and heterogeneous nature at the nanoscale.¹ This has long prevented experimentalists to directly observe the nucleation event in real-time. Statistical approaches such as counting nucleation rate under various crystallization condition have been a major experimental method to circumvent the difficulty.^{1,2} The discrepancy between the nucleation rate obtained from the statistical experiments and the one predicted by classical nucleation theory (CNT) has led to a general agreement that crystal nucleation is a more complex phenomenon than how it is described by CNT.²⁻⁵

A real-time observation of nucleation dynamics has been long desired as a critical step in establishing its microscopic picture. For example, crystallization of colloidal particles has been used as a model system to mimic the dynamics of crystallization because the assembly process of particles can be easily visualized in real-time by optical microscopy.^{11,12} Recent breakthrough in tackling this problem was the development of cryo- and liquid cell transmission electron microscopy (TEM) for visualizing crystallization of atomic/molecular systems.¹³⁻¹⁵ The presence of morphologically "featureless" or "amorphous" clusters was confirmed in these TEM studies,^{7,16} and in-situ TEM results caught the moments that the clusters became well-defined crystals.^{6,17,18} More and more studies show the involvement of pre-nucleation aggregates in crystal nucleation process and confirm the complexity of crystal nucleation process in contrast to CNT. A key question is to understand the structural dynamics of pre-nucleation aggregates toward the phase transition.

Optical spectroscopy could be a powerful technique to extract the molecular level details of the structural dynamics of pre-nucleation aggregates towards the phase transition. The application of optical spectroscopy on crystal nucleation problem has been, however, limited because the stochastic and heterogeneous nature of the nucleation process is detrimental to the interpretation of the spectroscopic signals. If one probes a large volume of a sample to capture a nucleation event that can occur anywhere

at any time, the obtained spectroscopic signal is obscured by the average of various species in solution (*e.g.* monomers, aggregates, and crystals). Although not applied in this field yet, there is a well-established powerful concept to deal with a stochastic, complex and heterogenous system: *Single molecule spectroscopy*.¹⁹ The key to bring optical spectroscopy with its full potential is to probe *single* nucleation event at a time, only if we can predict precisely where a nucleation occurs.

Herein, we report the first demonstration of single nucleus spectroscopy (SNS) which spectroscopically probes crystal nucleation process one nucleus at a time. SNS is based on a novel extension of optical trapping Raman micro-spectroscopy, a well-established tool to study a trapped single object.^{20–22} In this work, optical trapping was used to induce a crystal nucleation from a focused laser spot instead of as a tweezer to trap an object. Optical trapping induced crystallization (OTIC) was first demonstrated by Sugiyama *et al.* in 2007 by focusing a near infrared (NIR) laser in supersaturated glycine/D₂O solution.²³ Since the discovery, OTIC has been applied to a variety of systems,^{24–26} and furthermore high quality single crystals can be prepared while the polymorphs can be controlled by laser polarization.^{26,27} Our approach applies OTIC to spatially control a single crystal nucleation event (*i.e.* at a focused laser spot), so that a probe beam can be placed at the same position to track the nucleation process. We achieved measuring Raman spectral evolution of a *single* glycine crystal formation in solution with 46 ms time resolution at room temperature. The fast spectral acquisition allowed us to extract the Raman spectrum of pre-nucleation aggregates and its kinetics during a nucleation. The comparison between the experimental and simulated Raman spectra of glycine aggregates provided new insights into the glycine crystallization dynamics such as the formation of loosely-bound linear chains toward the nucleation.

Results and Discussion

The *in-situ* SNS setup to track *single* crystal nucleation dynamics was custom built based on an inverted microscope (Fig. 1a). While OTIC has been performed using a NIR laser in the past,^{23–28} we employed a 532 nm laser that served the dual role of inducing a crystal nucleation and Raman excitation (Fig. 1b). This design has two major benefits: i) the setup is as simple as conventional confocal microscope; ii) high laser power (> 1W at a sample) required to induce crystallization produces high

Raman signal (*c.f.* typical Raman spectroscopy is performed using ~ 10 mW with long accumulation time), which increases the temporal resolution of Raman spectroscopy (46 ms time resolution in this report). The other benefits of a 532 nm laser compared with a NIR laser is that Raman scattering is more efficient and the temperature of water at laser focus does not increase (~ 2 mK W^{-1}).²⁹ The polarization of the laser beam was randomized by a depolarizer to minimize any laser polarization effect on crystallization process to mimic a “natural” crystallization occurring in solution. This also simplified the interpretation of Raman spectroscopy by eliminating Raman spectral variation due to polarization selection rules.

