SUPPORTING INFORMATION FOR

Using Microfluidics to Observe the Effect of Mixing on Nucleation of Protein Crystals

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Preparation of Solutions:

Protein: Thaumatin (Sigma) was dissolved in 0.1 M pH 6.5 buffer of N-(2-acetamido)iminodiacetic acid (ADA buffer) to make a stock solution of high concentration. The stock solution was centrifuged at 13.2 krpm for 30 seconds (Eppendorf, centrifuge 5415D), and filtered with a 0.22 μm Millipore filter. The precise concentration of the stock solution was determined by measuring the UV-Vis (Agilent, 8453 UV-Visible system) of the solution after dilution of 30 times using an extinction coefficient of 28270 $L \cdot mol^{-1} \cdot cm^{-1}$ at 280 nm and a molecular weight of 22,000 Dalton. The stock solution was then diluted to the desired concentration with filtered ADA buffer. All thaumatin solutions were freshly made several hours before the experiment.

Buffer stream: 0.1 M pH 7.0 buffer of N-[2-hydroxyethyl]piperazine-N'-[2-ethenesulfonic acid]) (HEPES buffer, Sigma) was used.

Precipitant stream: 2.0 M sodium potassium tartrate (Sigma) in 0.1 M pH 7.0 HEPES buffer was used.

Carrier Fluid: the carrier fluid was a 10: 1 (v/v) mixture of FC 3283 (3M) : 1H,1H,2H,2H-perfluoro-1-octanol (Acros Organics).

Experimental details for each figure:

Figure 1: The microfluidic devices were fabricated using soft lithography (McDonald, J. C.; Whitesides, G. M. *Accounts Chem. Res.* **2002**, *35*, 491-499.) as described previously (*Angew. Chem. Int. Ed.* **2004**, 43, 2508-2511). The devices used consisted of two parts (refer to Figure 1a for a schematic of the device): the first part was a polydimethylsiloxane (PDMS) microfluidic device with channels of 200 × 200 μm^2 cross-sectional dimensions; the second part was an X-ray capillary with ID 180 μm and OD 200 μm (Hampton Research). The capillary was cleaned with chromic acid before use. The capillary was connected to the PDMS channel and the junction was sealed by partially cured PDMS. The partially cured PDMS was allowed to fully cure immediately afterwards in a 60°C oven for 10 min. The inner wall of the PDMS channels and the capillary was treated with N_2 flow (100 mm Hg) containing the vapor of tri(decafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) for 30 min, flushed with FC 3283 (3M) and baked in a 60°C oven for at least 2 hours. Before each experiment, the aqueous inlets of the device was made hydrophilic by filling the inlets with 5% BSA solution in PBS buffer for 10 seconds and sucking the solution out with vacuum. This procedure helped to maintain the flow stability.

The mixing experiments were performed in an 18°C room. Plugs of a mixture of protein, buffer and precipitant were formed and mixed in the microfluidic channel and transported to the glass capillary connected to the channel by injecting solutions of carrier fluid, thaumatin protein, buffer, and precipitant into the device. We used Harvard Apparatus PHD 2000 Infusion pumps and Hamilton Gastight syringes to inject solutions. 50-μL Hamilton Gastight syringes (1700 series, RN) with removable needles of 27-gauge

were used with 30-gauge Teflon tubing from Weico Wire & Cable to drive aqueous flows. 1-mL Hamilton Gastight syringes (1700 series, TLL) were used with 30-gauge Teflon needles with one hub from Hamilton for carrier fluid. All the solutions were filtered with 0.22 μm Millipore filters right before the experiment.

After the glass capillary was filled with plugs, the capillary was cut off from the PDMS device with the solutions still flowing. The capillary was sealed at two ends with capillary wax (Hampton Research) and kept in an 18°C incubator for the protein to crystallize in plugs. Every 3 hours the capillary was taken to the 18°C room to check for crystals that appeared in each plug.

The flow rates of each solution used in Figure 1 are listed in the table below:

	Carrier fluid (μL/min)	Protein (μL/min)	Buffer (μL/min)	Precipitant (μL/min)
Slow	3.6	0.40	0.04	0.40
Fast	20.0	6.00	0.60	6.00

Figure 3a: The error bar for each data point in Figure 3a indicates +/-- one standard deviation of ~ 300 plugs from 5~6 different experiments.

The device used in Figure 3a was the same as the devices in Figure 1, except that a Teflon capillary was used in place of a glass capillary. The Teflon capillary (Zeus, NY) had an OD of 254 μm and an ID of 200 μm. The fabrication and surface treatment of the channels were also the same as in Figure 1.

