nature portfolio

Peer Review File

Direct observation and control of non-classical crystallization pathways in binary colloidal systems

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a very nice paper with a very clear demonstration of complexity pertinent to crystallization. It captures nicely all the stages, though (as in many other works) the nucleation remains enigmatic. It can be published after addressing the following:
1. Direct analogy with atomic/molecular solids is problematic, and while it is relevant, it must be tuned down.
2. More quantitative analysis must be done a the data allow it. Thus, order parameter can be employed, and other methods as well. It may help to see the nucleation events. In particular, analysis of the SEM of intermediate structures would be important, and will be more convincing than multiple cartoons.
3. Citing the works of Rybtchinski et al. on protein crystallization and Vekilov/Rimer on organic crystallization is needed in light of its direct relevance to the observed phenomena.
4. There is a tendency to present a lot of simulations (which are nice) and less SEM. It would be important to present more SEM data.
5. Fig. E2 is not serving the flow of the text properly.

Reviewer #2

(Remarks to the Author)
The authors of this paper present yet another fantastic study on colloidal crystallization under highly controlled conditions. The use of highly monodisperse particles species with finely tunable attractive interactions, combined with density and refractive index matching allows visualizing previously unattainable conditions. Moreover, the authors use a very simple, yet highly controlled salt-diffusion gradient to slowly modulate local salt concentration and obsereve a wealth of different crystal structures under controlled conditions.
The experimental data are supported by numerical simulations close matching the data and adding insights on the proposed mechanisms for crystal nucleation and growth.

While the manuscript does not necessarily bring new physiscs in, it is nonetheless an impressive study showing the details of binary crystal growth under controlled conditions and certainly deserves to be published.

The paper is well-written with captivating illustrations and full details to reproduce experiments and simulations. I do not really have anything to add to it, other than a simple curiosity concerning the unidentifed dendritic structure. It would be very interesting to see an SEM image of the dendrite and to read the authors' opinion on the origin of its formtaion and on its properties.

Reviewer #3

(Remarks to the Author)
This manuscript presents a study of the nucleation and growth of binary colloidal crystals. The combination of experimental studies and MD simulations reveals a predominant non-classical nucleation pathway accompanied by multiple growth habits. Furthermore, the authors correlated various experimental parameters, such as bonding strength, volume fraction, and size ratio. The experiments are extensive, and the figures are beautiful. Several issues need to be addressed before publication.

1. It is not clear what is the motivation of the work since the work seems to be written in a highly condensed form (no regular introduction section but just directly went into the results after the abstract). Is it because the electrostatic interaction driven binary colloidal crystals have some unique properties from other colloidal crystals? Why does crystal morphology matter? Are there any fundamental knowledge gaps in the field? At least the authors need to review and comment on current literatures.
2. The field of colloidal crystallization has well-established quantitative methods for distinguishing different phases, such as global/local order parameters, local density, and bonding stability. In particular, during the nucleation stage, these analyses are essential to differentiate phase evolution (e.g., amorphous vs. crystalline). However, the current manuscript relies primarily on the look of time-lapse confocal optical microscopic images. At least some quantitative method should be employed to characterize phase transitions rigorously.
3. The manuscript describes two experimental setups: static and dialysis. It appears that open lattice structures arise only in the dialysis setup, potentially due to kinetic effects influencing local particle/ion diffusion and saturation. These structures are not replicable under static equilibrium conditions. The authors need to either address this limitation or present the detailed mechanistic reasoning and provide real-time, real-space data on the formation process (e.g., classical nucleation pathways) using the dialysis setup.
4. Figure 4 is quite out of place with the rest of the manuscript. The manuscript is mostly about non-classical crystallization pathways. What is the logical connection of Figure 4 with the rest of the text? For example, the content in Figure E3 appears more relevant to the paper's topic than Figure 4.
5. In Figure 1, the structures are predominantly polycrystalline without clear facets. In contrast, Figure 2 depicts monomer-by-monomer addition starting from a single crystal. Why is there such discrepancy?
6. The morphology of the blobs in Figure 2 is very similar to that of the nuclei in Figure 1. How do the authors differentiate between these structures?

