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Mechanisms of Crystallisation

Mesoscale Clusters in the Crystallisation of Organic Molecules

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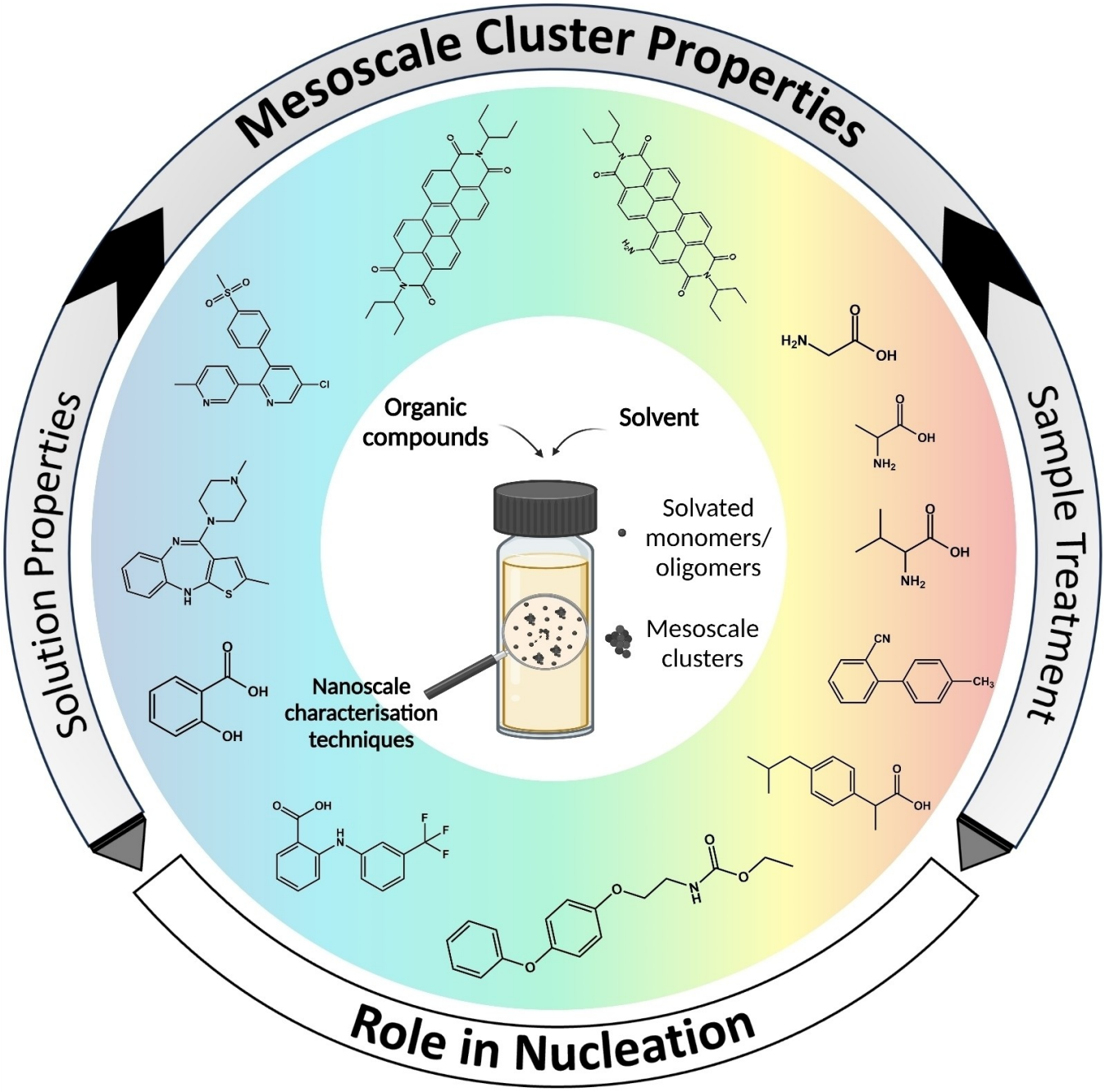

Abstract: The early stages of the molecular self-assembly pathway leading to crystal nucleation have a significant influence on the properties and purity of organic materials. This mini review collates the work on organic mesoscale clusters and discusses their importance in nucleation processes, with a particular focus on their critical properties and susceptibility to sample treatment parameters. This is accomplished by a review of detection methods, including dynamic light scattering, nanoparticle tracking analysis, small angle X-ray scattering, and transmission electron microscopy. Considering the challenges associated with crystallisation of flexible and large-molecule active pharmaceutical ingredients, the dynamic nature of mesoscale clusters has the potential to expand the discovery of novel crystal forms. By collating literature on mesoscale clusters for organic molecules, a more comprehensive understanding of their role in nucleation will evolve and can guide further research efforts.

1. Introduction

Crystallisation underpins the formation of structured materials integral to medicine, agriculture, and biology in the form of pharmaceutical treatments, pesticides, and biomineralisation.[1] A crystallisation process can be broadly divided into two steps: nucleation and crystal growth.[2] The pre-requisite for both is the formation of a supersaturated solution, as the new phase can only appear and grow when a system is in a non-equilibrium state.[2] Early stages of crystallisation play a decisive role in determining properties of the resulting crystals such as crystal structure, size distribution, shape, physicochemical and mechanical properties.[3] Hence, to achieve higher levels of control over crystallisation, a significant understanding of the fundamentals of nucleation is needed.

For decades, the classical nucleation theory (CNT) was central to developing an understanding of how nucleation occurs.[4] Though an oversimplification of the nucleation route, it considers that in a supersaturated state, density and structure fluctuations occur simultaneously with the addition and subtraction of monomer units to unstable pre-critical clusters possessing crystalline characteristics (Figure 1, top pathway).[4] Nucleation occurs when the radius of these crystalline clusters exceeds a critical size (termed the critical nucleus), overcoming unfavourable interfacial free energy and primarily driven by the favourable bulk free energy.[5] However, as the field has advanced and more experimental evidence has been produced, it has been found that the nucleation pathways of many materials do not adhere to the assumptions of the CNT.[4]

The nucleation pathways for various compounds have been scrutinised using a breadth of experimental techniques leading to the proposal of alternative pathways falling under the umbrella term of nonclassical nucleation.[1,3,6] Wolde and Frenkel proposed the first nonclassical nucleation pathway, two-step nucleation, for proteins based on simulation studies.[7] In this pathway, crystalline order is preceded by the formation of a dense, pre-crystalline liquid phase. This phase over time acts as the source for structure fluctuation to occur leading to the formation of the nucleus within this liquid-like cluster (Figure 1, middle route).[4] A second non-classical nucleation pathway incorporating a similar intermediate phase, the pre-nucleation cluster (PNC) pathway, has been hypothesised from a study of calcium nucleation in a biomineralisation process (Figure 1, bottom pathway).[1,8] Ultimately, the general trend of these non-classical pathways is that the intermediate and ambiguous species lack a crystalline order, a distinct interface, and thus cannot be explained solely by CNT theory. Many reviews that have emerged in recent years have focused on inorganic systems[1,9] or proteins[10] with only a handful of reviews on organic compounds.[3–4]

When an organic compound is dissolved in a solvent, there should be no observable large structures or density fluctuations identified in either static or dynamic liquid light

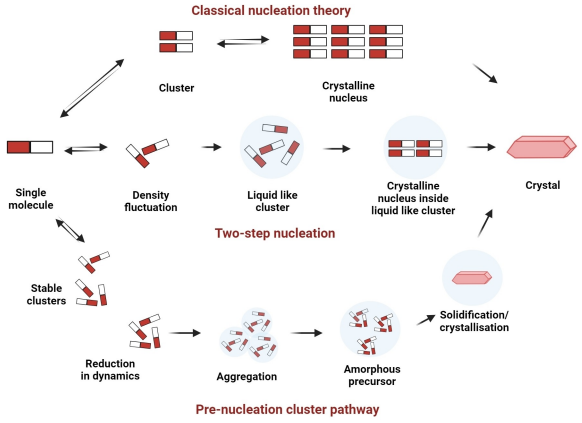

Figure 1. Alternative nucleation theories in literature. Top: Classical nucleation theory, Middle: Two-step nucleation (Adapted from Ref. [4] with permission. Copyright © 2013 WILEY-VCH), Bottom: Pre-nucleation cluster pathway (Adapted from Ref. [8] with permission. Copyright © 2022 American Chemical Society) (Created with BioRender.com).