The mixing experiments were performed using almost the same procedures as in Figure 1. Instead of transporting plugs to the glass capillary connected to the PDMS channel, plugs were transported to a piece of Teflon capillary via a glass coupler (Inner-Lok^{TM}, Polymicro). The surface of the glass coupler was made hydrophobic by cleaning

with chromic acid, treating with N_2 flow (100 mm Hg) containing the vapor of tri(decafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Inc.) for 30 min, flushing with FC 3283 (3M) and baking in a 60°C oven for at least 30 min.

The Teflon capillary containing crystallization plugs was inserted into a glass capillary and sealed at two ends with capillary wax to prevent evaporation. The capillaries were kept in an 18°C incubator and the crystals in each plug were counted every 3 hours in an 18°C room. The crystals grew rapidly at first and then gradually stopped growing after reaching a certain size (~ 100 to 200 μm). We could not use the crystal size to determine conclusively whether nucleation happened during mixing or after mixing.

The solutions used in Figure 3a were: protein: 22.0 mg/mL thaumatin in 0.1 M ADA buffer pH 6.5; buffer: 0.1 M HEPES pH 7.0; precipitant: 2.0 M sodium potassium tartrate in 0.1 M HEPES buffer pH 7.0. The flow rates of solutions for each data point in Figure 3a are listed below:

Total flow rate (μL/min)	Carrier fluid (μL/min)	Protein (μL/min)	Buffer (μL/min)	Precipitant (μL/min)
67.30	40.0	13.0	1.3	13.0
30.50	20.0	5.0	0.5	5.0
14.0	10.0	1.9	0.19	1.9
7.89	6.0	0.90	0.09	0.90
4.48	3.6	0.42	0.042	0.42

Figure 3b: The devices were fabricated using the same procedures as described in Figure 1. The devices with winding channels were the same as those used in Figure 1. The devices with straight channels had the same dimensions and inlet designs with the

devices of winding channels, except that the winding channels were replaced with straight channels.

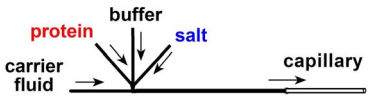

Figure S 1 A schematic of the device with straight channel used in Figure 3b**.**

The mixing experiments, crystallization and data collection were performed following the same procedures as Figure 1.

The solutions used in Figure 3b were: protein: 40.5 mg/mL thaumatin in 0.1 M ADA buffer pH 6.5; buffer: 0.1 M HEPES pH 7.0; precipitant: 2.0 M sodium potassium tartrate in 0.1 M HEPES buffer pH 7.0. The flow rates of solutions used in Figure 3b are listed below:

	Carrier fluid (μL/min)	Protein (μL/min)	Buffer (μL/min)	Precipitant (μL/min)
Low flow rate	4.0	2.0	1.0	1.0
High flow rate	30.0	15.0	7.5	7.5

Visualizing the flow patterns using hemoglobin.

Hemoglobin (Sigma) was dissolved in 0.1 M ADA buffer to ~50 mg/mL. The solution was centrifuged at 13.2 krpm for 30 seconds (Eppendorf, centrifuge 5415D), and filtered with a 0.22 μm Millipore filter before use. The buffer streams used were 0.1 M pH 7.0 HEPES.

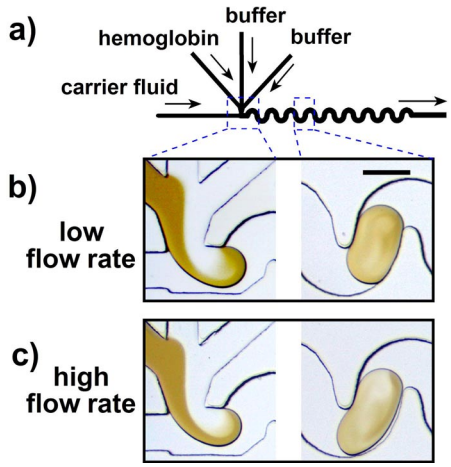

Figure S 2 a) A schematic of the microfluidic device; b), c) microphotographs of the plugs traveling in the microfluidic channels at low flow rate (b) and high flow rate (c). The scale bar is 200 μm.

Footnote 11

1) Definitions in equation $J \propto \exp[-C/(\ln S)^2]$

J is the number of nuclei formed per unit volume and per unit time; C is a parameter that depends on temperature, surface tension of the nuclei and molar volume of the growth units. One can find more detailed discussion of this equation in Garcia-Ruiz, J. M. *J. Struct. Biol.* **2003**, *142*, 22-31 (ref. 10 in the manuscript)

2) Measuring the solubility of thaumatin in salt solutions

The solubility of thaumatin was measured by both dissolution of thaumatin crystals and crystallization of thaumatin solution. To measure the solubility by crystallization, 15mg/mL thaumatin solution, HEPES buffer and precipitant (all were prepared as in the "preparation of solution" section) were mixed in an Eppendorf tube and kept in the 18°C incubator for the protein to crystallize. To measure the solubility by dissolution, thaumatin crystals were first obtained from crystallization experiments, washed a few times with the precipitant solution of the desired composition, transferred to an Eppendorf tube with the precipitant solution and kept in the 18°C incubator. The

Eppendorf tubes were wrapped with Parafilm to prevent evaporation. After incubation for several weeks, an aliquot of the supernatant solution was taken from the tube to determine the concentration of thaumatin in the solution by UV-Vis as described in the "preparation of solutions" section. The aliquot of the supernatant solution was centrifuged at 13.2 krpm for 30 seconds and filtered with a 0.22μm Millipore filter before UV-Vis measurement. When the concentrations of thaumatin in solutions from dissolution and crystallization were the same, we took the concentration as the solubility of thaumatin under the particular precipitant concentration.

