Separating nucleation and growth in protein crystallization using dynamic light scattering

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A means of controlling crystallization is to separate the phases of nucleation and growth. Methods to achieve this, other than seeding, involve lowering the supersaturation by changing the temperature or diluting drops after incubating them for a given time at nucleation conditions. However, by the time nuclei or crystals are visible under the microscope too many nuclei will have formed. Dynamic Light Scattering was applied practically, to determine the most likely time for nucleation-growth decoupling to be performed successfully. The time at which DLS showed a significant change in the size-distribution of species in solution, corresponded to that optimal time.

Keywords: protein crystallization, nucleation, microbatch, dynamic light scattering, biological macromolecules, crystallogenesis

1. Introduction

One way to control the crystallization process is to separate the phases of nucleation and growth, i.e. to start the process at conditions which induce nucleation and then transfer the system to metastable conditions, which promote optimal growth. Methods to achieve this other than seeding involve changing the temperature (Rosenberger *et al*., 1993; Haire, 1996) or diluting microbatch drops after incubating them for a given time at spontaneous nucleation conditions (Saridakis et al., 1994).

It has been shown that the optimum time for dilution of microbatch drops was long before the appearance of the first visible microcrystals (Saridakis *et al*., 1994). The dilution method has recently been adapted by Saridakis & Chayen (2000) to vapour diffusion, with similar results. As in the case of temperature shifts, these techniques yielded improved crystals, but were very time-consuming, since many processes of trial and error were required to determine the right time at which to dilute (the time scale could only be guessed at by reference to the time which it took to see the first crystals). Consequently this method has not yet been adopted for routine use.

The most effective moment to intervene with a crystallization experiment is soon after the formation of the first critical size nuclei which will eventually form the crystal. By the time nuclei or crystals can be observed under laboratory microscopes, they have already reached a size of approximately 5 µm, and by then it is probably too late to act, since the nucleation process is too far advanced (Saridakis *et al*., 1994).

Dynamic light scattering (DLS) offers a size resolution of "particles" in optically transparent aqueous samples some three orders of magnitude below an optical microscope and consequently forms a useful tool for an early, non-invasive, *in-situ* observation of a crystallization event. A laser is focussed onto the protein solution and the light scattered by the particles within the solution, such as protein molecules or aggregates, is collected. The events recorded in the scattering volume of approx. 50 µm x 50 µm are bulk representative as has been confirmed experimentally (see Materials and Methods). The time constant(s) of the second order Auto-Correlation Function (ACF) of the scattered light intensity deliver the diffusion coefficient(s), and hence the hydrodynamic radii of the particles present. DLS is sensitive to variations in particle size (in the range of approx. >1nm) and interactions of protein molecules in solution (Schmitz, 1990). DLS is routinely used in many labs to assess sample mono-dispersity using dilute protein samples (D'Arcy, 1994; Ferré-D'Amaré, 1994; Bergfors, 1999). DLS can be used as an indicator of the induction time for nucleation, i.e. the time at which post-critical nuclei start their existence (Malkin & McPherson, 1993; Ataka, 1998). It has also been used successfully with lysozyme to show an increase in hydrodynamic radius as supersaturation proceeds (Mikol *et al*., 1990; Malkin *et al*., 1993; Malkin & McPherson, 1993; Georgalis *et al*., 1995; Peters *et al*., 1998; Schueler *et al*., 1999). In this study we have monitored the crystallization of proteins mixed with their crystallising agents by DLS, so as to get an indication as to when to dilute the trial in order to lead it out of the nucleation into the growth phase. This was achieved using a DLS-apparatus which is able to handle a crystallization sample in standard cuvettes of 20-30 µl.

We have set out to test whether changes in the aggregation profile of a supersaturated protein sample at nucleation conditions as a function of time, can be used as an indicator of when to reduce the supersaturation of the trial. By diluting, at various times after set up, batch drops set at the same (nucleation) conditions as the solution from which DLS data are being recorded, this can be qualitatively verified. Indeed, if the dilutions result in the solution being brought to metastable levels of supersaturation, solutions that contain post-critical nuclei at the time of dilution will sustain their growth into visible crystals, whilst solutions containing only pre-critical aggregates will remain clear. We have therefore monitored the end results of microbatch crystallization experiments, where the solution was diluted to (known) metastable conditions at various times after set up, and compared these results with changes in the size-distribution time-profiles as resolved by DLS from an identical solution, at corresponding times.