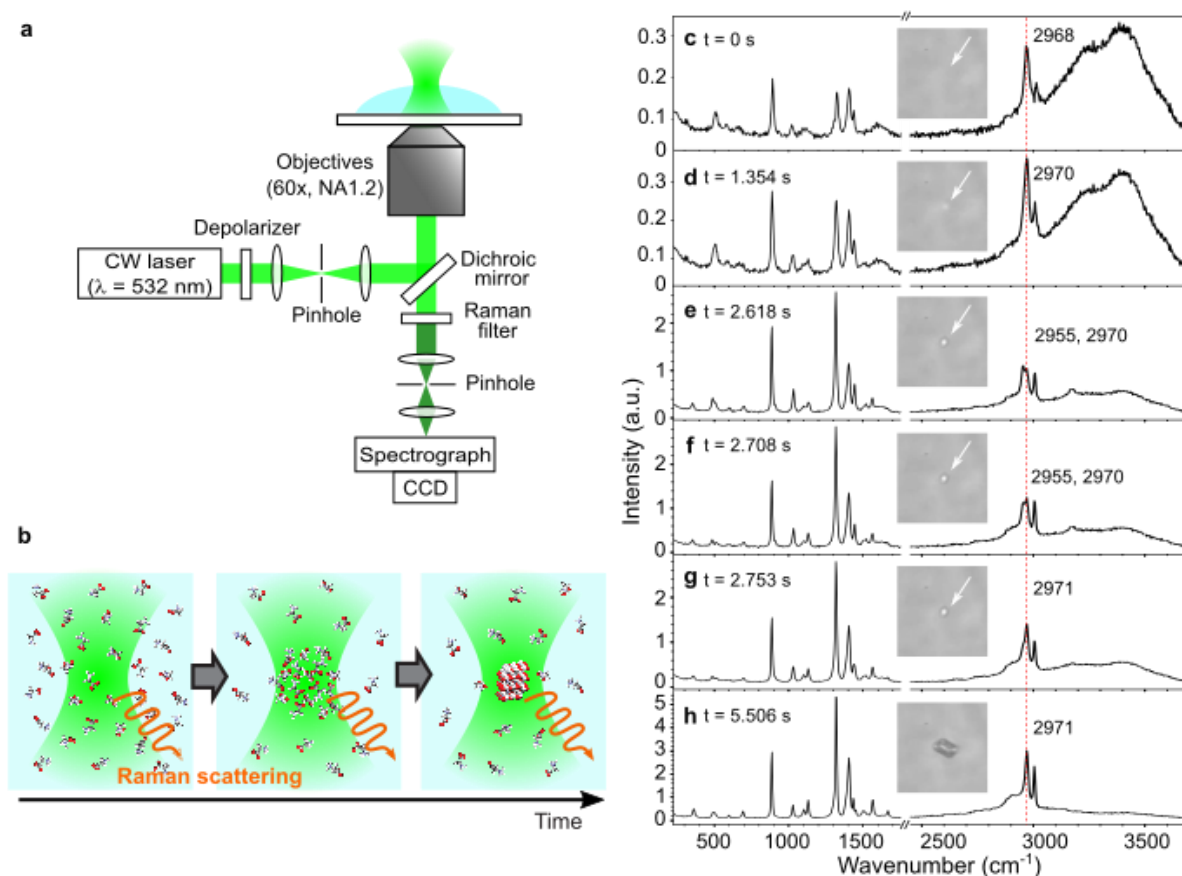


Fig. 1 Single Nucleus Spectroscopy (SNS): *In-situ* Raman spectroscopy during α -glycine crystallization at single nucleus at a time. **a**, A simple scheme of the home-built SNS setup to achieve *in-situ* Raman measurement during *single* crystal nucleation. **b**, A cartoon representing the dual role of 532 nm CW laser. A tightly focused CW laser induces crystallization at the focus while it serves as Raman excitation laser to track the crystallization dynamics. **c-h**, Snapshots (46 ms time resolution) of Raman spectral evolution during α -glycine crystallization. Insets are the bright field microscopy images ($16 \times 16 \mu\text{m}$) taken at the corresponding time to each acquired spectrum.