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scattering experiments if complete solute dissolution and molecular solvation is ensured, i.e., a homogeneous solution is formed.[11] However, with growing interest in alternative non-classical nucleation theories and advances in analytical techniques, it has been well established that solutions of organic compounds, even below saturation concentrations, are inhomogeneous and host regions of higher and lower solute concentrations.[12] The terminology used to describe these inhomogeneities within the solution, particularly in relation to crystallisation, appears to be ambiguous in the literature. In retrospect, the initial report of such irregularities pertaining to an organic compound can be traced back to the research conducted by J. W. Mullin et al. in 1969.[13] They ascribed the observed concentration gradients across an undisturbed column of highly supersaturated aqueous solutions of citric acid under isothermal conditions to clustering occurring within citric acid molecules without use of any characterisation technique.[13] Subsequently, over the following decades, with the advent of nanoscale characterisation techniques, for organic molecules, a plethora of terms, such as spherical supramolecular aggregates,[14] metastable clusters,[15] nanodroplets,[16] precrystalline clusters,[17] mesospecies,[18] mesoscale clusters,[19] molecular clusters,[19] and prenucleation clusters[20] have all been used to describe the same or similar entities. Hence, for the purpose of this review, a common term, "mesoscale cluster", will be utilised to refer to associations of molecules in solution, that could be solute alone or a combination of solute and solvent, with sizes in the mesoscale range of 10 nm to 1000 nm.[21]

At present, the exact role of mesoscale clusters in organic molecule crystallisation is not completely understood. However, as the properties of mesoscale clusters evolve, it will become clearer whether control over cluster structure can be harnessed to promote control over the

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Prof Sarah Hudson has a BA Hons in Natural Sciences (Chemistry) and an M.Sc. from Trinity College Dublin as well as a Ph.D. in biocatalysis from the University of Limerick in Ireland. She did two postdoctoral positions in MIT, Boston, USA and WIT, Waterford, Ireland in drug delivery and pharmaceutics before taking her current academic position at the University of Limerick in Ireland. Current research interests include small molecule and protein crystallisation, pharmaceutical formulation, and drug delivery.

Michael Svärd obtained his Ph.D. in 2011 at the KTH Royal Institute of Technology, Stockholm. After a postdoctoral stay at the University of Limerick, he obtained a docent degree (associate professor) at KTH in 2018. He is Swedish representative in the European Federation of Chemical Engineering Working Party on Crystallisation. Current research interests include increasing the fundamental understanding of crystal nucleation and solid–liquid phase equilibria for improved control of various crystallisation processes.

Jennifer Cookman completed her Ph.D. in 2017 from University College Dublin specialising in electron microscopy techniques to investigate crystallisation mechanisms and nanomaterial properties. Jennifer was awarded an SFI Pathways Fellowship to begin her own research group in 2022 at the University of Limerick. Her research interests include investigating crystallisation mechanisms of organic compounds, specifically pharmaceuticals, using liquid phase transmission electron microscopy coupled with analytical chemistry methods.

Åke Rasmuson, professor emeritus of chemical engineering at UL and KTH, has been working in the area of industrial crystallisation since 1985. His work includes fundamentals of nucleation and crystal growth, thermodynamics of solid-solution systems, cocrystallisation, nanocrystallisation, population balance modelling and hydrodynamics of agitated tanks. More recently his work has focused on rationalising the influence of solute and solvent properties on crystal nucleation, and on the relation to molecular clustering in solution.

structure of the nucleus required to promote polymorphic purity or selectivity. Publications in the field of crystallisation discuss the impact of solution properties, such as solute concentration,[22] temperature,[23] and solvent type,[24] and sample treatment conditions, including agitation,[25] filtration,[26] and thermal pre-treatment,[15] on the crystallisation outcome. This review focuses on the relationship between these solution properties, sample treatment and mesoscale cluster properties, as well as the subsequent role of mesoscale clusters in nucleation.

Four aspects are considered in the review. Firstly, the critical properties of clusters investigated thus far are discussed with a focus on size, number concentration, kinetic stability, and internal structure. These properties are discussed in relation to their dependence on solution properties and sample treatment parameters. Secondly, the experimentally understood role (thus far) mesoscale clusters play in nucleation is covered. The third section outlines considerations for different measurement methodologies specific to the characterisation of mesoscale clusters. To conclude, the fourth and final section maps out the future research direction that should be taken to rationalise the system dependent cluster attributes and their role in nonclassical nucleation pathways of organic molecules.

2. Critical Cluster Properties

The size, number concentration, kinetic stability, and internal structure of mesoscale clusters in solution are deemed key to understanding their role in crystallisation processes. In nonclassical nucleation, it is believed that the mesoscale cluster serves as a reservoir of molecules for the formation of the nucleus.[16] A larger cluster size could therefore possess a greater mass for nucleation and a correspondingly higher likelihood of nucleation. Furthermore, a higher number concentration of mesoscale clusters could assist nucleation.[27] As yet, it is unclear whether mesoscale clusters are thermodynamically stable and if they maintain a steady population or if they are metastable moieties that vary over time. Moreover, the internal structure of mesoscale clusters has sparked curiosity, since it has been revealed that the underlying structure of clusters could aid in manipulating subsequent crystal assemblies.[4] As will be described in the following sections, these critical cluster properties depend on parameters such as solvent, solute concentration, and temperature, and are influenced by sample treatment conditions, e.g., agitation, filtration, and thermal pre-treatment.

2.1. Cluster size

Liquid light scattering techniques such as dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) have proven powerful for the direct determination of the mesoscale cluster size in organic compounds particularly considering the solvodynamic diameter (hydrodynamic for aqueous solutions). The majority of studies that use DLS to report the size of mesoscale clusters indicated the presence of a bimodal distribution.[16,18–19,28,29] This has been corroborated by analysing the correlation graphs obtained in parallel with the cluster size distribution. Figure 2 displays a typical correlation graph that results in a bimodal distribution for an undersaturated aqueous glycine solution.[19] The two decays in the correlation graph correspond to the decay rates of two populations, one population that will diffuse faster and one that will diffuse slower. The fast decay is attributed to hydrated glycine monomers and dimers. The slower decay is caused by the presence of inhomogeneous solute distributions, resulting in peak distributions with a size range of 10–1000 nm, defined as mesoscale clusters.[19]

Cluster size has been reported to depend on solution properties such as solute concentration,[14,16,19,30] temperature,[15] and solvent pairing.[30–31] The investigation of undersaturated solutions of organic compounds by means of DLS and NTA has generated considerable attention among the studies that have been published. All these studies have demonstrated the existence of mesoscale clusters below the solid–liquid equilibrium, i.e., undersaturated solutions. The existence of clusters in undersaturated solutions has been demonstrated for *DL*-alanine,[18] fenoxycarb,[29–30] salicylic acid,[30] and 2-cyano-4′-methylbiphenyl (OTBN).[31] Zimbitas et al. investigated the size of mesoscale clusters in undersaturated aqueous glycine solutions (Figure 3A), finding little to no change in size with increasing solute concentration.

In the literature, the correlation between increasing solute concentration and cluster size has thus far produced contradictory findings.[19,29] Zong et al. observed that mesoscale clusters of OTBN were only detected at concentrations beyond a certain threshold in chloroform (>0.21 M) and methanol (>0.28 M) as shown in Figure 3B. Below such concentrations, just solvated monomers/oligomers were detected. Cluster sizes in the mesoscale range were unaffected by further solute concentration increases (Fig-

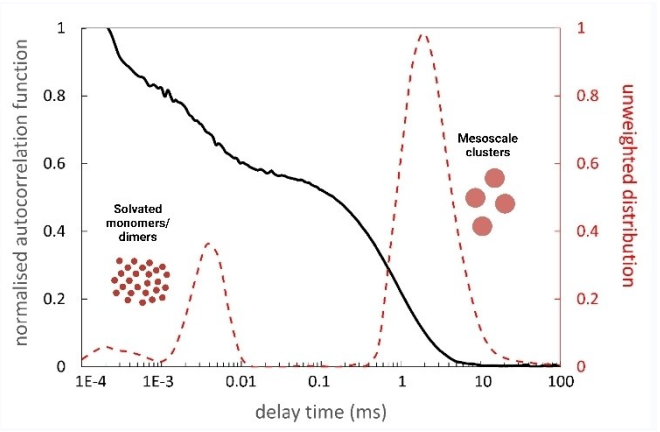

Figure 2. DLS autocorrelation function (black, solid line) showing two distinct decays representing a bimodal distribution as displayed by the corresponding delay time distribution (red, dashed line) for an undersaturated glycine aqueous solution. (Adapted from Ref. [19] with permission. Copyright © 2019 Elsevier Ltd) (Created with BioRender.com).

