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Nucleation and polymorphism explored via an easy-to-use microfluidic tool

M. Ildefonso [a], Eve Revalor [a], P. Punniam [a], J.B. Salmon [b], N. Candoni [a], Stéphane Veesler [a,*]

[a] Centre Interdisciplinaire de Nanosciences de Marseille, CNRS, Aix-Marseille Université, CINaM-UPR 3118, Campus de Luminy, Case 913, 13288 Marseille Cedex, France
[b] Laboratoire du futur, unité mixte Rhodia/CNRS/Bordeaux-1, 33608 Pessac Cedex, France

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ABSTRACT

We present an easy-to-use microfluidic set-up, easily transferable to the laboratory and determine an accurate method for metastable zone width measurement. We clearly define a zone in the phase diagram where nucleation is mononuclear. We nucleate a single crystal of metastable phase, which turns out to be stable. This approach holds promise for the control and the study of crystallization processes.

1. Introduction

Miniaturization of tools to crystallize organic molecules and proteins is a hot topic. Novel methods such as crystallization robots for high-throughput screening [1] and emulsion-based methods have been developed. Examples of the latter include spherical crystallization by the quasi-emulsion method [2,3], methods using the miscibility gap in the phase diagram [4] or microfluidic methods [5]. Working with small volume systems has already proved valuable and holds great promise for the control and the study of crystallization processes, for instance, producing crystals of controlled size [6]. In practice, this makes it possible to increase the number of experiments and decrease the amount of material used [7–9].

In this paper, we focus on nucleation, with an easy-to-use and easily transferable to the laboratory (this technology can be implemented in standard laboratory environments) microfluidic set-up developed by Laval et al. [10,11]. Hundreds of droplets can be formed and stored in a microfluidic chip. These droplets are independent micro-reactors in which the confinement makes it possible to dissociate crystal nucleation from growth, allowing nucleation to be studied and controlled. The high number of droplets in the microfluidic chip yields a large sample of independent crystallization events, a prerequisite for statistical analysis of a stochastic process such as nucleation. Currently, the supersaturation range over which experiments can be performed is rather limited due to the metastable zone. In addition, the nucleation rate J can only be measured in the vicinity of the metastable zone limit [12]. Thus, determination of the metastable zone width is a key step in nucleation studies. Here, we develop a method to measure metastable zone width. By using it to examine a protein, the lysozyme, we isolate and observe a metastable polymorph.

2. Materials and methods

2.1. Protein solutions

Hen-egg white lysozyme (14600 Da, $pI=11.2$) was purchased from Sigma (batch 057K7013 L 2879) and was used without further purification. The purity of lysozyme was checked by molecular sieving. A suitable amount of lysozyme was dissolved in pure water (ELGA UHQ reverse osmosis system) to obtain the stock solutions required. A 1.4 M NaCl solution was also prepared. The different solutions were buffered with 80 mM acetic acid, adjusted to pH=4.5 with NaOH (1 M) and filtered through 0.22 μm Millipore filters. The pH was checked with a pHmeter (Schott Instrument, Prolab 1000) equipped with a pH microelectrode. Lysozyme concentrations were controlled by optical density measurements (Biochrom, Libra S22) taking an extinction coefficient of 2.64 mL cm^{-1} mg^{-1} at 280 nm. In all the crystallization experiments hereunder, the NaCl concentration was fixed at 0.7 M at pH=4.5.

2.2. Droplet generation and storage

The basic microdevice design and construction was previously described [10]. In our adaptation, shown in Fig. 1, microfluidic chips are divided into 2 parts: the plug factory zone for the generation of droplets and the droplet storage zone.

Plug factory: protein and salt solutions are injected (points 1 and 2 in Fig. 1a) at a rate of 300 μL/h with syringe pumps (Bioseb BS800) and thus mixed by diffusion. Droplets are generated at the intersection between the silicone oil (Sigma oil AP 100, viscosity 940 cSt) and

* Corresponding author. Tel.: +336 6292 2866; fax: +334 9141 8916.
E-mail address: veesler@cinam.univ-mrs.fr (S. Veesler).