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have no further comments, except for one - it seems that the new references were not properly added. I suggest double-checking the citations and adding the references.

Reviewer #2

(Remarks to the Author)
I thank the authors for the careful revision of the manuscript. At this stage, I have nothing to add and I am happy to recommend publication

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments and I support its publication in Nature Communications.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response to Reviewers

We sincerely thank all reviewers for their thoughtful and constructive feedback, which has helped us improve the manuscript. We appreciate that the reviewers found that "The experiments are extensive, and the figures are beautiful" (#3), that this is "very nice paper with a very clear demonstration of complexity pertinent to crystallization" (#1) and "yet another fantastic study on colloidal crystallization" (#2).

Below, we provide a detailed response to each comment and describe the revisions we made. Given the strongly positive response of the referees and these changes, we feel our work is now suitable for publication in Nature Communications".

Reviewer #1

This is a very nice paper with a very clear demonstration of complexity pertinent to crystallization. It captures nicely all the stages, though (as in many other works) the nucleation remains enigmatic. It can be published after addressing the following:

1. Direct analogy with atomic/molecular solids is problematic, and while it is relevant, it must be tuned down.

Response: We appreciate the Reviewer's feedback regarding the use of analogies with atomic and molecular solids. After reviewing the manuscript, we identified four instances where such analogies were made. While we believe that these comparisons can be insightful and engaging, we acknowledge the Reviewer's concern. Since these analogies are not essential to the core message of our work, we have modified those pieces to instead emphasize the universal principles underlying our findings.

Specific changes to the manuscript:

Analogy #1: "*While classical theories have long described crystallization as a monomer-by-monomer addition (whether involving atoms, ions, molecules or colloids).*"
This sentence now reads : "*While classical theories have long described crystallization as a monomer-by-monomer addition.*"

Analogy #2: "*This process, which is believed to play significant roles in a range of atomic and colloidal systems, begins with the rapid formation of 'particle blobs'- a condensed liquid-like phase containing both positive and negative colloidal ions.*"
This sentence now reads : "*This process, which is believed to play significant roles in a range of systems, begins with the rapid formation of 'particle blobs'- a condensed liquid-like phase containing both positive and negative colloidal ions.*"

In this paragraph, we also added references to the work of Rybtchinski and Vekilov/Rimer as suggested by the reviewer (see Comment 3 below).

Analogy #3: "*These waves are reminiscent of wetted step-edge growth in atomic systems, where atoms or molecules from a liquid phase deposit onto the edges of crystal steps and then integrate into the crystal lattice.*"
This sentence now reads : "*These waves are reminiscent of wetted step-edge crystal growth, where particles from a liquid phase deposit onto the edges of crystal steps and then integrate into the lattice.*"

Analogy #4: "*We hypothesized that the strength and range of particle interactions plays a crucial role in modulating the crystal formation pathways we observed, a phenomenon also noted in various other colloidal and molecular systems.*"
This sentence now reads : "*We hypothesized that the strength and range of particle interactions plays a crucial role in modulating the crystal formation pathways we observed, a phenomenon recognized in a growing number of particle systems.*"
In this paragraph, we also added references to the work of Rybtchinski and Vekilov/Rimer as suggested by the reviewer (see Comment 3 below).

2. More quantitative analysis must be done a the data allow it. Thus, order parameter can be employed, and other methods as well. It may help to see the nucleation events. In particular, analysis of the SEM of intermediate structures would be important, and will be more convincing than multiple cartoons.

Response: We appreciate the Reviewer's suggestion to incorporate more quantitative analysis. In principle, such analysis would require precise spatial and temporal data at the single-particle level. However, due to the small particle size, achieving single-particle resolution is not feasible for most experimental samples. While SEM imaging provides the necessary spatial resolution, the preparation process introduces temporal delays that are difficult to quantify.