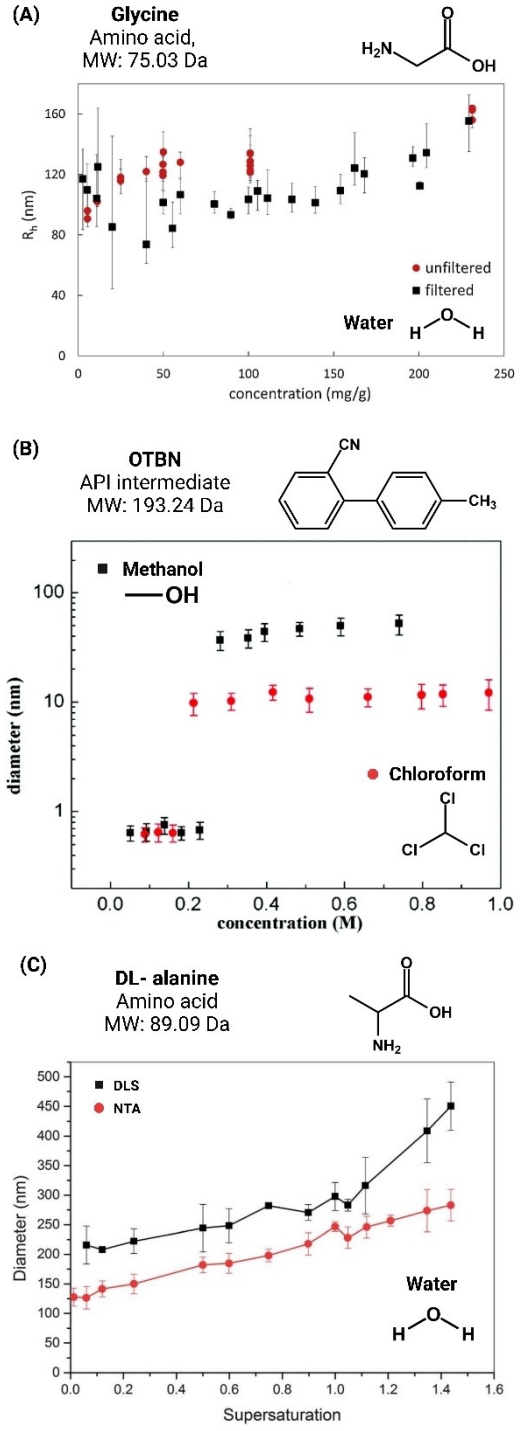

Figure 3. (A) Hydrodynamic radius (R_h) of mesoscale clusters plotted against undersaturated aqueous glycine solutions. Red circles ″Unfiltered, Black squares″ 1 μm PTFE filter. (B) DLS results for the identification of mesoscale clusters of OTBN in methanol and chloroform displaying a negligible variation in diameter with increasing concentration. (C) DLS and NTA results for aqueous *DL*-alanine solutions showing the increasing size of clusters from undersaturated to supersaturated conditions (A) Adapted from Ref. [19] with permission. Copyright © 2019 Elsevier Ltd; B) Adapted from Ref. [31] with permission. Copyright © 2020 Royal Society of Chemistry; C) Adapted from Ref. [18] with permission. Copyright © 2013 Royal Society of Chemistry) (Created with BioRender.com).

ure 3B).[31] A similar trend was found with glycine[19] and olanzapine.[32] However, Jawor-Baczynska et al. and Kakkar et al. found a strong dependence of the mesoscale cluster size on the solute concentration in their work with aqueous *DL*-alanine[18] (Figure 3C) and salicylic acid and fenoxycarb,[30] respectively. Hence, the relationship between cluster size and solute concentration appears to be a system-dependent property, making the study of different solvent-solute systems critical for consolidating the knowledge.

The dependence of cluster size on the solvent is another important factor to evaluate the solvent-solute relationship for solutions that host mesoscale clusters. Given the importance of solvent choice on nucleation kinetics,[33] it has received remarkably little attention, with only a few studies focusing on this aspect.[30–31] Such studies include relating the mesoscale cluster size with solubility of the organic compound in the same solvent. For example, the cluster size in OTBN-methanol solutions is larger than those in chloroform at the same mole fractions (Figure 3B) where OTBN is known to be more soluble in chloroform than methanol.[31] Fenoxycarb and salicylic acid display the same trend where the clusters are smaller in solvents where the solubility is higher.[30] As more systems are studied this relationship can be developed further to understand the role of clusters in nucleation kinetics.

The influence of sample treatment has garnered great interest as agitation, filtration, and thermal pre-treatment have been shown to change the nucleation behaviour.[26,34–35] In the reports evaluating the impact of agitation on nucleation kinetics, it has been reported that cluster aggregation can be promoted through agitation, induced by shear forces.[34] This behaviour, can accelerate nucleation and has been substantiated through experimental validation with corresponding studies supporting this phenomenon.[25,36] Zong et al. investigated the effect of varying agitation times on cluster sizes of OTBN in chloroform and methanol.[31] Cluster sizes evaluated by DLS increased by a factor of four after an agitation time of six minutes, beyond which the sizes remained constant (Figure 4). These observations were further corroborated by NTA which showed the size distribution shifting towards larger sizes as a result of agitation time and remaining relatively constant after six minutes.[31]

It is of significance to investigate whether there exists a correlation between the size of clusters and the size of nuclei, and consequently, the rate of nucleation. CNT theory was used to determine the different nucleation parameters for salicylic acid and fenoxycarb which were further correlated with cluster size.[30] The investigation revealed that, for both compounds, the solutions exhibiting a higher nucleation rate were also the ones in which larger sized clusters were detected. (Figure 5A). Additionally, there was an inverse relationship between cluster size and the interfacial energy, i.e., a higher interfacial energy corresponded to a lower size (Figure 5B).[30] However, it should be noted that the critical nucleus and interfacial energy values were obtained through utilisation of the CNT equation, which is a commonly employed methodology but is not without its

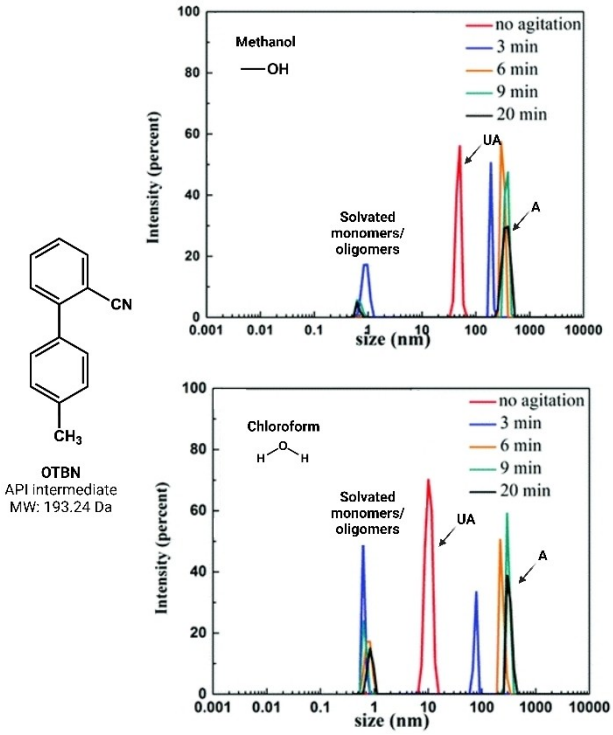

Figure 4. DLS based size distribution analysis of the effect of agitation on mesoscale clusters of OTBN in methanol and chloroform. Agitation (A) up to 20 minutes promoted the growth of mesoscale clusters compared to the unagitated (UA) solutions. (Adapted from Ref. [31] with permission. Copyright © 2020 Royal Society of Chemistry) (Created with BioRender.com).

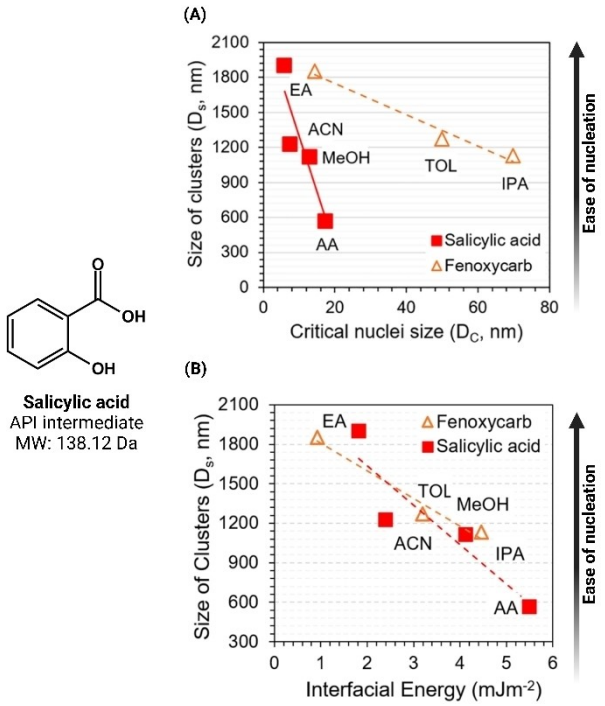

Figure 5. Mesoscale cluster size at 48 h for salicylic acid at a supersaturation of 1.05 in a variety of solvents plotted against, (A) Critical nuclei size, and (B) Interfacial energy. Solvent acronyms: EA = ethyl acetate, ACN = acetonitrile, MeOH = methanol, AA = acetic acid, TOL = toluene, IPA = isopropan-2-ol. (Adapted from Ref. [30], with permission (Created with BioRender.com).

inherent constraints when dealing with nonclassical nucleation mechanisms.