2. Materials and methods

2.1. DLS device

A DLS apparatus (DIMINIGON-A, Dierks and Partner, Hamburg, Germany) and software package also commercially available from the same company was used, as described by Dierks *et al.* (1999) and references therein. All data presented here were collected at an angle of 90°. Temperature stabilization (at 20 +/- 0.05°C) of the cuvette holders, data evaluation and selection of measurement times were computer-controlled. The instrument is designed to take reliable measurements from as little as 20-30 µl of solution, contained in a glass cuvette which can be covered with light paraffin oil, thus closely mimicking the conditions of a microbatch experiment. The software uses CONTIN (Provencher, 1982) to analyse the ACF of the intensity fluctuation.

2.2. Proteins

Two proteins were used in this study. Porcine pancreatic trypsin type IX (cat. no. T-0134) and thaumatin from *Thaumatococcus danielii* (T-7638) were purchased from Sigma (Steinheim, Germany) and used without further purification. Furthermore, hen egg-white lysozyme (Sigma L-6876) was used to compare the obtained

hydrodynamic radii with the ones published to allow for confirmation of appropriate values for variables such as refractive index and viscosity for quiescent systems, and to assess the reliability of the size estimates given by the apparatus. As outlined above, the reliability, accuracy and reproducibility was good if the supersaturated solution was filtered through 0.1 µm mesh size filters. All salts and buffers were purchased from Sigma.

The solutions from which DLS measurements were taken were dispensed (30 µl) in the glass cuvettes that are furnished with the apparatus, whereas parallel microbatch experiments were set up in Terasaki-type plates (Nunc, Denmark) covered with light paraffin oil (BDH, U.K.). Many identical 5 µl crystallization drops were set up for each protein, at conditions known to promote nucleation and the rapid growth of fairly small crystals. The same conditions were used for the DLS measurements.

These conditions were: (a) 20 mg/ml protein, 34% (sat at 20°C) ammonium sulphate, 100 mM Tris pH 8.4 for trypsin, and (b) 32 mg/ml protein, 0.45 M sodium potassium tartrate, 50 mM PIPES pH 6.7 for thaumatin.

For each protein, 100 µl of solution at the above conditions was prepared and filtered through 0.1 µm mesh size micro-centrifuge filters (Ultrafree-MC, Millipore, Bedford, USA). The solution was then distributed between the DLS cuvette and the wells of a Terasaki plate.

A series of auto-piloted DLS measurements, with 20 sec ACF acquisition time for each measurement and 1 sec stand-by between measurements was recorded, starting as soon as possible (approx. 5 to 15 minutes) after the mixing and filtering of the ingredients, when the solution had calmed. Further series were recorded at regular time intervals thereafter, as detailed below. Each series consisted of at least 20 measurements. This ensured that distorted ACFs or ones with overflows could be identified and dismissed**.** Measurements were taken in different parts of the cuvette (by slightly modifying its vertical position and orientation with respect to the laser beam) to ensure that the scattering volume was representative of the sample bulk.

At the same regular intervals, microbatch drops were also diluted (as described below; two drops per interval) with filtered buffer solution, to metastable conditions, which had been determined beforehand for each protein by establishing the supersolubility curve around published conditions (Christopher *et al.*, 1998; Chayen *et al.*, 2001). A supersolubility curve separates the spontaneous nucleation zone of a crystallization phase diagram from the zone at which the solution remains clear (metastable or unsaturated). The metastable zone is then the area of conditions below the supersolubility curve and above solubility, where nuclei transferred from the spontaneous nucleation zone (e.g. by dilution) will continue to grow. The method is described in more detail in Saridakis *et al.* (1994) and information on the proteins studied here was available to us from previous work (Chayen *et al*., 2001). In this case, one metastable condition for each protein was sufficient. These were: (a) 18 mg/ml protein, 30% (sat at 20°C) ammonium sulphate, 100 mM Tris pH 8.4 for trypsin, and (b) 20 mg/ml protein, 0.28 M sodium potassium tartrate, 50 mM PIPES pH 6.7 for thaumatin.

Both the nucleation and metastable conditions were chosen previous to and independently of any DLS-related measurement or consideration, being the pairs of conditions that yielded the larger and morphologically best crystals in preliminary dilution experiments.