Glycine was chosen as a model system in this study. While glycine has been widely studied as it is the simplest amino acid, its crystallization process has been shown rather complex and is under active debates.^{30–36} Fig. 1c-f shows a series of Raman spectra obtained *in-situ* during a *single* crystal nucleation of glycine which highlights the dynamic nature of crystal nucleation (see the movie SI1 for the whole experiment). Over the course of a nucleation, broad Raman bands around 3200-3500 cm⁻¹ due to O-H vibration of water molecules decreased. This coincides with the appearance of a small blurry object in the bright field image starting from the frame Fig. 1d. These observations can be interpreted that the local concentration of glycine molecules increased and pushed water molecules out of the confocal detection volume. The formation of a crystal is clearly captured with the increase of Raman intensity and the appearance of new peaks (Fig. 1e) even if the bright field image remains blurry. Among various spectral changes upon nucleation, the appearance of the Raman peak at 2955 cm⁻¹ caught our immediate attention because α -glycine (C-H stretching mode: ~2970 cm⁻¹) was expected to form in water. From the comparison of the feature with the reference spectra measured on the same setup from α -, β - and γ -glycine (Fig. SI1), the species was identified as β -glycine. The Raman spectral feature of β -glycine was short-lived and quickly became that of α -glycine (Fig. 1f-g).

The spectral evolution during a glycine nucleation was analyzed using non-negative matrix factorization (NMF), a type of unsupervised principal component analysis used in machine learning analysis (Fig. 2).³⁷ NMF has been used to deconvolute spectra in the field of Raman spectroscopy, and a powerful aspect of this analysis algorithm is that a set of partial Raman spectrum (PRS) as well as the amplitude of each PRS during the spectral evolution can be obtained without any assumption concerning the spectral shapes. The number of PRS used for the deconvolution is the only pre-determined parameter. We first applied NMF analysis with two PRS to the spectral series in which clear feature of β -glycine was observed for several seconds before converting to α -glycine, to minimize the complexity of the crystallization dynamics (Fig. 2a). The analysis with two PRS did not reach good fitting results (Fig. SI3), which indicates that the glycine crystal nucleation does not occur through CNT mechanism where the whole spectral evolution should be reproduced by a linear combination of the spectrum of solution and crystal. Instead, good quality fit was achieved with three PRS (Fig. 2b-f). One

of the PRS (PRS-2) matches well with the Raman spectrum of β -glycine. Interestingly, the spectra before the appearance of β -glycine were deconvoluted to two PRS (PRS-1 and 3).