To address these challenges, we performed quantitative analyses on our computer simulation data. These simulations, in conjunction with our optical and electron microscopy data, provide a comprehensive and accurate representation of the nucleation process. Additionally, we would like to clarify that the "cartoons" in our manuscript are not schematic illustrations but rather 3D renderings derived directly from our simulation data.

3. Citing the works of Rybtchinski et al. on protein crystallization and Vekilov/Rimer on organic crystallization is needed in light of its direct relevance to the observed phenomena.

Response: We appreciate the Reviewer's suggestion and thank them for pointing out these relevant references. We have now incorporated two additional citations to the works of Rybtchinski on protein crystallization and Vekilov/Rimer on organic crystallization (some of their work was already cited in the previous version of the manuscript). At the same time, in accordance with Comment #1, we have minimized direct analogies to atomic and molecular systems to maintain the focus on universal principles

Specific changes to the manuscript: We have added the following references:

- Houben, L.; Weissman, H.; Wolf, S. G.; Rybtchinski, B. *A mechanism of ferritin crystallization revealed by cryo-STEM tomography*. Nature 2020, 579, 540–543.
- Ma, W.; Balta, V. A.; Pan, W.; Rimer, J. D.; Sullivan, D. J.; Vekilov, P. G. *Nonclassical mechanisms to irreversibly suppress ß-hematin crystal growth*. Communications Biology, 2023, 6, 783.

4. There is a tendency to present a lot of simulations (which are nice) and less SEM. It would be important to present more SEM data.

Response: We appreciate the Reviewer's emphasis on the importance of SEM data, as it provides critical structural details at the microscopic scale. From the outset, we have aimed to strike a balance between different characterization techniques, including SEM, optical microscopy, and simulations. However, obtaining high-quality SEM images of these delicate structures is extremely challenging, and we have made every effort to document as many samples as possible.

That said, we are somewhat puzzled by the Reviewer's request, as SEM images are already included in all main figures and in five of the eight supplementary figures, which we believe represents a substantial amount of data. The only structure for which we had not previously included SEM images was **Structure V in Figure 3h**, as it was too fragile to be reliably fixed and imaged. However, motivated by the Reviewer's comment, we have now successfully obtained SEM images of this elusive crystal and have included them as a supplementary figure in the revised manuscript.

Specific changes to the manuscript: We have now included Extended Figure E9, which shows the SEM image of the only crystal structure that had not previously been imaged.

5. Fig. E2 is not serving the flow of the text properly.

Response: Upon careful reading of this section, we believe this may refer to our original manuscript going back and forth between experiments under static conditions and dialysis.

Specific changes to the manuscript: We have now swapped Extended Figures E2 and E3 and rearranged the text such that all static experiments proceed all dialysis experiments, and feel this figure now synergizes with the revised flow of the paper.

Reviewer #2

The authors of this paper present yet another fantastic study on colloidal crystallization under highly controlled conditions. The use of highly monodisperse particles species with finely tunable attractive interactions, combined with density and refractive index matching allows visualizing previously unattainable conditions. Moreover, the authors use a very simple, yet highly controlled salt-diffusion gradient to slowly modulate local salt concentration and observe a wealth of different crystal structures under controlled conditions.
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Response: We thank the Reviewer for their positive feedback and interest in the unidentified dendritic structures. We share this curiosity and have made multiple attempts to isolate and image these crystals. However, their thin and fragile nature makes them difficult to preserve during the fixing process required for SEM imaging.

Following the Reviewer's suggestion, we dedicated additional effort to capturing these structures and have now included an additional Extended Figure E9 in the revised manuscript. While this provides some visual insight, the formation mechanism and properties of these structures remain unclear and will be the focus of future studies.

Specific changes to the manuscript: We have added the additional Extended Figure below.