2.2. Number Concentration

DLS and NTA are the two techniques most used to examine cluster number concentration which is correlated to and reported as scattered intensity for DLS, and particle count per unit volume for NTA measurements for each population. Unlike cluster size, the dependence of number concentration on solute concentration can be generalised whereas little is known about its dependence on solvent choice and temperature. Studies with aqueous glycine solutions[19] and *DL*-alanine[18] have shown that mesoscale cluster number concentration increases as solute concentration increases, regardless of the detection method used. This can be seen in Figure 6A where, using both DLS and NTA, the number concentration of glycine mesoscale clusters increases as the solute concentration increases.[18]

When considering filtration and thermal pre-treatment, although several studies have attempted to correlate these treatment parameters with size, the most notable observations have been made for number concentration, determined using NTA.[31] For the investigated systems, an increase in duration or temperature of thermal pre-treatment, decreases the likelihood of primary nucleation.[15,35] A possible explanation for this might be found in the susceptibility of cluster concentration to be detrimentally influenced by thermal pre-treatment, whereby the cluster concentration decreases with exposure to temperatures above ambient. This has been experimentally demonstrated by Svärd et al. for fenoxycarb-isopropanol (IPA) solutions.[29] They observed a significant decrease in mesoscale cluster concentration (Figure 6C) measured with NTA after incubation at 40 °C compared to freshly dissolved solutions and solutions exposed to near ambient conditions (22 °C).[29]

There have been some reports on the modulation of mesoscale cluster populations due to filtration, a vital step in processing samples for crystallisation.[16,19,29] There exists two aspects of filtration with regards to mesoscale clusters: the impact of pore size and the impact of the membrane material. Zimbitas et al. used DLS and NTA to demonstrate that the impact of filtration pore sizes on the cluster concentration for undersaturated aqueous glycine solutions.[19] Even though the mean cluster size was smaller than the pore size (1 μm), the number of clusters significantly decreased after filtration (Figure 6A) indicated by a decrease in scattered intensity in DLS, with no change in solute concentration after filtration. The authors hypothesised that the shear stress during filtration would break or trap the large mesoscale clusters population, resulting in the observed decrease. However, when measured with NTA, the change in cluster concentration was negligible, (Fig-

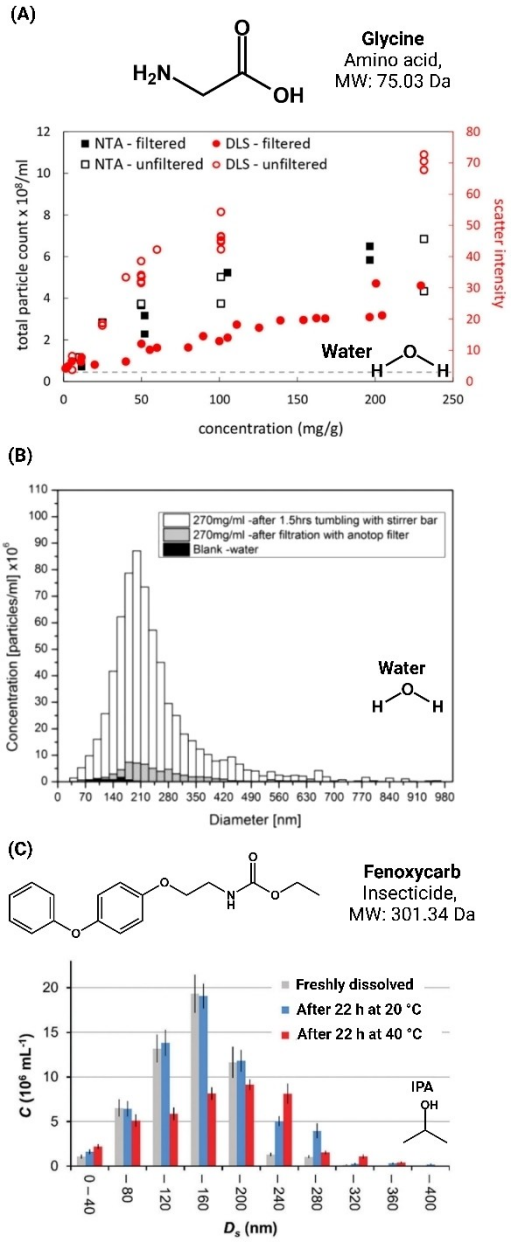

Figure 6. (A) Impact of solute concentration and filtration (1 μm PTFE filter) on cluster number concentration for aqueous glycine solutions measured with DLS and NTA (NTA = Black square, DLS = Red circle); (B) Impact of filtration (20 nm Anotop filter) and agitation on the aqueous glycine solutions measured with NTA Note: Blank water was also filtered through 20 nm Anotop filter; (C) Impact of thermal pre-treatment on the cluster number concentration of fenoxycarb in isopropanol measured with NTA (A) Adapted from Ref. [19] with permission. Copyright © 2019 Elsevier Ltd; B) Adapted from Ref. [16] with permission. Copyright © 2013 American Chemical Society; C) Adapted from Ref. [29], with permission) (Created with BioRender.com).

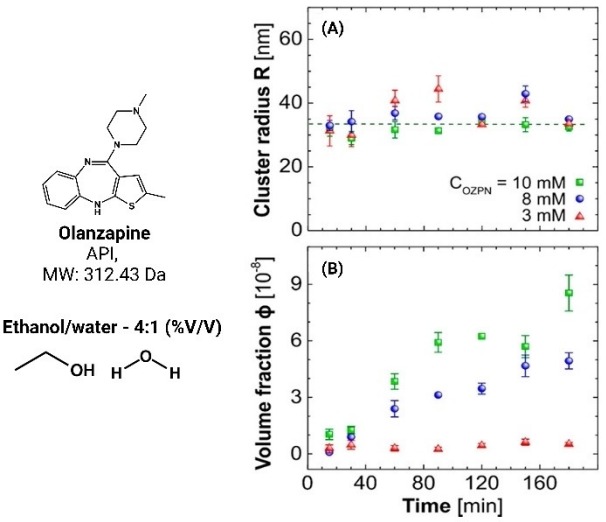

Figure 7. Evolution of (A) cluster radius, and (B) volume fraction against time for olanzapine in ethanol/water mixture (Adapted from Ref. [32] with permission. Copyright © 2017 American Chemical Society).

ure 7A). This is because, unlike DLS, which is sensitive to larger sized particles, number-based distributions measured by NTA do not account for minor fluctuations in the populations.[19] For the evaluation of impact of different filter membrane material, Jawor-Baczynska et al. investigated the impact of filtering supersaturated aqueous glycine solutions using two types of filter membranes; polytetrafluoroethylene (PTFE) with 100 nm pore size and Anotop (aluminium oxide) with 100 nm and 20 nm pore sizes.[16] In the post-filtered samples, the hydrophilic aluminium oxide filter significantly reduced the cluster number concentration, whereas the hydrophobic PTFE filter had a negligible effect on the cluster population. No solute concentration change was observed for any sample post-filtration indicating the fragile nature of mesoscale clusters.[16]

It is widely accepted that mechanical disturbances can accelerate the onset of nucleation in supersaturated solutions.[34] Jawor-Baczynska et al. investigated the effect of agitation on cluster populations and nucleation, in their work on supersaturated aqueous glycine solutions.[16] They discovered that agitated solutions easily formed hundreds of crystals in a few hours, whereas unstirred solutions took days to nucleate a few crystals. They discovered that agitating the vial with a free-to-tumble stir bar induced a large mesoscale cluster population (Figure 6B), which accelerated nucleation.[16] Their following study with aqueous *DL*-alanine solutions in concentrations ranging from undersaturated to supersaturated showed that filtration with a 100 nm Anotop membrane removed the mesoscale cluster population from the size distribution on DLS.[18] Subsequently, 120 h in a free-to-tumble stir bar, induced cluster reformation to comparable levels as the unfiltered solutions proving the ability of agitation in overcoming the kinetic barrier that was thought to exist for clusters to reform.[18]

2.3. Kinetic Stability

Liquid light scattering techniques have been used for gaining understanding of the cluster stability primarily due to its

short analysis time. Contrasting results have been obtained where some reports have indicated mesoscale clusters to be stable[18,31] while others have reported them to be metastable.[21,29] In the case of OTBN-methanol and chloroform solutions, the size of clusters remained stable for several days in the absence of agitation as measured by DLS and NTA.[31] A similar trend was also observed for undersaturated aqueous glycine solutions.[18] On the other hand, for an undersaturated olanzapine-ethanol:water solution, even though the size was stable over 3 h, the volume fraction increased due to the increased cluster concentration (Figure 7).[32] Note that in the example of olanzapine, the cluster number concentration measured with NTA was converted into volume fraction.