Terasaki plates were examined by light microscope every few days for more than one month after each experiment. The results were then compared with the corresponding series of DLS data showing the size distribution profile of different species in solution at the given time. Such experiments were performed twice for each protein. In addition, various incomplete experiments performed at

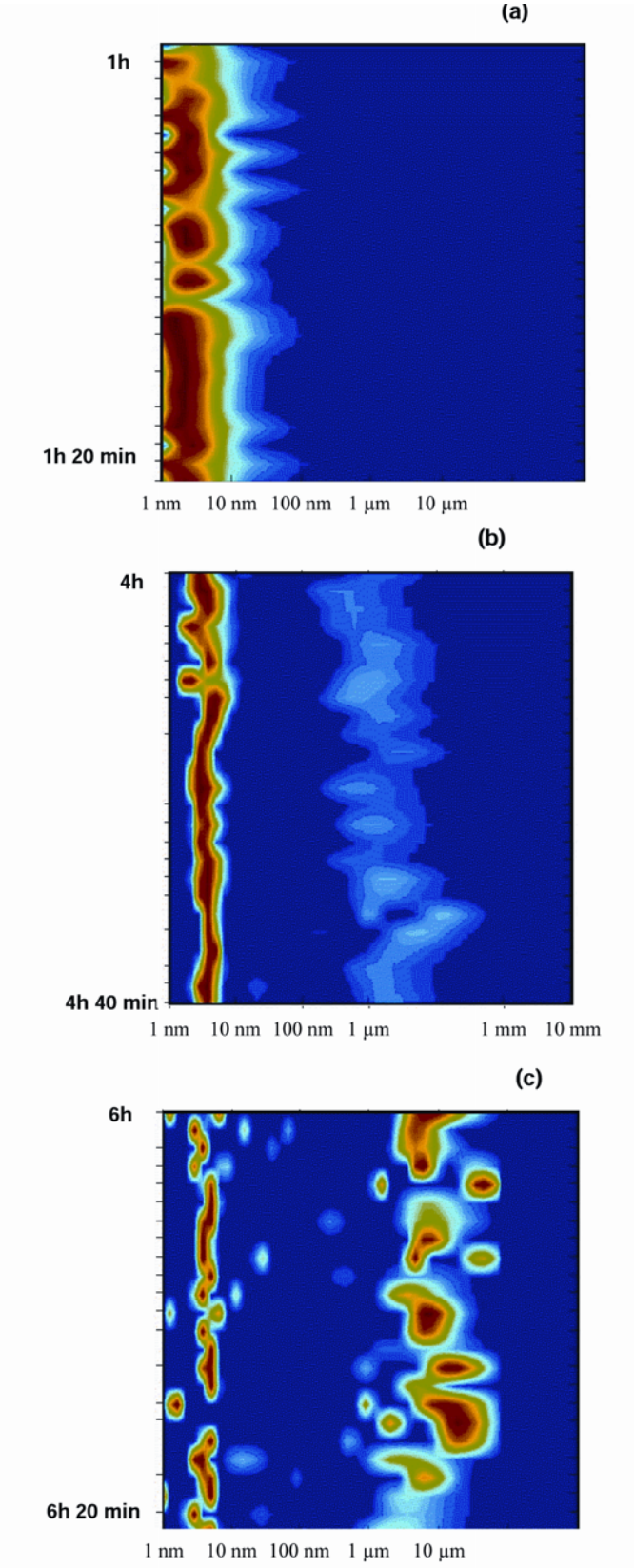

Figure 1
DLS measurements showing size of particles in solution *versus* time for a trypsin crystallization solution collected at various times after set up and filtration of the solution: (a) 1h to 1h 20 min; (b) 4h to 4h 40 min; (c) 6h to 6h 40 min.

Table 1 Numbers and sizes of crystals of undiluted trials, compared with dilutions at several times.

	Undiluted controls	Diluted between 0 - 3h 30 min	Diluted between 4h - 4h 30min	Diluted between 5h - 5h 30 min	Diluted at 6h	Diluted at 7h	Diluted at 8h
Trypsin	Usually clusters. Occasional single crystal (max. 200 x 200 x 50 μm)	drops clear	drops clear	drops clear	1 - 5 crystals. Typically 500 x 200 x 200 μm	1 - 5 crystals. Typically 500 x 300 x 200 μm	Clusters. Occasionally single crystals up to 200 x 200 x 50 μm
Thaumatin	20 - 100s crystals. Max. 200 x 100 x 100 μm, but mainly microcrystals	drops clear	5 - 20 crystals. Typically 400 x 200 x 200 μm	5 - 20 crystals. Typically 400 x 200 x 200 μm	100s of microcrystals	100s of microcrystals	100s of microcrystals

other times (e.g. consisting of dilutions only, or for checking the repeatability of the DLS measurements) were in generally good agreement with the "full-scale" experiments.