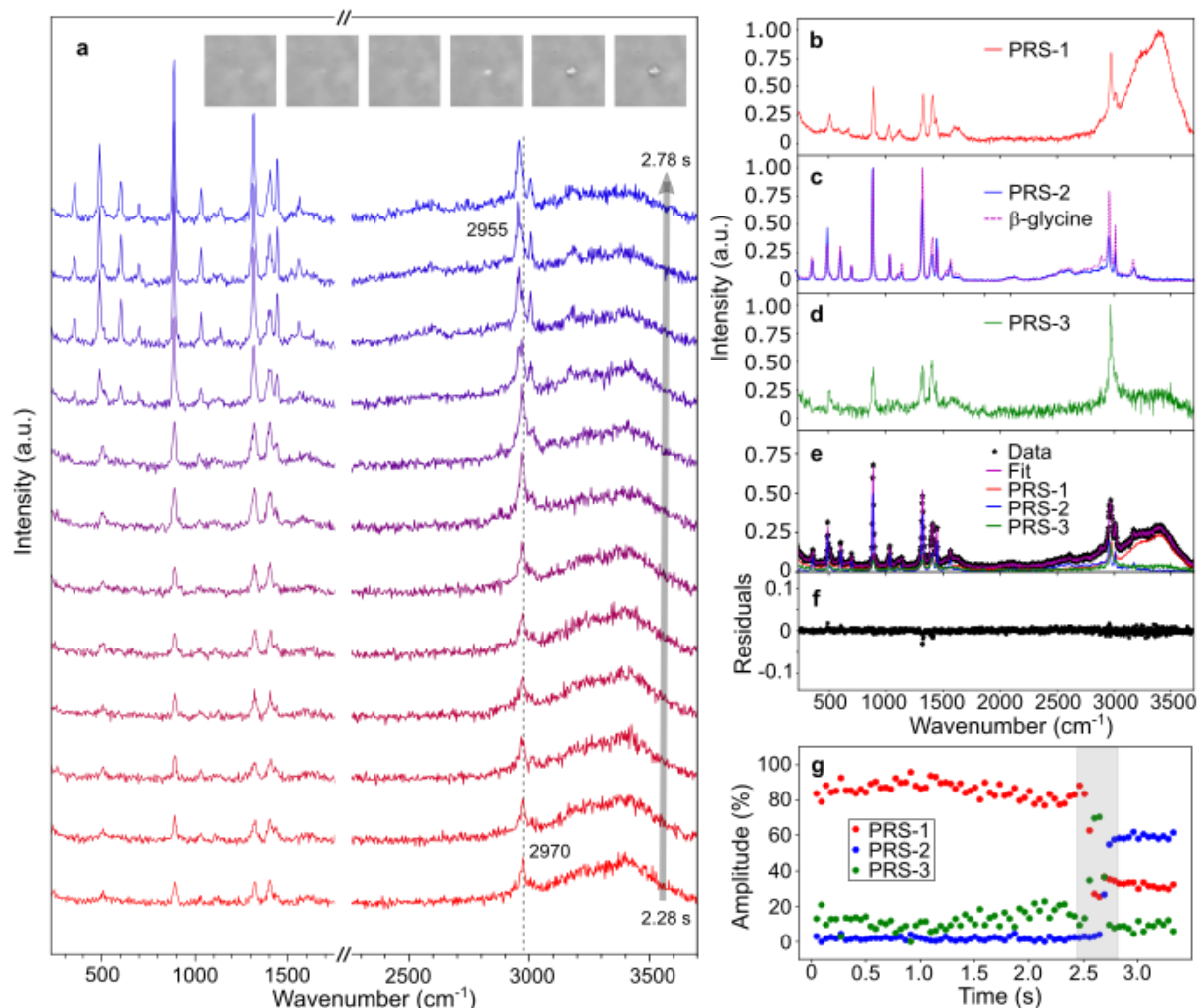


Fig. 2 Raman spectral evolution during a β -glycine crystal nucleation and its non-supervised data decomposition analysis. **a**, Snapshots of Raman spectra showing the phase transition from solution (bottom) to crystal (top) with the bright field microscopy images (16x16 μm) corresponding to every other spectrum (at 2.28 s to 2.78 s from the left to right) as insets. **b-d**, Three spectra obtained by non-supervised data decomposition of the series of spectra in the panel **a** (PRS: Partial Raman Spectrum 1-3). **e**, An example of the fit to the data (the frame at 2.692 s) by three constituents and **f**, the residuals of the fit. **g**, Temporal evolution of each constituent amplitude during the crystal nucleation.

If we assumed that crystal nucleation occurred via non-classical nucleation pathway, PRS-1 can be interpreted as the Raman spectrum of monomers and PRS-3 as that of aggregates. This assumption fits well with the temporal evolution of each PRS amplitude (Fig. 2g). Initially, the spectra

were composed of 80~85 % of PRS-1, 0 % of PRS-2 and 15~20 % of PRS-3. Just before the nucleation occurred at ~2.65 s (the sudden increase of the amplitude of PRS-2), the amplitude of PRS-1 decreased while that of PRS-3 increased (highlighted by grey color). Once a crystal formed, the amplitude of PRS-3 dropped down to almost zero. PRS-1 does not go down to zero at this time range, because there are remaining solution in the focal volume as the size of a crystal is still too small to occupy the whole focal volume. This kinetics was reproduced qualitatively in many experiments although there are some variances between the experiments due to the stochastic nature of crystallization (Fig. SI4 and Fig. SI5 for more examples).