Alternatively, the metastability of clusters has been demonstrated in studies involving fenoxycarb[29] and *DL*-valine.[21] On cooling an undersaturated *DL*-valine-water: IPA solution from 55 °C to 30 °C, the authors observed an increase in cluster number concentration (Figure 8A), however, upon reaching the desired temperature (30 °C), the number concentration and size of the clusters decreased with time (Figure 8A), indicating its metastable nature.[21] For the range of concentrations investigated from undersaturated to supersaturated states for fenoxycarb-IPA solutions, it was discovered that the size and the derived count rate (a measure of the scattered intensity), increased over time (in the absence of agitation) (Figure 8B).[29] Overall, for the three distinct systems, it appears that the stability of clusters, in terms of size and population, is system dependent.

These studies raise the question of how large the mesoscale clusters can grow, and what kind of stability mechanisms are at play in the solutions given that CNT theory predicts that critical nuclei are in the nanometre range.[30] However, the solute-solvent systems presented are diverse, in structure, solvent pairing, and concentrations, therefore, further exploration of the kinetic stability of clusters is needed to better comprehend the relationship. In addition, the influence of sample treatment on kinetic stability has not yet been investigated for mesoscale clusters, even though it is known to influence nucleation kinetics.[26,35]

2.4. Internal Structure

The current level of understanding of the structure of mesoscale clusters of organic molecules - the presence or gradual emergence of domains of order, and how this affects the composition - is very limited. The bulk of the current knowledge is based on small angle scattering of X-rays (SAXS)[16,18,31] or neutrons (SANS).[15,37] The major challenge associated with the elucidation of structure is the small percentage of molecules involved in clusters along with the nanometre size of the clusters.[4] Primarily, the internal

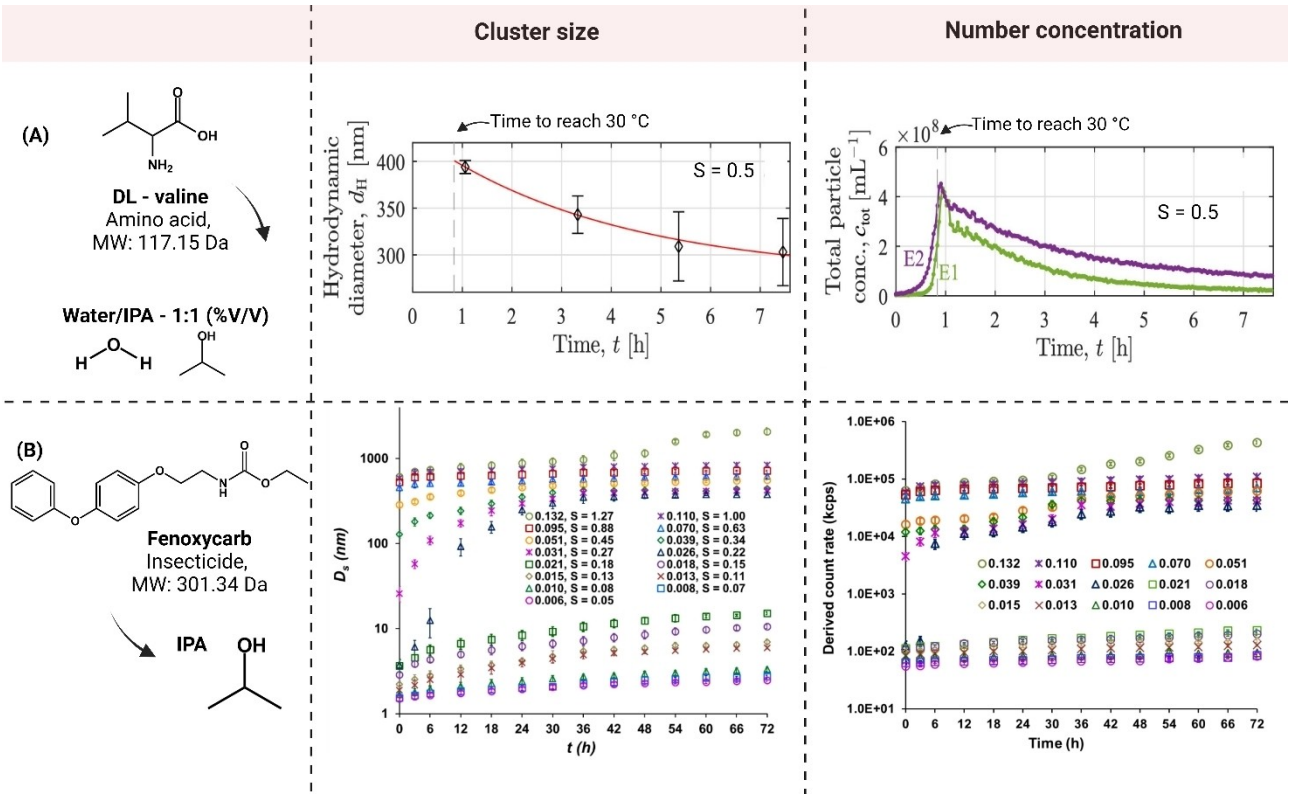

Figure 8. Evolution of cluster size (Diameter = d_H, D_s) & number concentration against time (t) for (A) aqueous solution of DL-valine Note: E1 and E2 represent the data from the two different experiments, S = Supersaturation & (B) fenoxycarb in isopropanol (IPA) (A) Adapted from Ref. [21] with permission. Copyright © 2022 American Chemical Society; B) Adapted from Ref. [29], with permission) (Created with BioRender.com).

structure of mesoscale clusters has been studied in relation to increasing solute concentration. Zimbitas et al. and Jawor-Baczynska et al. attempted to reveal the structure of mesoscale clusters by fitting SAXS scattering data from undersaturated[19] and supersaturated aqueous glycine solutions[16] to a smooth sphere model and a mass fractal model (Figure 9A). A weak fit with the smooth sphere model suggested the absence of a well-defined interface. An excellent fit was obtained with the mass fractal model, indicating that the cluster population consisted of non-spherical, irregularly shaped compact domains.[16,19] The change in cluster structure across glycine concentrations justified the minimal dependence of cluster size on increasing concentration previously observed for the aqueous solutions of glycine.[16,18] It was proposed that there was a change in the density of mesoscale clusters and hence scattered intensity, instead of an increase in the size. In another study, Ward et al. indicated the presence of non-centrosymmetric symmetry within clusters of aqueous urea solutions at near saturation levels using second-harmonic scattering in an attempt to probe internal structure with increasing solute concentration.[38] Although these results are promising,[16,19,38] further verification of internal structure in mesoscale clusters is required.

Only one study has evaluated differences in cluster structure in relation to different solvents. Zong et al. investigated the effect of the solvent on the cluster structure for OTBN in chloroform and methanol.[31] The pair-distance distribution function (PDDF) curve derived from the Fourier transformation of the scattered intensities were used to obtain insight into the cluster structure. On the basis of the PDDF curves (Figure 9B), the OTBN mesoscale clusters were predicted to have a globular structure in methanol and a core–shell structure in chloroform.[31] This is a first-of-its-kind insight into structural differences based on the solvent, but a study such as this requires further validation of a larger number of systems and improved resolution which could be offered by synchrotron sources. Possibly, insights into the internal structure of mesoscale clusters in organic systems could be obtained from work conducted on calcium carbonate, where distinct proto-crystalline motifs have been reported,[39–42] with further information available in some excellent reviews on such inorganic systems.[1,9] It remains to be seen, however, whether such structural characteristics can be identified in mesoscale clusters of organic molecules.