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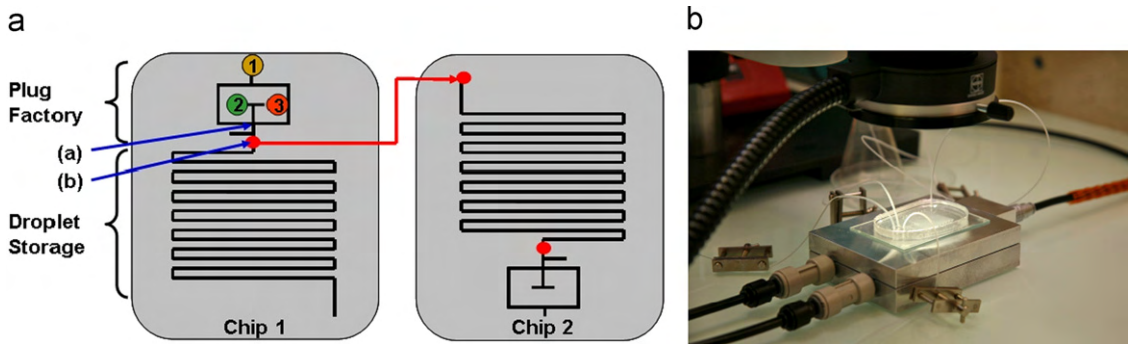

Fig. 1. (a) Design of the microdevice: oil, protein and NaCl solutions are injected in inlets (1), (2) and (3), respectively, (channel width 500 μm) and (b) microfluidic experimental set-up with thermostatted sample-holder.

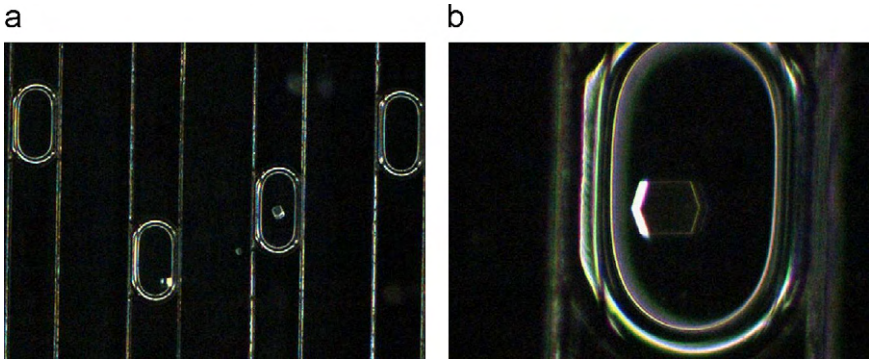

Fig. 2. (a) and (b) Droplets of lysozyme solutions (20 mg/mL—0.7 M NaCl—pH=4.5) observed after stored 20 h (channel width 500 μm).

Table 1
Experimental conditions for metastable zone width determination.

Protein concentration (mg/mL)	Temperature (°C)			
15	15	20	25	30
20	20	25	30	35
30	25	30	35	40
50	30	35	40	45

aqueous streams (point (a) in Fig. 1a), the total flow rate for the oil being 1.4 mL/h. Each droplet has a volume of 250 nL with a volume polydispersity of a few percent [11,13].

Up to 200 droplets are stored, either in the same chip or in another; in this latter case chip 2 is connected to the outlet of the chip 1 plug factory zone (point (b) in Fig. 1a) using micro-tubing (SCI, BB311-24) of 584 μm inner diameter. This procedure allows us to fill several chips with the same chemical composition.

2.3. Determining metastable zone

In order to determine the metastable zone width for lysozyme solutions at pH=4.5, we fill 4 microfluidic devices with the same solution and store them at different temperatures for 20 h. Then we observe and count crystals that have nucleated in each drop with a stereomicroscope (Wild Makroskop) equipped with a CCD camera (imaging source, DFK 31BF03). During observation the device is thermostatted by Peltier elements (Anacrismat, France) (Fig. 1b). Typical images at different magnifications are shown in Fig. 2. Different concentrations were tested; experimental conditions are summarized in Table 1.

3. Results and discussion

3.1. Metastable Zone (MZ) width

The metastable zone limit is characterized by the maximum temperature above which the supersaturated solution can stay for the required time, here 20 h, without losing its metastability [14]. This temperature (at which there is no crystal), T_{MZ}, is extrapolated (interpolation) from a plot of the average number of crystals versus temperature (Fig. 3a). Experimental results are summarized in Table 2, i.e. average number of crystals versus temperature for different protein concentrations. Note that we nucleated solely tetragonal lysozyme after 20 h for temperature ranging from 15 to 40 °C.