It is worth noting that the amplitude of PRS-3 was steadily 15~20 % before the nucleation event. This could mean that the aggregates of glycine molecules are abandoned in a supersaturated aqueous solution. To test this hypothesis, we measured Raman spectrum of glycine aqueous solution as a function of concentration (Fig. 3a). To minimize an effect of optical trapping on the local concentration, a low laser power (50 mW) was used for this series of measurements. The intensity of Raman spectrum increased as the concentration, as expected. The intensity, however, did not increase proportionally as a function of the concentration but rather showed saturation behavior (Fig. SI6b). This suggests that the spectrum is composed of more than one species at higher concentration. In fact, NMF analysis with two PRS resulted in an excellent fit to the series of Raman spectra (Fig. 3b, PRS-1' and 2', Fig. SI7). The amplitude of PRS-1' is dominant at low concentration, and it decreases as the concentration increases while that of PRS-2' increases (Fig. 3c). From the concentration dependence on the amplitude, we assign PRS-1' as the spectrum of glycine monomers (dilute phase) and PRS-2' as that of glycine aggregates.

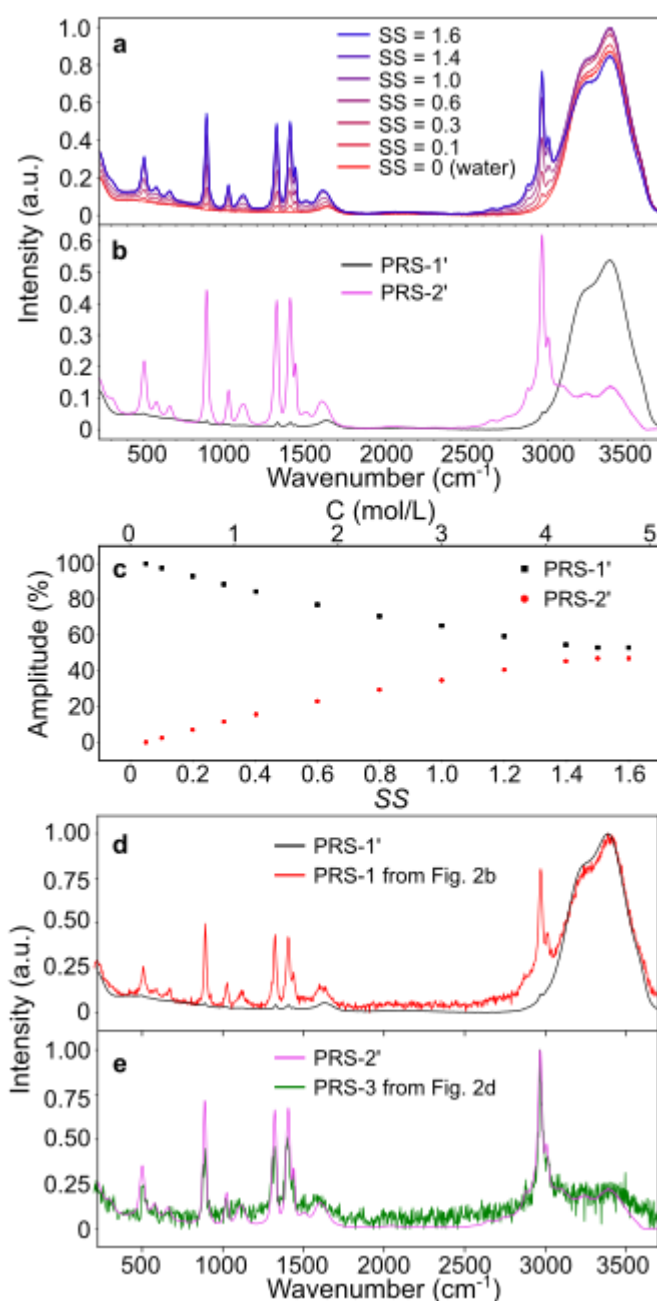


Fig. 3 A series of Raman spectra of glycine solution at different concentration and its NMF analysis revealing the spectrum of glycine monomers and aggregates. a, Raman spectra of glycine solution at different concentration. **b**, Two PRS constituting the series of spectra in the panel **a** (PRS 1' and 2'), obtained from the NMF analysis. **c**, The amplitude of each component as a function of the concentration. **d**, The overlay of PRS-1' and PRS-1 obtained from the nucleation dynamics (Fig. 2b), and **e**, PRS-2' and PRS-3 (Fig. 2d).