3. Results

3.1. Trypsin

Results representative of the data obtained for these sample systems for a run of DLS measurements with trypsin are presented in Figures 1 a - c. This mode of presentation displays the (apparent) size of the species on one axis and successive measurements in the series spread along the other. A colour (grey) scale represents the relative change of each species contained in the solution as a function of time. However, without further assumptions derived from specific tests, it is not possible to assign a specific scale (e.g. concentration or number of particles) to this representation. The scale is "normalised" with the species contributing most to the scattering being always assigned the maximum value of 1. The more precise values for the peak centres and widths that are stated in the text have been read from histograms (not shown here) obtained directly from CONTIN.

Data collected very shortly after mixing show a strong majority of size distribution for a small component (peak at 1.8 ± 0.4 nm), and a much smaller population of very large size aggregates (peak in the μm size range), that vanishes very quickly. This situation remains practically unchanged (Fig. 1a) until the series ending at t = 4h 40 min. A very weak peak corresponding to a few larger size aggregates (hundreds of nm to μm range; Fig. 1b) has however appeared in the t = 4h - 4h 40 min series. In the following series, starting at 6 hours after set up (Fig. 1c), the scattering is now dominated by very strong peaks in the μm size range and signs of more transient new peaks at tens to hundreds of nm sizes are evident. This situation remains relatively stable afterwards.

A repeat measurement run for trypsin (not shown here) under identical conditions, presents a very similar picture.

In the microbatch drops, a dilution time of 6 to 7 hours after set-up reproducibly yielded larger crystals than the undiluted controls. Dilutions performed later usually resulted in crystals no larger or morphologically better (by visual inspection) than the controls, whereas those performed earlier generally resulted in clear drops (Table 1).

Two other series of measurements, under identical starting conditions, were also run, in which the solutions inside the DLS cuvettes were themselves diluted at the determined optimum decoupling time. Care was taken not to move the cuvette inside the DLS apparatus when diluting. In both cases, within 40 min from dilution time, a weakening of the large size peak and a corresponding strengthening of the small size peak were observed, i.e. a partial reversion of the size-distribution profile to starting time values.

3.2. Thaumatin

The discontinuous change in the size-distribution profiles is less pronounced in the case of thaumatin, so a more sensitive method was used to display and assess the results (Fig. 2 shows one of the runs). Curves showing the normalised scattering intensity *versus* (apparent) hydrodynamic radii of species in solution were plotted separately for each measurement of the series, and compared. For a series of measurements starting at 10 min, 30 min, 1h 30 min, 2h 30 min, 3h 30 min, 4h 40 min after set up, almost every measurement in the series yields a curve very close to the typical ones shown in Fig. 2a, b (for clarity, three curves per series only are shown). These correspond to two resolvable populations in solution, the most numerous corresponding to a peak at 1.9 ± 0.2 nm and the less numerous corresponding to a peak initially at 55 ± 10 nm but slowly shifting to larger size.

In the series measured at t = 5h 30 min (Fig. 2c), various extra peaks make their appearance and approximately half the measurements in the series now yield very different size-distribution profiles, the original peaks remaining nevertheless dominant. The profiles remain fairly unchanged thereafter, with a slow progression of the small component resolved species towards larger sizes (Fig. 2d for t = 6h 30 min).

The microbatch drops set up in identical conditions remained clear when diluted between 30 min and 3h 30 min after set up and yielded single crystals larger than those in the undiluted controls when diluted between 4 and 5h 30 min after set up. Those diluted later than 6 hours after set up yielded small crystals, no better (often worse) in terms of size and morphology than those in the controls (Table 1).

4. Conclusion

This study has focused on separating the nucleation and growth phases by diluting crystallising solutions from nucleation to metastable conditions. Dilution is a good method to use either when a protein is not temperature-sensitive or to avoid handling crystal seeds.