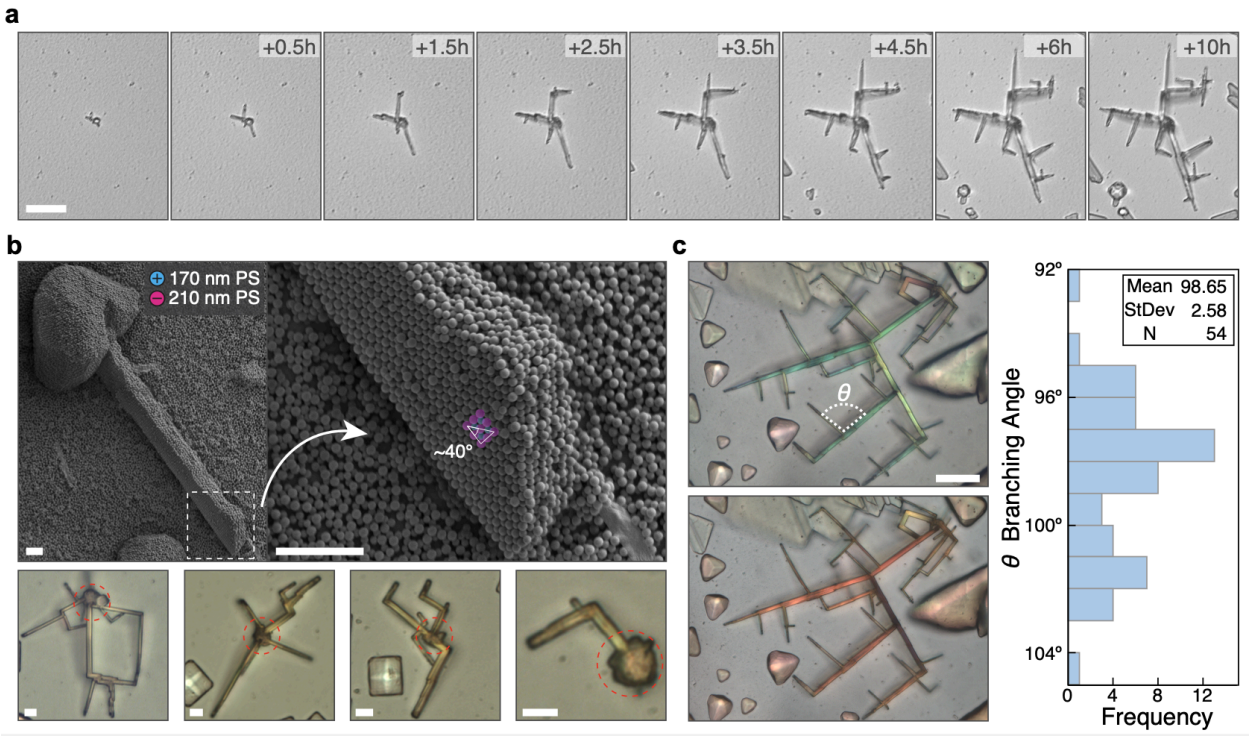

Figure E9: **Branched crystals**. **a**, Time-lapse bright-field microscopy images showing the nucleation and growth of branched crystals from a central seed (see also Extended Data Movie 7). This sample consists of a 1:1 mixture of 170 nm (+) PS and 210 nm (–) PS particles in a dialysis setup. Scale bar, 50 μm. **b**, (Top) SEM image showing a fragment of a crystal that survived the fixing process. This image suggests that these branched structures may form via heteroepitaxial growth from a central seed crystal. While the central seed's habit resembles the Th3P4 structure, the rest of the crystal appears to share the same structure as the flag-like crystal shown in Extended Data Figure E7e. Scale bar: 2 μm. (Bottom) Bright-field images showcasing various branched crystals, all featuring a central seed crystal circled in red. Scale bar: 10 μm. **c**, (Left) Polarized light microscopy images of a branched crystal extending over hundreds of micrometers and exhibiting strong color birefringence. (Right) Measurements of the branching angles θ reveal a relatively constant value of 98.6°, suggesting anisotropic growth due to facet-controlled growth or twinning. Scale bar: 50 μm.