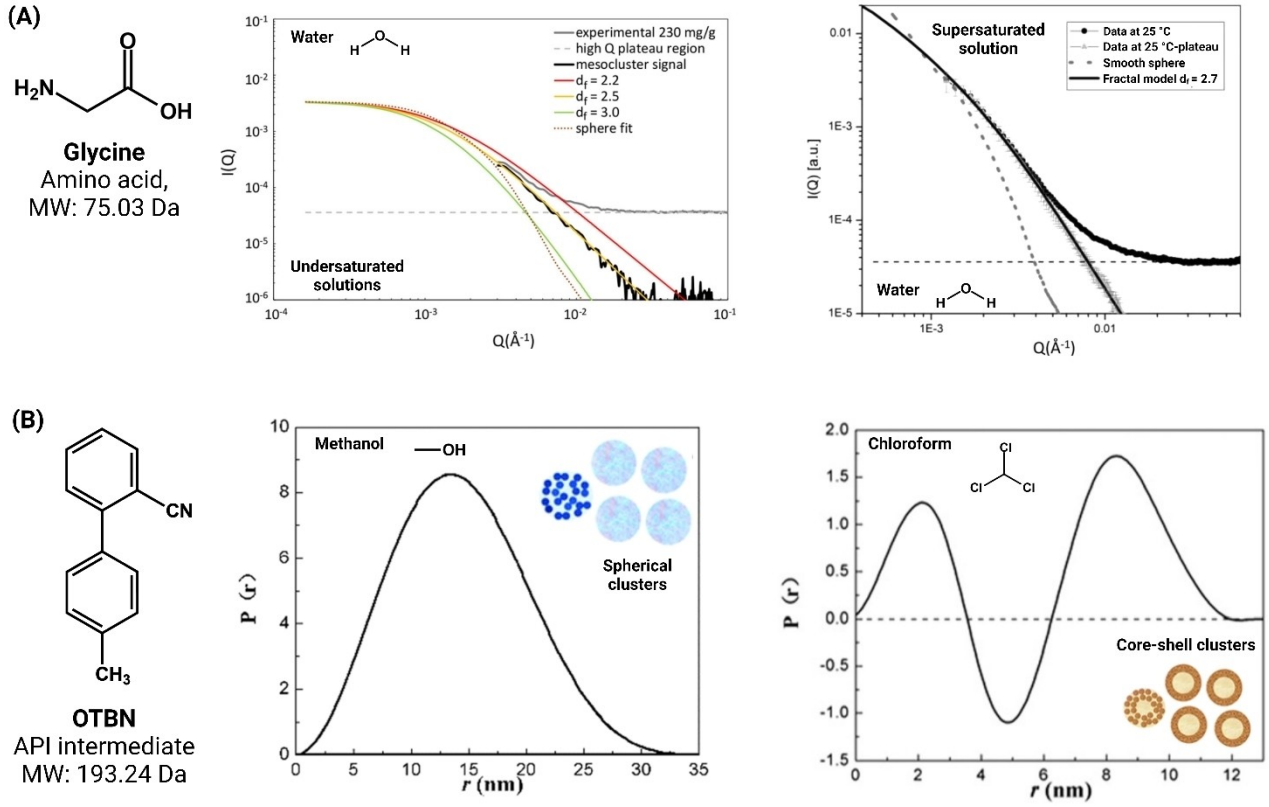

Figure 9. (A) SAXS experimental data for undersaturated glycine solution (230 mg/g) (and supersaturated glycine solution (270 mg/g) fitted to different models Note: I(Q) = Scattered intensity, Q(A^{-1}) = Scattering vector, d_f = Fractal dimension; (B) PDDF curve with corresponding predicted cluster structure for OTBN in methanol and chloroform Note: P(r) = PDDF, r(nm) = real-space distance. (A) Adapted from Ref. [19] with permission. Copyright © 2019 Elsevier Ltd. Adapted from Ref. [14] with permission. Copyright © 2013 American Chemical Society; B) Adapted from Ref. [31] with permission. Copyright © 2020 Royal Society of Chemistry). (Created with BioRender.com).

3. Role in Nucleation

Perhaps the most important reason for learning more about the critical properties of mesoscale clusters of organic molecules is to probe the impact of these properties on subsequent nucleation pathways. A considerable portion of the literature employed a liquid light scattering methodology to indirectly examine the function of mesoscale clusters in the process of nucleation.[16,31] The principal determinant behind the widespread adoption of this approach is its ease of use in sample and data analysis, availability, and suitability for benchtop experimentation. It has been noted that the application of mild agitation to supersaturated aqueous glycine solutions, assessed by NTA, led to the coalescence of glycine mesoscale clusters.[16] It was postulated that this larger size population of mesoscale clusters possesses a higher quantity of glycine, thereby accelerating the glycine crystallisation process (Figure 10A).[16] A comparable observation was made for nucleation of OTBN-chloroform and methanol solutions (Figure 10B).[31] According to the report, the agitation led to the aggregation of mesoscale clusters and subsequent size increase, which was concomitant with accelerated nucleation compared to solutions lacking such larger mesoscale clusters.[31]

The proposed role of mesoscale clusters in the nucleation pathways based on experimental findings discussed above is depicted in Figure 10. The initial phase comprises aggregation or growth of mesoscale clusters promoted by agitation in all three cases (Figure 10A–C). It is worth noting that mesoscale clusters were observed to be in a state of equilibrium with the solvated monomers/dimers. Though agitation promoted coalescence of these clusters as hypothesised by the authors,[16] a scenario for the growth of these clusters could be argued as the solutions under investigation were supersaturated based on the work involving fenoxycarb.[29] A school of thought exists that distinguishes the mesoscale cluster pathway from the two-step pathway, in that the former involves pre-existing mesoscale clusters in the solution, while the latter involves the "nucleation" of a dense liquid phase during the crystallisation process.[3] As highlighted in the critical properties section (number concentration), with filtration it can be possible to eliminate the population of mesoscale clusters on account of their fragile nature.[16,18] However, subsequent agitation of the solution has been shown to result in the reformation of a cluster population level comparable to that of an unfiltered solution[18] showing semblance to the two-step pathway. This provides additional evidence in favour of consolidating the proposed pathways and striving to establish common characteristics.

Transmission electron microscopy (TEM), on the other hand, allows direct visualisation of the nucleation pathway's progression, providing a more thorough picture of the mesoscale clusters' unique involvement in nucleation. Through the capture of time-resolved micrographs, cryo-TEM has shown to be very successful in offering significant insights into the crystallisation process.[20,43–44] This is accomplished by rapidly vitrifying an aliquot at pre-set time-points during the crystallisation process. Tsarfati et al. pioneered this procedure for organic molecules using cryo-TEM imaging to investigate the transformation of mesoscale clusters into crystals for organic dyes, perylene diimides (PDIs), in water/tetrahydrofuran (THF) solvent mixtures.[20] In the case of two of the dyes, within a few minutes of

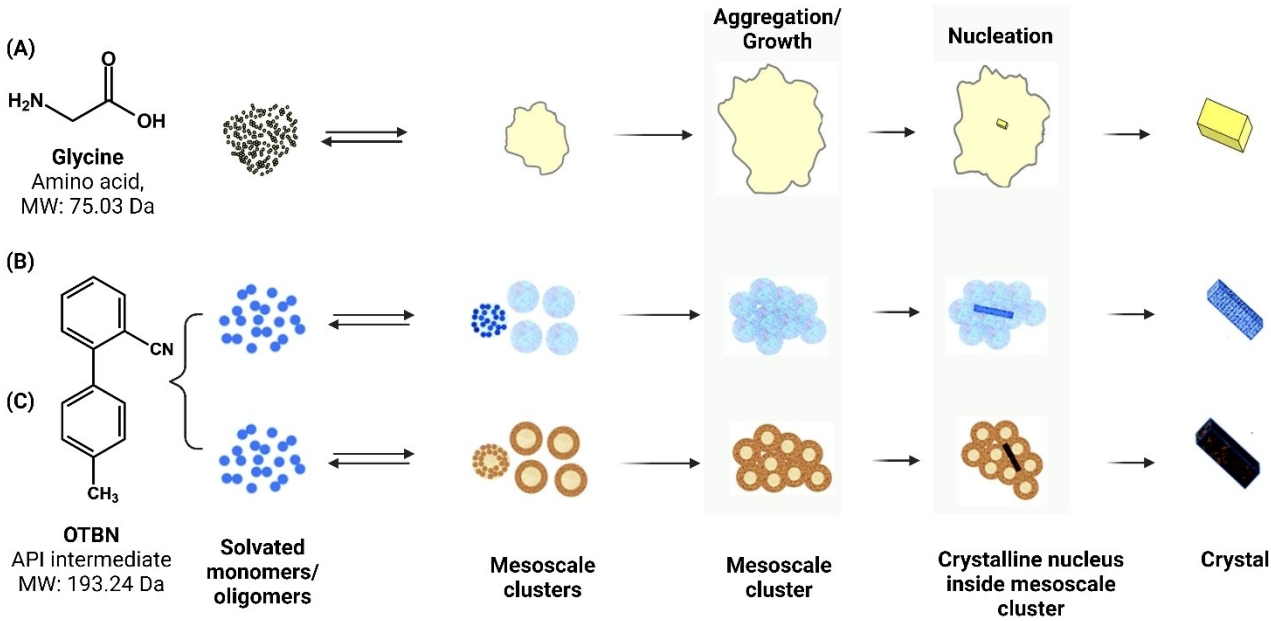

Figure 10. Schematic representation of crystallisation pathway reported for (A) Glycine (where the aggregation/growth stage is through agitation), (B) OTBN in methanol (C) OTBN in chloroform. Note that aggregation/growth stage is induced by agitation. (A) Adapted from Ref. [16] with permission. Copyright © 2013 American Chemical Society; B) and C) Adapted from Ref. [31] with permission. Copyright © 2020 Royal Society of Chemistry) (Created with BioRender.com).

mixing the THF solution and water, mesoscale clusters could be identified (Figure 11B–C). With increased ageing time, there was a tendency towards the development of crystalline order within mesoscale clusters, culminating in crystal formation (Figure 11B–C).[20] Furthermore, mesoscale clusters were found to be hollow, which was ascribed to the existence of solvent inside the clusters.[20] Despite their structural similarities, both dyes had visibly distinct cluster structures that were rich in solvent and formed the final crystal via a common pathway. A follow-up study by Tsarfati et al. analysed two APIs, ibuprofen (IbuH) and etoricoxib (ETO) also by cryo-TEM.[43] A similar mechanism was observed while also reporting a diverse spectrum of crystallisation intermediates which hinted towards a pathway amalgamation of classical and nonclassical modes, which they called a "continuum crystallisation model".[43]