Remarkably, the PRS assigned as monomer (PRS-1') and aggregates (PRS-2') from the concentration series matches the spectral feature of PRS-1 and 3 obtained from β -crystal nucleation dynamics (Fig. 3d-e). This strongly supports our interpretation of the observed nucleation dynamics (Fig. 2) discussed earlier. Crystal nucleation of glycine occurs in non-classical pathway where pre-

nucleation aggregates are formed and converted to a crystal. While there are always some aggregates present under the optical trapping laser, the rapid increase in the amplitude of aggregates which leads to crystal nucleation starts rather stochastically. It remains unclear what is the critical difference between the aggregates abandoned in solution and the pre-nucleation aggregates that convert to a crystal. As their Raman spectra matched well (Fig. 3e), aggregates that are present in solution and pre-nucleation aggregates may be structurally similar, except that we can only compare the time-averaged spectrum over 46 ms. It will be crucial to track the structural fluctuation at faster time scale to gain further insight. The differences between individual aggregates and “pre-nucleation aggregates” will be certainly an important aspect to be spectroscopically explored in the future.

While the mechanism of glycine crystallization from aqueous solution has been under intense debates last decades, the direct comparison between simulated and experimental results has been challenging. This is the first time that Raman spectrum of pre-nucleation aggregates was identified, and this provides us a crucial link to the theoretical investigation of its structure. Recent studies based on molecular dynamics simulation suggested the presence of aggregates at high concentration of glycine aqueous solution,^{34,38,39} which agree with our observation. In this work, we were determined to gain an insight into the conformations of the pre-nucleation aggregates. To this end, we ran molecular dynamics simulations at two concentrations: low (3.3 mol L⁻¹) representing the initial concentration in the experiments and high (5.2 mol L⁻¹) modeling a putative concentration before nucleation. We have analyzed the sampled configurations (Fig. 4a-b) and performed Quantum Chemistry calculations to obtain Raman spectra from glycine clusters of varying sizes. The agreement between the simulated and the experimental Raman spectra (see Fig. 4c-e) has formed the basis for our interpretation of the computational results.

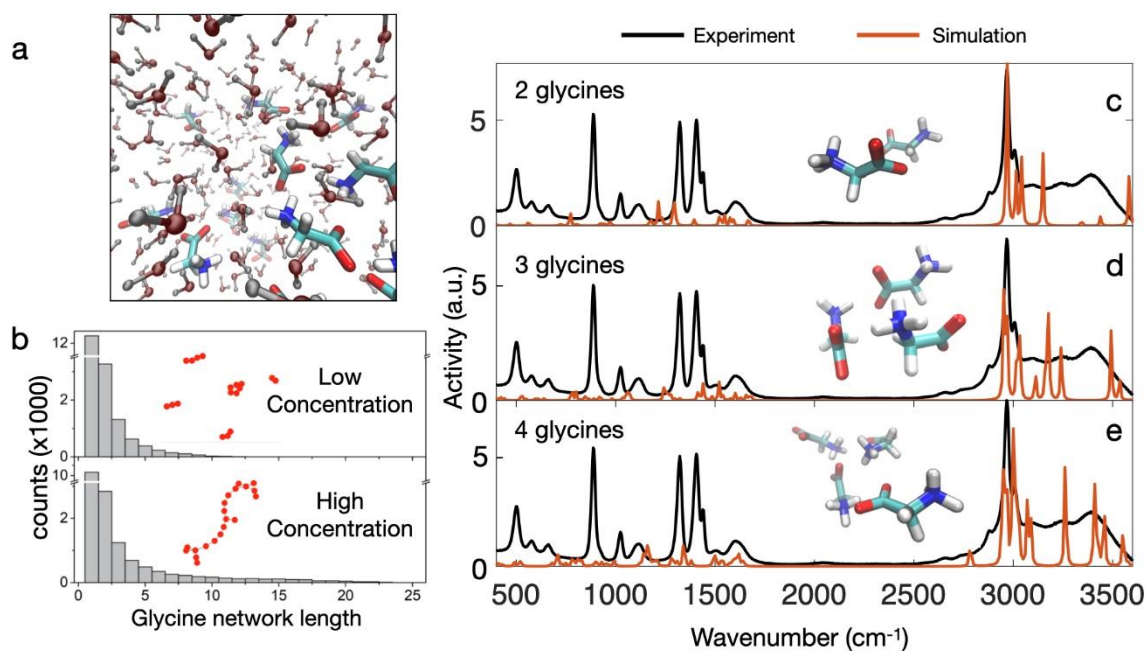


Fig. 4 Molecular dynamics (MD) simulation of glycine solutions and Quantum Chemistry calculation of the sampled glycine clusters. **a**, A snapshot from a MD trajectory of glycines in water; **b**, the effect of concentration on the formation of glycine-glycine contacts: at low concentration glycine predominantly exist as monomers, and as the concentration increases loosely-bound linear chains form. The insets are showing samples from the network visualizations: red circles are individual glycines, and the lines between them indicate contacts. Disconnected glycines are omitted. **c-e**, (red) simulated Raman spectra from glycine clusters of increasing sizes sampled using MD: **c**) two , **d**) three , and **e**) four glycines; the experimental PRS2'assigned to aggregates is shown in black.