Reviewer #3

This manuscript presents a study of the nucleation and growth of binary colloidal crystals. The combination of experimental studies and MD simulations reveals a predominant non-classical nucleation pathway accompanied by multiple growth habits. Furthermore, the authors correlated various experimental parameters, such as bonding strength, volume fraction, and size ratio. The experiments are extensive, and the figures are beautiful. Several issues need to be addressed before publication.

1. It is not clear what is the motivation of the work since the work seems to be written in a highly condensed form (no regular introduction section but just directly went into the results after the abstract). Is it because the electrostatic interaction driven binary colloidal crystals have some unique properties from other colloidal crystals? Why does crystal morphology matter? Are there any fundamental knowledge gaps in the field? At least the authors need to review and comment on current literatures.

Response: Our primary motivation in this work is to gain a deeper understanding of the mechanisms governing the crystallization of binary colloidal systems. By elucidating these mechanisms, we aim to develop better strategies for controlling both the formation process and the resulting crystal structures.

In response to the reviewer's suggestion, we have expanded the introductory paragraphs to provide a clearer context for our study.

Specific changes to the manuscript: The following text has been added to the introduction:

"*Charged colloidal particles are well-known model systems for studying self-assembly, as their interactions can be finely tuned through surface charge density and surrounding ionic conditions. When oppositely charged colloidal particles interact, they can form ordered binary structures akin to atomic crystals.(12,13) While such electrostatically driven assemblies have been extensively investigated in the context of simple crystalline lattices,(14) the detailed mechanisms governing their nucleation and growth remain largely unexplored. Previous studies on binary colloidal crystals have primarily focused on equilibrium structures and phase behavior, often assuming direct monomer-by-monomer attachment.(15) However, accumulating evidence suggests that electrostatically driven crystallization follows more complex pathways, involving*

intermediate amorphous phases, aggregation-driven nucleation, and particle reorganization.(16–18) Despite theoretical predictions and computational models supporting these non-classical pathways,(10,19–21) direct observation has remained limited, particularly in systems where interaction potentials can be dynamically controlled. Here, we investigate the formation of ionic binary colloidal crystals under conditions where electrostatic interactions dictate crystallization pathways."

2. The field of colloidal crystallization has well-established quantitative methods for distinguishing different phases, such as global/local order parameters, local density, and bonding stability. In particular, during the nucleation stage, these analyses are essential to differentiate phase evolution (e.g., amorphous vs. crystalline). However, the current manuscript relies primarily on the look of time-lapse confocal optical microscopic images. At least some quantitative method should be employed to characterize phase transitions rigorously.

Response: We appreciate the Reviewer's suggestion regarding the use of quantitative methods to distinguish different phases during crystallization. While such analyses are well-established in colloidal crystallization studies, their application relies on the ability to accurately track individual particles. In our system, the small particle size limits single-particle resolution, making it infeasible to extract precise quantitative data from experimental nucleation stages.

To overcome this limitation, we have performed quantitative analyses on our simulation data, where individual particle trajectories can be fully resolved (see extended Figures E4-E6). We believe that these simulations, in combination with our experimental time-lapse optical and electron microscopy data, still provide a comprehensive picture of phase evolution and nucleation dynamics.

3. The manuscript describes two experimental setups: static and dialysis. It appears that open lattice structures arise only in the dialysis setup, potentially due to kinetic effects influencing local particle/ion diffusion and saturation. These structures are not replicable under static equilibrium conditions. The authors need to either address this limitation or present the detailed mechanistic reasoning and provide real-time, real-space data on the formation process (e.g., classical nucleation pathways) using the dialysis setup.