Cookman et al. were the first to use liquid phase TEM (LPTEM) to study the nucleation of organic compounds.[45] Their study of undersaturated flufenamic acid (FFA) nucleation in ethanol offered a temporal description of each step, similar to nonclassical nucleation.[45] Under the influence of an electron beam, FFA nucleation occurred in a solution where the average concentration was 25× below the solubility. The use of high temporal resolution imaging aided in the discovery of many steps resembling nonclassical nucleation pathways (Figure 11A). The nucleation process involved the formation of coalesced mesoscale clusters from aggregation which served as a nucleation site. This was evident by the formation of an electron dense entity within the cluster which then developed into the final crystal.[45] Figure 11 displays the typical phases discovered by the two electron microscopy methods, each with distinct stages for the appearance of mesoscale clusters and nucleation within

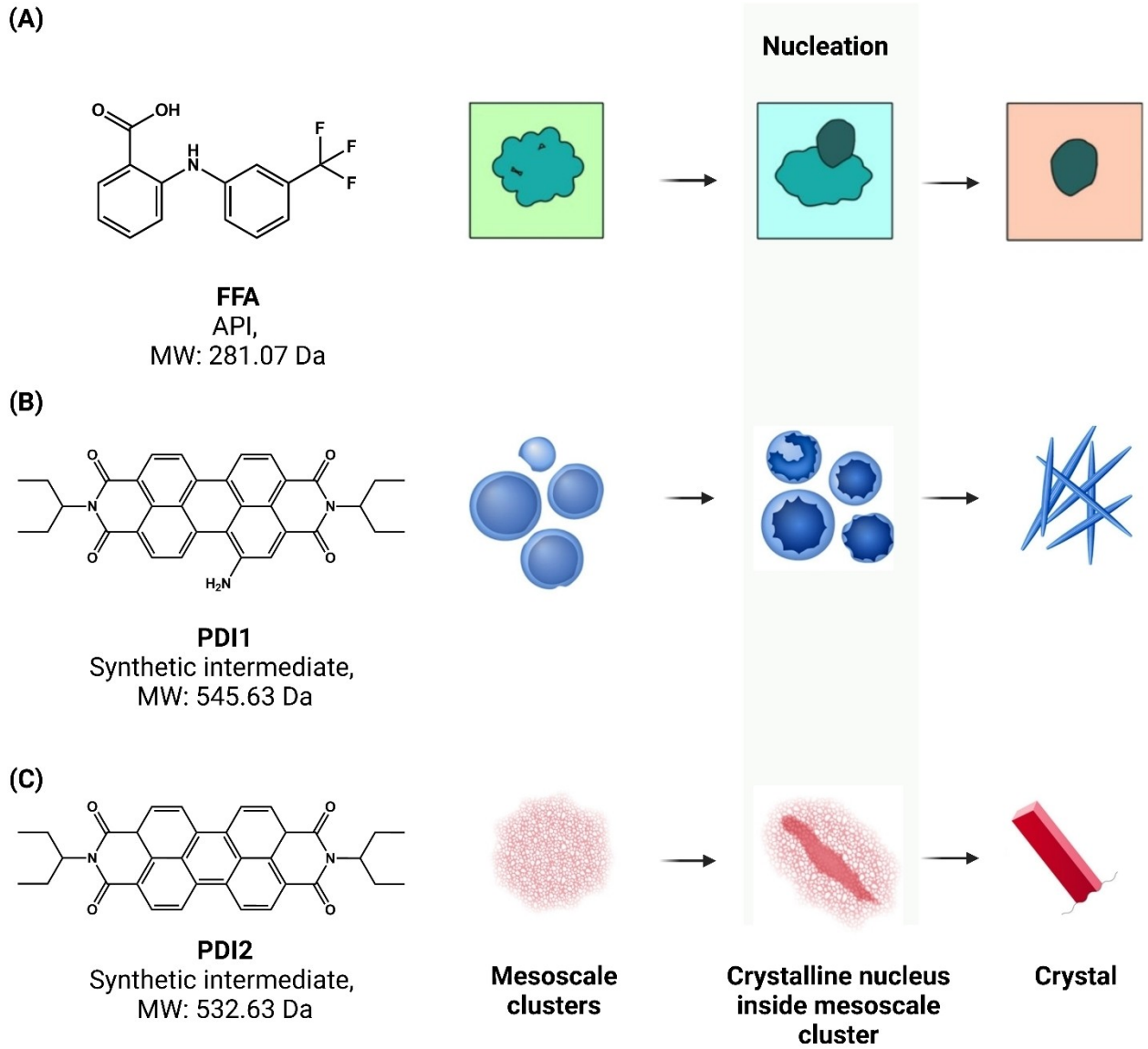

Figure 11. Schematic representation of crystallisation pathway reported for (A) FFA, (B) PDI1 and (C) PDI2 (A) Adapted from Ref. [45] with permission; B and C) Adapted from Ref. [20] with permission, Copyright © 2018 American Chemical Society) (Created with BioRender.com).

them. It should be noted that electron microscopy methods have the ability to record the process in real time,[20,44,46] and hence the reported pathways appear to have a larger number of sub steps.

Based on the previously discussed literature, a common mechanism for the envisioned role of mesoscale clusters in nucleation can be summarised (Figure 12A). Mesoscale clusters form spontaneously as a result of solute dissolution, and the likelihood of nucleation occurring in these solute-rich mesostructured species is relatively high due to the high local solute concentration environments.[16] Any type of agitation can help accelerate the collision and shear forces between clusters, promoting the formation of larger clusters and thus increasing the rate of nucleation.[16,31] This is because they offer relatively larger solute domains that will accelerate the process of mass transfer and reordering of the molecules needed to form a crystalline nucleus.[16] However, in absence of agitation, larger sized mesoscale clusters eventually will form in supersaturated solutions, resulting in a significantly slower rate of nucleation.[16,29]

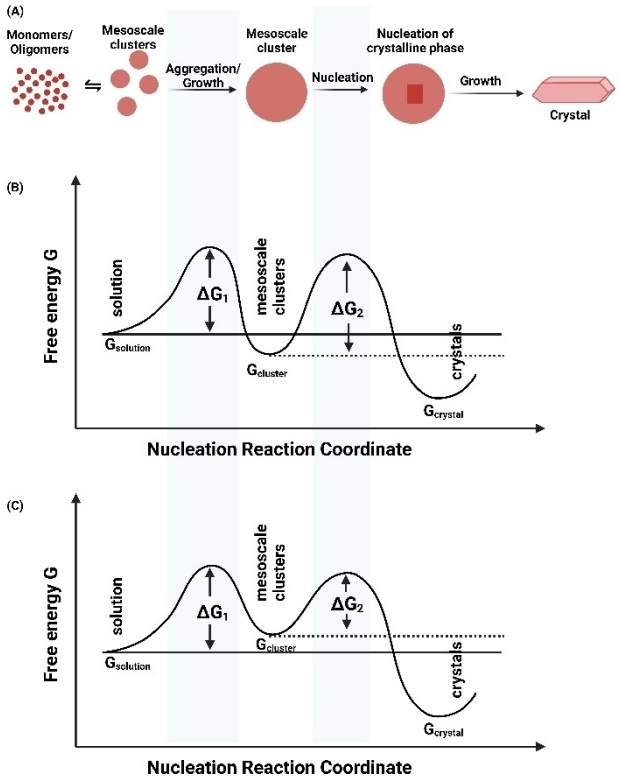

Figure 12. (A) Schematic for nonclassical nucleation pathway involving mesoscale clusters (where the aggregation/growth stage is through agitation) with corresponding free energy diagram for two cases of (B) Mesoscale cluster being stable in relation to solution, (C) Mesoscale clusters being metastable in relation to solution. Note: $G_{solution}$, $G_{cluster}$, and $G_{crystal}$ represent the free energy of solution, mesoscale clusters, and final crystal, respectively. ΔG_1 = barrier to formation/aggregation/growth of mesoscale cluster; ΔG_2 = barrier to nucleation with the mesoscale cluster (Adapted from Ref. [6] with permission, Copyright © 2010 Royal Society of Chemistry) (Created with BioRender.com).