Snapshots obtained from the MD simulation of glycine aqueous solution (Fig.4a, Fig. SI8) showed that glycine forms loosely bound hydrogen-bonded linear chains within the simulation box. We call these networks “loose” in the sense that doubly hydrogen bonded cyclic dimers⁴⁰ are almost never observed. Fig. 4b shows the histograms of the lengths of glycine networks in our simulations. The histogram on the top shows that at low concentrations monomers and loose dimers dominate the solution. As the concentration increases larger clusters emerge. The insets show the results of the network visualization analysis: red circles are individual glycines and the lines between them indicate contacts (2.5 cut-off was used). The calculated Raman spectra for the clusters of two, three, four glycines (Fig. 4c-e) show that the peaks gradually ‘fill in’ the broad spectroscopic feature observed in experiments (black), especially in the region of 2500–3500 cm⁻¹ as the size of the clusters increases. A broadening of Raman spectrum is resulting from the heterogeneity of the conformation of individual

glycines in the hydrogen bonded clusters (See movie SI2 and SI3). The agreement between theory and experiment indicates that the structure of pre-nucleation aggregates may be loosely-bound linear chains.

Recent studies based on X-ray diffraction and solid-state NMR on flash cooled glycine aqueous solution proposed a new hypothesis that α -glycine crystallization occurs via glycine dihydrates and β -glycine.^{33,35,41} The presence of glycine dihydrates in water at room temperature is yet to be confirmed. Our results suggest another nucleation pathway that loosely-bound linear chains of glycine clusters may be the precursors to constitute the hydrogen bonded networks observed in the crystal structure of β -glycine.

In summary, we demonstrated a novel *in-situ* optical spectroscopy to study crystallization dynamics of glycine one nucleus at a time. Spectral dynamics obtained during single glycine nucleation event from aqueous solution at room temperature along with non-supervised spectral decomposition analysis provided new evidences to understand the nucleation dynamics: i) glycine crystallization occurs via non-classical nucleation pathway where pre-nucleation aggregates grow and nucleate; ii) the comparison between the experiments and simulations using the Raman spectrum of pre-nucleation aggregates suggests that glycine molecules form loosely-bound linear chains which may be the precursor to β -glycine crystal. Experimental investigation of crystal nucleation by optical spectroscopy has been hampered by the stochastic and heterogenous nature of the nucleation. An *in-situ* spectroscopic approach presented in this study, “single nucleus spectroscopy”, can be regarded as a basic platform where more advanced spectroscopy techniques can be coupled to. We believe this is a crucial breakthrough to bring more optical spectroscopy in to further accelerate the investigation of crystal nucleation.

Methods (3000 words max)

Sample preparation

Glycine (Sigma-Aldrich) was used without further purification. Glycine aqueous solution was prepared using a ultrapure water (MilliQ). Glycine was dissolved at 80 °C using a ultrasonication, and

filtered with a 0.2 μm Target2TM Nylon syringe filter (Thermo Scientific), and then slowly cooled down (~3 hours) to room temperature in a dry bath (Thermo Scientific). The degree of supersaturation (SS) was calculated using the solubility of glycine in water at 20 °C (0.225 g ml⁻¹).⁴²

For SNS, a sample was assembled by placing a silicone isolator sheet (0.25 mm thick, Grace Bio-Labs 664475) with a 10 mm hole on a piranha cleaned cover glass. The silicone sheet was cleaned in methanol before the use. 10 μL of glycine aqueous solution (SS = 120 %) was dropped in the hole which resulted in a thin film of glycine solution (100 μm thickness). Another cover glass was placed on top to prevent the evaporation of water. No spontaneous crystallization was observed for ~1 hour.