Response: While we have not observed the formation of the L_3S_4 crystal under static conditions in our experiments, our simulations clearly demonstrate that this structure can form under such conditions. We believe there are key technical reasons that make it challenging to observe this phase experimentally.

First, our results suggest that the L_3S_4 crystal forms only within a very narrow window of salt and particle concentrations. Identifying this precise region through static experiments is challenging, whereas in dialysis conditions, the continuous variation of salt concentration along the capillary naturally facilitates access to this regime, leading to its reproducible formation.

Second, real-time imaging of the formation process is severely limited by the constraints of 3D optical imaging. Such imaging requires particles with an optical refractive index matched to the solvent, which, in practice, necessitates the use of heavier materials than polystyrene (PS). The increased prevalence of gravitational effects in these denser systems likely disrupts the formation of the open L_3S_4 structure, further complicating direct visualization.

Following the reviewer's suggestion, we have explicitly discussed these experimental limitations in the manuscript.

Specific changes to the manuscript: The following statement was added to the main text: "*So far, we have only observed* L_3S_4 *formation under dialysis conditions, whereas simulations reproducibly assembled* L_3S_4 *under static conditions, suggesting that its formation might occur only within a very narrow range of* λ_D *values.*"

4. Figure 4 is quite out of place with the rest of the manuscript. The manuscript is mostly about non-classical crystallization pathways. What is the logical connection of Figure 4 with the rest of the text? For example, the content in Figure E3 appears more relevant to the paper's topic than Figure 4.

Response: Figure 4 presents the discovery of a previously unreported low-density crystal structure. While this finding could stand alone, we included it to emphasize that non-classical crystallization pathways can lead to entirely new structures. We still feel that this was an inspiring way to conclude the manuscript, highlighting the broader impact of our findings. As noted above, we have rearranged the text referring to E2 and E3 which better incorporates the data in (old Figure E3) into the flow of the paper, and we now transition to our study of L_3S_4 by providing motivation for investigating this structure in more detail, including highlighting the possibility that non-classical crystallization pathways could increase the likelihood of the hetero-epitaxial growth observed on top of this structure.

5. In Figure 1, the structures are predominantly polycrystalline without clear facets. In contrast, Figure 2 depicts monomer-by-monomer addition starting from a single crystal. Why is there such discrepancy?

Response: We appreciate the Reviewer's observation regarding the apparent discrepancy between Figures 1 and 2. The key distinction is that Figure 1 presents various stages of crystal formation through a non-classical crystallization pathway, whereas Figure 2a shows a sample that nucleated via a classical crystallization pathway and is undergoing growth via monomer-by-monomer addition.

Additionally, crystals forming via non-classical pathways tend to appear polycrystalline at early nucleation stages (as seen in Figure 1) and develop well-defined facets only at later stages, once most of the amorphous-like "blobs" are incorporated into the growing structure (as illustrated in Figure 2b). To clarify this point, we have revised the text to better highlight these differences.

Specific changes to the manuscript: We have revised the caption of Figure 2 which now reads: "*Sequential time-lapse microscopy images capturing the growth of CsCl-like colloidal crystals.* ***a****, Well-defined faceted crystals formed via a classical nucleation pathway and growing through direct monomer-by-monomer addition from the gas phase (see also Extended Data Movie 2).* ***b****, Similar crystals formed via a non-classical crystallization pathway, growing through the attachment and absorption of amorphous blobs (see also Extended Data Movie 3).*"

6. The morphology of the blobs in Figure 2 is very similar to that of the nuclei in Figure 1. How do the authors differentiate between these structures?

Response: As explained in the main text, we refer to 'particle blobs' as a condensed liquid-like phase containing both positive and negative colloidal ions. Throughout all figures in the manuscript, the term 'blobs' consistently denotes this same condensed phase. Its amorphous structure has been characterized to the best of our ability using confocal and electron microscopy, as well as computer simulations.

Reviewer #4

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We appreciate the reviewer's time and effort in evaluating our work.