The ability of the CNT to express nucleation through thermodynamic factors via the capillary assumption, which is not the case with nonclassical nucleation theories, is one of its advantages.[1] This is accomplished by assuming that the nanosized nuclei have the same structure as the bulk structure and thus exhibit corresponding interfacial energetics. On the other hand, there are currently no mathematical expressions of the underlying energetics of mesoscale clusters due to a lack of proper understanding of the structure of the clusters and, as a result, the interfacial contribution. Figure 12B and C depict commonly envisioned energetics for nonclassical nucleation via intermediates. Figure 12B represents the case where the mesoscale clusters are in equilibrium with the solution containing solvated monomers or oligomers, with their stability ($G_{cluster}$) lower than the free energy of the solution ($G_{solution}$). This is a commonly observed case where mesoscale clusters are spontaneously formed on solid dissolution and is thought to be caused by the splaying off of the nanoscale domains by the penetration of the solvent into an outer crystal lattice.[16] Figure 12C represents the case where mesoscale clusters are formed during the pre-nucleation phase and are considered metastable.[21] They will have similar energetics, with their stability ($G_{cluster}$) being higher than the free energy of the solution ($G_{solution}$), which would be attributed to system-dependent behaviour varying between compounds.

4. Considerations for Different Measurement Methodologies

Due to its stochastic nature, the study of the nucleation of organic molecules presents a formidable challenge,[47] as does the investigation of the pre-nucleation phase. The combination of poor light and electron scattering contrast inherent to organic molecules, coupled with the presence of loose clustering of solute and solvent molecules with no defined structures, makes this domain a challenging research topic.[1,3] A recent highlight discusses critical factors to consider when selecting techniques, such as sample preparation difficulties and instrument accessibility.[3] In the following paragraphs, these techniques will be evaluated based on important considerations when analysing mesoscale clusters.

DLS and NTA have been widely used in academic research to analyse cluster size, number concentration, and stability. However, the resolution limitations of the liquid light scattering methods must be considered. Single-angle DLS has a resolution threshold of 3:1, which means clusters must differ in size by a factor of three to be accurately resolved.[48–49] Therefore, it is difficult to identify slight differences within the cluster population arising out of variation in solution properties and sample treatment. In contrast, although NTA allows for the identification of size distributions through number-based analysis relatively more sensitively than that of DLS,[50] it does not allow for the identification of monomers/oligomers, therefore any change in the equilibrium between the two populations will remain unclear if used alone. Another criterion to examine is the

method in which cluster size is determined. In the case of DLS, it is critical to monitor the sample's corresponding correlogram as any variations arising in the cluster size would be reflected in the changes in decay time.[51] This ensures the validity of the reported observations and data quality. The number of cluster tracks monitored, plays a key role in judging the quality of the size distributions acquired by NTA.[52] Additionally, it is worth noting that NTA is prone to analyst variability, emphasising the need for consistency in parameter selection across all samples.[53]

In theory, SAXS can provide information on the size, shape, and surface of mesoscale clusters.[16,19] It has been used in the literature to extract information about the structure of mesoscale clusters from scattered intensities. Fitting scattered intensities to novel geometric models (fractal,[16,19] core–shell,[31] and globular[31]) has provided qualitative insights into the probable structure of mesoscale clusters. The proposed prediction models for cluster structure, however, need to be validated using direct observation methods such as electron microscopy. Furthermore, there are a number of problems in terms of beamline and experimental setup that must be addressed.[3] The species of interest are organic in nature, with no phase boundary, and polydisperse with weakly scattering populations. Even with a high-intensity source, the measurement period is long in order to acquire a suitable signal-to-noise ratio.[54] This could be a significant barrier in the case of time-sensitive (or irradiation sensitive samples), which is often the case with mesoscale cluster investigations. Furthermore, in the case of supersaturated metastable solutions, there is a risk of nucleation occurring during the measurement period, posing an additional hurdle for researchers.

Despite the numerous benefits of cryoTEM, there are limitations in the processing of extracted aliquots vitrified for analysis, particularly in terms of sample treatment.[55] As a standard procedure in the vitrification process, humidity modulation is employed to minimise the evaporation of an aqueous sample during incubation on the specimen grid.[55] The droplet is subsequently blotted until the grid is extracted from the chamber and immersed in liquid ethane to begin the vitrification process. It has been found that reported studies on non-aqueous samples do not account for the potential miscibility of the water-rich humid atmosphere and the non-aqueous droplet, for example for perylene diimides in water/THF mixtures,[20] etoricoxib in methanol,[43] and carbamazepine in ethanol.[44] The tiny droplet of a few microliters deposited on the specimen grid prior to vitrification is susceptible to interference from the aqueous-based humid environment, which can disrupt the carefully regulated solvent/anti-solvent ratio or the mother crystallising solution. In addition, it is essential to consider that the act of aliquoting a supersaturated solution entails the risk of initiating nucleation.

In contrast to cryo-TEM, LPTEM has the potential to observe liquid phase nucleation events both temporally and spatially.[45] Nonetheless, its applicability to the study of mesoscale clusters is in its infancy. While LPTEM appears to be a promising method for elucidating cluster kinetics and crystallisation mechanisms, its utility is limited by the potential for electron-beam interactions to introduce artefacts or un-controlled reactions.[45] Although these problems can be mitigated, they cannot be eliminated entirely. Radiolysis is a crucial factor that must be considered in electron microscopy-based research. This is due to the various interactions, such as hydrocarbon contamination, heating, electrostatic charging, and ionisation, that can impact the examined materials.[56] Due to the liquid environment, LPTEM is more susceptible to detrimental radiolysis events, resulting in a series of side-reactions that generate anions, cations, gases, and radical species.[57] This process can lead to pH changes, interfacial formation, local heating, and the emergence of secondary nucleation sites.[57] In contrast to these negative effects of radiolysis, the effects of the electron-beam have been exploited to modify the local chemistry to investigate the crystallisation mechanisms of active pharmaceutical ingredient (API) solutions in greater detail, where the average concentration was below the solubility.[45–46]

5. Summary and Future Outlook

Overall, there is abundant experimental evidence for the existence of large solute-rich domains in solutions of various classes of compounds. The suggested use of the unambiguous term "mesoscale clusters" to define these entities, especially for organic compounds, offers a common terminology for all work involving such species. This review highlights the recent surge in the relationship between mesoscale cluster properties, sample treatment, solution properties and the role of mesoscale clusters in nucleation. Nanoscale characterisation is key to achieving a more holistic understanding of the aforementioned relationship.

As expounded by this review, it is evident that the bulk of mesoscale cluster research until now has focused on amino acids and intermediates, as being suitable model compounds due to their small size and convenient chemical structure. Although certainly these studies have substantially advanced our knowledge of mesoscale clusters, it is necessary to now shift focus to larger, more flexible, organic molecules. These could include many APIs currently under development that are hindered by their inability to solubilise in aqueous environments, typically used in drug processing. There are two intrinsic difficulties with such APIs: conformational flexibility and high molecular weight, both of which have been demonstrated to increase the difficulty of crystallisation.[58] This makes the exploration of their mesoscale cluster properties even more alluring. It is worth noting that compounds with conformational flexibility are more likely to exist in many polymorphic forms.[59] Little to no research has been conducted on the properties of the mesoscale clusters for such systems. The bulk of the molecules discussed in this review had a molecular weight of less than 200 Da.

Although a picture is beginning to emerge for the critical properties of mesoscale clusters, several key theories need further experimental clarification. The differentiation of mesoscale clusters from other phenomena such as oiling

out[60] and liquid-liquid phase separation[61] is one of the grey areas. Furthermore, although NTA has been used to acquire an idea of the volume fraction, a more accurate determination is still lacking. Although it is known that these clusters consist of solute-rich regions, differentiation between clusters and particles of comparable size is challenging using nanoscale characterisation techniques. Moreover, even though a number of organic compounds have been reported to form mesoscale clusters, the universality of the phenomenon needs to be explored. Finally, it would be fascinating to investigate whether information on undiscovered polymorphs may be obtained through interactions between solute molecules inside clusters.

In conclusion, rational assembly and design of materials depends on our ability to control the nucleation process, which will necessitate rigorous predictive capabilities derived from a deeper comprehension of solution behaviour and mesoscale cluster fundamentals. Nonclassical nucleation via mesoscale clustering is essentially a qualitative theory currently while in contrast, CNT can be used to model and quantify a number of thermodynamic and kinetic parameters. Future development of mathematical models for the nonclassical nucleation pathways will enable rationalisation of the system-dependent cluster attributes to the physicochemical properties of the system under investigation.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Mesoscale Cluster · Nanoscale Characterisation Methods · Nonclassical Nucleation · Organic Compounds · Pre-Nucleation

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Minireviews

Mechanisms of Crystallisation

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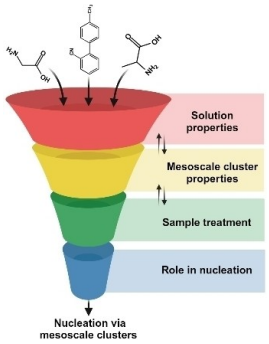
Mesoscale Clusters in the Crystallisation of Organic Molecules



There is a surge in research on mesoscale clusters of organic molecules in light of the limitations of classical nucleation theory. This mini-review offers a fresh perspective on calling upon nanoscale characterisation techniques to evaluate the interdependence of solution properties and sample treatment on cluster properties to go alongside their role in nucleation, all based on literature-based experimental evidence.