According to our experimental results the pair protein concentration—T_{MZ} is a point on the curve that limits the metastable zone. In Fig. 3b, a plot of these pairs for different protein concentrations is presented together with the solubility curve [15]. The metastable zone that we are interested in is situated between the solubility curve and the MZ limit. Note that here we obtained the MZ limit after only 4 experiments because we were able to fill several storage chips using only one generation chip (see part 2.2.). Moreover, the high number of reproducible droplets in the microfluidic chip permits an accurate statistical analysis. To summarize, we show here that this microfluidic method is an accurate and easy-to-use way to determine MZ width.

3.2. Mononuclear vs polynuclear nucleation

In the literature, mononuclear nucleation is often claimed for small volume systems, namely droplet methods [16] or microfluidic methods. However, this was recently questioned both

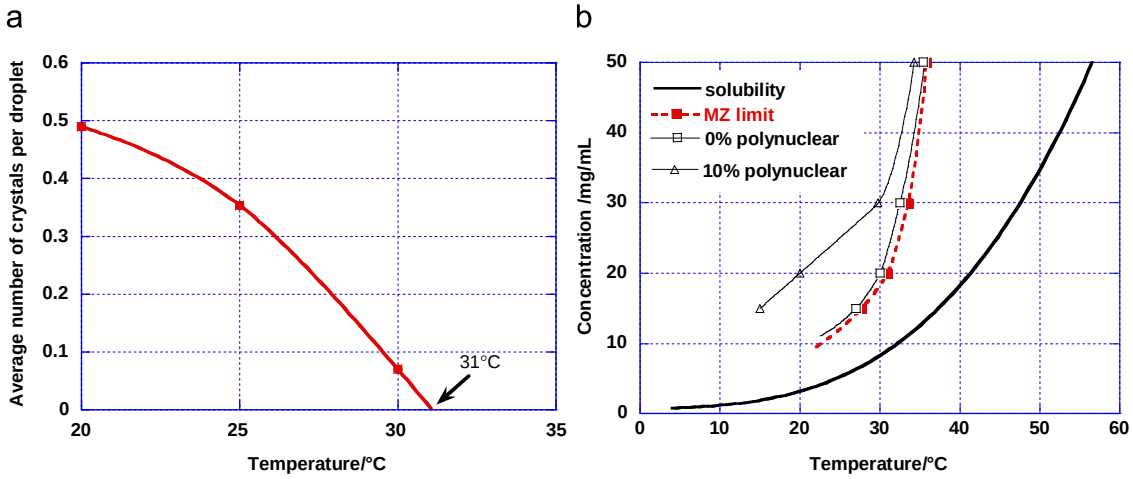

Fig. 3. (a) Average number of crystals per droplet versus temperature after 20 h, lysozyme at 20 mg/ml, NaCl 0.7 M and pH=4.5 and (b) tetragonal lysozyme (0.7 M NaCl—pH=4.5) solubility versus temperature according to [15], MZ limit and limit between mono and polynuclear mechanism (see text for explanation), lines improve legibility.

Table 2
Results for metastable zone width determination.

Protein concentration (mg/mL)	[a]	Temperature (°C)							
		15	20	25	30	35	40	T_{MZ} [b]	T_{polyN} [c]
15	0	57.05	64.52	90.97	99.20				
	1	31.29	27.74	4.17	0.8				
	>1	11.66	7.74	4.86	0			27.7	$25 < T < 27.7$
	$<n>$	0.601	0.471	0.201	0.008				
	N	163	155	144	125				
20	0		64.52	76.55	94.37	100			
	1		24.52	17.24	4.23	0			
	>1		10.96	6.21	1.41	0		31.0	30.0
	$<n>$		0.490	0.354	0.070	0			
	N		155	145	142	103			
30	0			69.62	59.84	100	96.49		
	1			27.85	31.50	0	3.51		
	>1			2.53	8.66	0	0	33.5	32.5
	$<n>$			0.342	0.496	0	0.035		
	N			158	127	122	114		
50	0				18.44	79.57	100		
	1				35.46	16.13	0		
	>1				46.10	4.30	0	35.8	35.6
	$<n>$				1.794	0.258	0		
	N				141	93	101		