Conventional crystallization was performed to obtain α -, β -, and γ -glycine to measure Raman spectrum of each phase (Fig. SI1). α -glycine was prepared by the slow evaporation of glycine aqueous solution. β -glycine was prepared by adding methanol to aqueous solution of glycine. γ -glycine was obtained by the slow evaporation of glycine aqueous solution with potassium nitrate as additives (see more details in Supplementary Information).

Single Nucleus Spectroscopy (SNS) setup

1) General scheme

The optical setup (Fig. 1a) was home-built based on an inverted microscope (Olympus IX73). A 532 nm CW laser (Laser Quantum, Opus 532) was used with a dual role for optical trapping and Raman excitation. The diameter of the laser beam was properly adjusted by a telescope to slightly overfill the back aperture of water-immersion objective lens (Olympus UPLSAPO60XW, NA1.2). Liquid crystal polymer depolarizer (Thorlabs, DPP25-A) was used to randomize the polarization of the laser beam. A periodic retardation pattern of the depolarizer generates two focus spots due to diffraction. The beam after the depolarizer was once focused by a lens to spatially filter one of the spots and then was recollimated (Fig. SI9). A dichroic beamsplitter (AHF analysentechnik AG, Raman beamsplitter RT 532 rdc, F78-535) and a 532 nm RazorEdge® ultrasteepest long-pass edge filter (Semrock, LP03-532RE-25) were used to remove the excitation beam from Raman scattering. The signal was spatially filtered at the conjugate plane using a 25 μm pinhole to remove the contribution of extrafocal volume. The size

of the pinhole adopted in this setup is smaller than the one in typical setups ($\sim 100\ \mu\text{m}$). Therefore, the contribution of Raman signal from the extrafocal volume is significantly reduced and the spectral sensitivity to the nucleation dynamics occurring at the laser focus is improved.⁴³ Raman spectrum was recorded by using a spectrograph (Andor, Kymera 193) and a CCD (Andor, iDus420) with a grating with a density of 900 l/mm blazed at 550 nm (Quantum Design AG, A-SR2-GRT-0900-0550).

2) Technical details of *in-situ* glycine crystallization

The depolarized laser beam was focused just below ($\sim 1\ \mu\text{m}$) the liquid-air interface of glycine solution. While it has been reported that the height of the liquid-air interface continuously changes during OTIC with a 1064 nm laser,⁴⁴ almost no change of the height was observed in the experiments with a 532 nm laser. This could simply be because there is no heating at the focus when 532 nm is used. OTIC with a 532 nm laser not only allows one to use aqueous solution instead of deuterated (D_2O) solution, but also reduces complications related with heat-induced flows around the focus spot. Although the height of the liquid-air interface was stable, we also verified that the spectral shape near the liquid-air interface does not change due to the focus position relative to the interface (Fig. SI10). When the beam is focused at or above the interface, Raman signal is lower because the half or more of the focus probes air. Besides the intensity, the spectral shape remained unchanged.

Data analysis

The raw data were noise-filtered by Singular Value Decomposition (SVD, see Fig. SI2). The cleaned spectra were analyzed by Non-negative Matrix Factorization (NMF) using the Scikit_Learn library in Python.⁴⁵ The python code and the data are available on Yareta. For more details, see the Supplementary Information.

Computational simulations

Molecular dynamics simulations were run on the zwitterionic form of glycine in a $8\ \text{nm}^3$ water box using the AMOEBA 2013 forcefield⁴⁶ implemented in the OpenMM toolkit⁴⁷ at two concentrations: $3.3\ \text{mol L}^{-1}$ and $5.2\ \text{mol L}^{-1}$. The Raman spectra calculations were performed on clusters obtained

from the MD trajectories using the Gaussian 16 suite of programs⁴⁸ with B3LYP/6-311G(d)³¹. For the complete technical discussion, please see the Supplementary Information Section SI2.

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Author Contributions

T.B.M.A. designed the initial experiments. T.B.M.A., O.U. and J.B. built the setup and finalized the design of the experiments. O.U. performed most of the measurements under supervision of T.B.M.A and J.B.. J.B. introduced NMF analysis, and O.U. and J.B. performed most of the NMF analysis. N.L and L.S. performed the computational simulations and analysis. All authors were involved in drafting the manuscript.

Competing Interests Statement

The authors declare no competing interests.

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