The measured solubility (mg/mL) of thaumatin at 18°C and different salt concentrations were listed below:

Experiment	Salt concentrations (M)			Solubility from dissolution (mg/mL)	Solubility from crystallization (mg/mL)
	KNa tartrate	HEPES	ADA		
Fig 1, Fig 3a	0.95	0.052	0.050	1.42	1.32
Fig 3b	0.50	0.050	0.050	5.84	4.96

Calculating supersaturation of thaumatin in Figure 1:

In Figure 1, the solubility of thaumatin was ~1.4 mg/mL. The supersaturation is defined as $S=c/s$, where c is the actual concentration of thaumatin, and s is the solubility of thaumatin. The supersaturation after complete mixing: s=(22.0mg/mL/2.1)/(1.4mg/mL)=7.5. At the interfaces where protein is not diluted, s=22.0(mg/mL)/(1.4mg/mL)=15.7.

Derivation of the qualitative scaling argument of the mixing effect

A detailed description of mixing inside plugs traveling through a winding channel can be found in a previous publication (*Appl. Phys. Lett.* **2003**, 83, 4664-4666). In plugs there are interfaces where the solutions interdiffuse (Figure 2). The interfaces stretch and

fold as the plug traveling through a winding channel. Each stretching and folding (one cycle of advection) doubles the interfacial area and decreases the diffusion distance by half. For a channel with width w [m] and flow velocity U [m/s], the time t_{conv} [s] required for the plug to undergo one cycle of advection is aw/U, where a is a unitless experimental parameter that depends on the device geometry. After n cycles of advection, the diffusive mixing time t_{diff} [s] (time required for the plug to be mixed by mere diffusion) becomes $t_{diff}=(w2^{-(n+1)})^2/D$. D [m^2/s] is the diffusion coefficient. Full mixing will be achieved when the time t_{conv} required for the plug to undergo another cycle of convection equals to diffusive mixing time t_{diff}, which is $t_{conv}=aw/U=(w2^{-(n+1)})^2/D= t_{diff}$. According to this equation, at the time the solutions are completely mixed (t_{mix}), $2^{n+1}=(wU/Da)^{1/2}$ (Equation 1).

With the assumptions we made in the manuscript, the total number (N) of nucleation events from mixing should be proportional to the product of the total interface area A (determined by t_{mix}) and the time t_{conv} required for the plug to undergo 1 cycle of advection, so $N \propto A(t_{mix})t_{conv}=A_0 2^n aw/U$ (Equation 2). Here n is the number of advection cycles at t_{mix}, and A_0 is the interface area before the first cycle of advection. Substituting Equation 1 into Equation 2 yields $N \propto (A_0/2)(wU/Da)^{1/2}aw/U=(A_0/2)(a/DU)^{1/2}w^{3/2}$. Considering that $A_0 \propto w^2$and discarding the constants, we get $N \propto w^{7/2}(DU)^{-1/2}$.

Removing the second assumption (every newly generated interface is fresh for only one cycle of convection) in the main text leads to a qualitatively similar result. Removal of this assumption assumes that nucleation happens at the interface until mixing in the plug is complete. The number (N) of nucleation events from mixing during a cycle of convection (corresponding to a time interval of $t_{conv}=aw/U$) will be proportional

to the product of the interfacial area $A(n)$ at the time and t_{conv}. The total number of nucleation (N) events from mixing is a sum of nucleation during all the convection cycles, $N \propto \int_0^m A(n) * (aw/U) dn$ (Equation 3), where m is the number of cycles of advection needed before mixing is complete. In Equation 3, $A(n)=A_0 2^n$. Integrating Equation 3 gives $N \propto A_0 awe^m/(U\ln 2)$. Combining this equation with Equation 1 gives $N \propto A_0(aw/U\ln 2)[(1/2)(wU/Da)^{1/2}]^{1/\ln 2} \propto (A_0 w/U)(wU/D)^{1/(2\ln 2)}$. Taking into account $A_0 \propto w^2$, $N \propto w^{3+1/(2\ln 2)}/(U^{1-1/(2\ln 2)} D^{1/(2\ln 2)})$. This equation predicts a slightly weaker dependence of nucleation rate (N) on flow velocity (U).