[a] 0, 1 and >1 correspond to 0, 1 and more than 1 crystal per droplet in percentage, $<n>$ is the average number of crystals per droplet and N the total number of droplets.
[b] T_{MZ} extrapolated temperature of metastable zone limit
[c] T_{polyN} extrapolated temperature for polynuclear nucleation

theoretically [17] and experimentally [18]. Here, we are able to determine a clear zone in the phase diagram where it was observed a single nucleation event, that is to say is mononuclear. From the data presented in Table 2, we can extrapolate a T_{polyN} from the evolution of number of crystals with temperature, at a fixed protein concentration. For this purpose, an extrapolation similar to the one used for T_{MZ} is applied to the percentage of droplets where more than one crystal is observed. At T_{polyN} and above, the probability of observing more than one crystal in a droplet is 0. Data are presented in the last column of Table 2 and plotted in Fig. 3b, leading to a polynuclear limit. The mononuclear zone is situated between the MZ limit and the polynuclear limit. Below this limit, the probability of obtaining more than 1 crystal increases. This mononuclear zone is very narrow (Fig. 3b); to widen it we plotted the 10% limit (Fig. 3b), i.e. where 10% of droplets contain more than 1 crystal. This strategy yields a greater experimental zone maximizing the number of droplets with 1 crystal.

3.3. Polymorphism

By controlling the nucleation mechanism, monodisperse particles can be produced [6]. This is useful in order to isolate a metastable phase. Indeed, it was recently shown that in small volume systems the probability of observing a metastable phase is increased [13,19]. Because of the mononuclear nucleation, the nucleated crystal of the metastable phase being alone cannot dissolve. Thus, small volumes are able to "freeze" the metastable phase [19]. Moreover, the higher supersaturations reached in small volume systems increase the probability of nucleating a metastable phase [20].

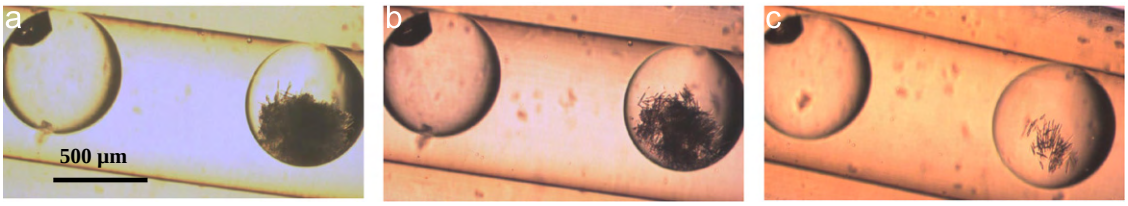

Fig. 4. Droplets of lysozyme solutions (20 mg/mL (0.7 M NaCl—pH=4.5) observed (a) at 20 °C after stored 20 h at 6 °C, after increasing temperature to 30 °C, (b) after 6 min and (c) after 12 min.

We tested this assertion with lysozyme crystallization for which different polymorphs are known [21,22]. In part 3.1. we showed that with temperatures from 15 to 40 °C we nucleated solely tetragonal lysozyme after 20 h. To nucleate a new phase, we therefore performed experiments at 20 mg/mL and 6 °C for 20 h and we observed (at T=20 °C) a different crystal habit (the sea-urchin habit) in 6 droplets out of 237 (Fig. 4). In a morphodrom of lysozyme crystals, at pH 4.6 and in NaCl solution, Tanaka et al.[23] previously observed the sea-urchin habit but did not assigned it to a new phase. Thus, varying temperature, we verified whether if the 2 crystal habits corresponded to 2 different phases [24] (Fig.4). We increased the temperature to 30 °C and observed the dissolution of the sea urchin-like habit (on the right in Fig. 4b–c) whereas the tetragonal lysozyme (on the left in Fig. 4b–c) was stable and probably continued growing. In addition, we estimate the concentration in the droplet. We assume that droplets are spherical with a diameter of 500 µm and that their initial concentration of lysozyme is of 20 mg/ml. Hence, if a cubic shape lysozyme crystal of 50 µm of edge emerges in a droplet, it decreases its concentration of 5%, that is to say the final concentration is around 19 mg/mL.

In fact, the equilibrium temperature of tetragonal lysozyme, in this experimental condition, is about 40 °C (Fig. 3b). Moreover, using the bracketing method [25] we estimate at $\pm$1 °C the equilibrium temperature to be 24 °C for the sea urchin phase in suspension in a 19 mg/mL lysozyme solution. Using this microfluidic method, therefore, we successfully isolate a metastable phase.

4. Conclusion

In this paper, we have shown how an easy-to-use microfluidic set-up, easily transferable to the laboratory (this technology can be implemented in standard laboratory environments), in conjunction with an appropriate method, enables the metastable zone width of a protein to be measured. The technique also enables a zone in the phase diagram where nucleation is mononuclear (one crystal per droplet) to be defined. We also demonstrate the nucleation of a single crystal of a metastable phase, which turns out to be stable; because the metastable phase is "frozen" in the droplet due to the high supersaturations reached in small volume droplets generated by the microfluidic system.

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