The results demonstrate that DLS can be applied in a very qualitative way to determine **the time** in which to intervene with a crystallization experiment and lead it from nucleation to growth conditions. We have shown, for two model proteins, that the time at which DLS showed a significant change in the size-distribution profile of species in solution, corresponded to the time at which the solution can be effectively transferred to metastable conditions, for optimal growth. The method presented here will therefore pinpoint the most likely time for such nucleation-growth decoupling to be performed successfully in a given system. Although this time may be found by systematic trials as has been described in the Introduction, the method described here will allow many laboratories, which lack automated crystallization facilities or dedicated personnel but have a

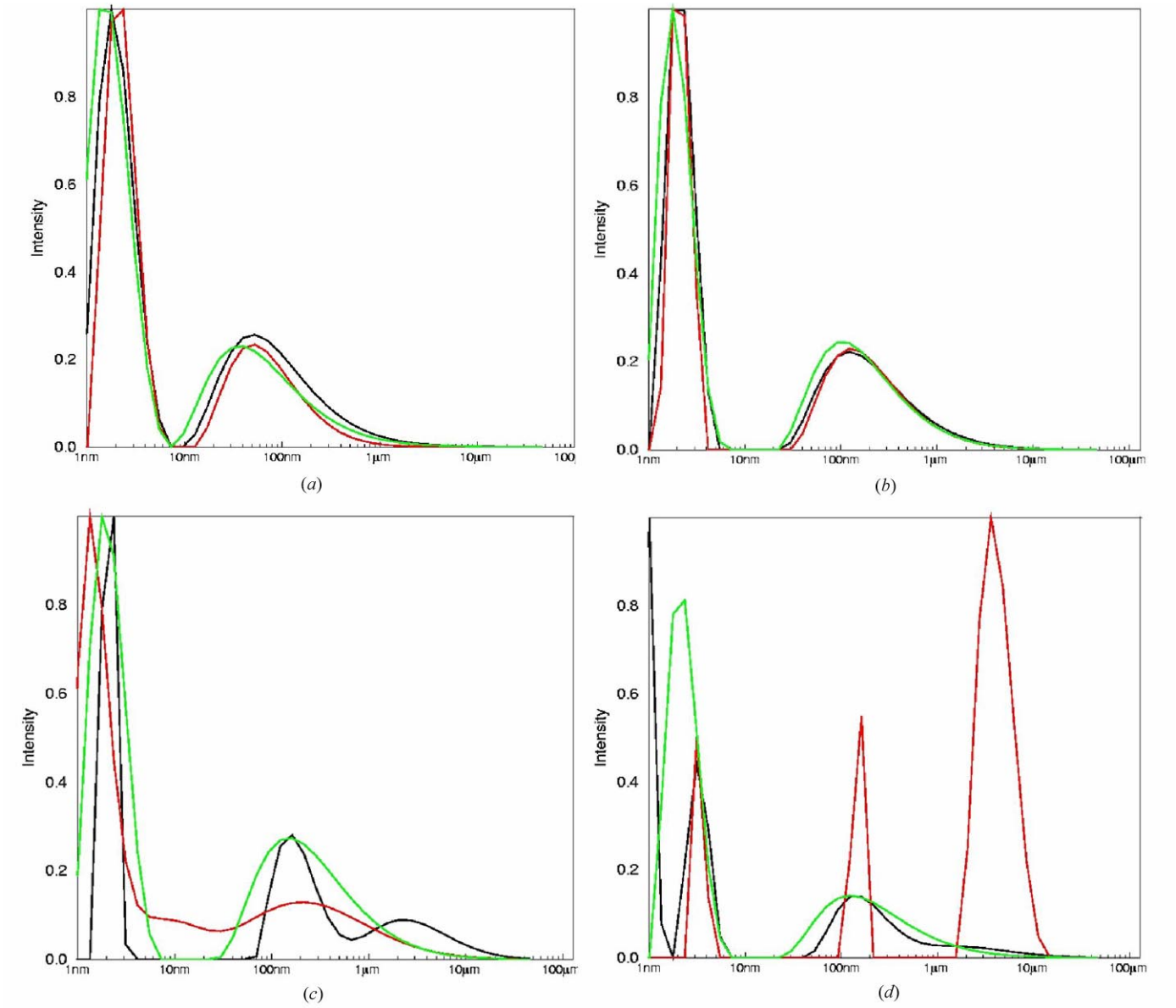

Figure 2
DLS measurements showing (relative) intensities of scattering for different size aggregates collected at various times after set up and filtration, for a thaumatin crystallization solution. 20 autocorrelations were performed for each series, corresponding to time intervals of ca. 8 min. For clarity, only three typical curves per starting time (corresponding to 3 different ACFs within these 8 min) are shown: (*a*) 10 min; (*b*) 3h 30 min; (*c*) 5h 30 min ; (*d*) 6h 30 min.

simple DLS machine at their disposal, to routinely perform crystal optimisation based on nucleation-growth decoupling.

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