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Spatially-controlled protein crystallization in microfluidic chambers

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ABSTRACT

We present a simple microfluidic device able to trigger the nucleation of the crystals at specific locations on the microchip for the statistical study of protein crystallization. The microsystem is an array of independent PDMS microchambers connected to a fluid-dispensing channel. The chambers are filled with a crystallizing aqueous protein solution and then sealed with a fluorinated oil phase. Each chamber presents a small oil/water interface at the connection with the main channel. The crystals most likely grow near the interface, allowing a microscopic observation of the nucleation events at specific positions on the chip. For the sake of demonstration, the method is applied to the crystallization of HEW lysozyme.

1. Introduction

Protein crystals are three-dimensional arrays of macromolecules in which every molecule or specific group of molecules has the same orientation and relationship to its neighbors. Starting from the first crystallization experiment of hemoglobin achieved by Hünefeld in 1840, the rationale of the fabrication of protein crystals has slowly shifted from purification needs towards the theoretical studies of crystallization and the structural determination of proteins and protein complexes [1].

The multidimensional phase diagram of a protein depends on a wide range of physico-chemical parameters such as the solution composition, the nature of the precipitating agent, the pH and the ionic strength, the temperature, etc. To crystallize, a protein solution should be in a supersaturated, metastable state of its phase diagram, in a region where nucleation and growth are permitted and amorphous precipitation forbidden [2].

Obtaining an acceptable protein crystal is an empirical, trial- and error based procedure, and several crystallization techniques have been developed so far (vapor-diffusion, free-interface diffusion, dialysis, etc.) [1,2] to allow the protein solution to follow different kinetic crystallization paths within the phase diagram.

Systems where the crystallizing solution is directly in contact with the surface of the vessel are likely to promote defect-induced heterogeneous nucleation [3–5] which can be detrimental for the quality of the crystals. To avoid this, the microbatch technique, a container-free, high-throughput crystal growth screening method [6] has been developed. In brief, a small aqueous droplet of supersaturated protein crystallizing solution, containing a precipitant, is encapsulated in an organic oil used as a sealant to avoid evaporation. The crystallization occurs in a molecularly smooth liquid vessel, and it has been shown recently that the nature of the oil greatly influences the apparition of crystals [7].

Microfluidics and more generally lab-on-a-chip technologies offer a wide range of possibilities in the domain of protein crystallization. The ability to manipulate fluids at the pico- to the nanoliter scale, using valves [8], droplets [9,10] or wells [11–13], makes possible the replication of the classical techniques with a high-throughput screening of the crystallization conditions, a lower product consumption and a greater control of the transport phenomena [14]. Microchips can be used for formulation purposes, i.e. construction of phase diagrams and crystal growth [8,9,11–13,15], or for fundamental studies of protein crystallization [16].

The apparition of a protein crystal is a statistical event and the measurement of the time distribution of the nucleation process necessitates the parallel study of several identical vessels of

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crystallizing solution. Well-based devices are usually simpler to design, to use and to analyze [17] but the defects of the walls increase the occurrence of heterogeneous crystallization. Droplet-based microdevices, however, show no surface-defects but are more difficult to fabricate since flow controls and specific surfactants [18] are necessary to create and stabilize the droplets.

In this article, we present a hybrid microfluidic device that takes the advantages of both of the above designs [19,20]: the microsystem is an array of 70 circular PDMS microchambers loaded with the crystallization solution and sealed by a surfactant-free fluorinated oil phase that sits within the main channel. Each chamber is closed by a small oil/precipitating solution interface that triggers nucleation in its vicinity and allows the spatial control of the crystal position on the chip with a sub-10 μm resolution.

2. Materials and methods

2.1. Protein crystallization solution

The protein solution contains 35 mg mL^{-1} of HEW lysozyme (EC Number 235-747-3) diluted in an acetate buffer (pH=4.5, 10 mmol L^{-1}). Sodium chloride (NaCl) was chosen as the precipitating agent at a concentration of 1 mol L^{-1}. The sealing oil is a fluorinated Fluorinert FC-40 oil ($C_{21}F_{48}N_2$). All chemicals were purchased from Sigma-Aldrich and were used as received, without further treatment nor purification. Ultrapure water (Millipore, 18.2 MΩ cm^{-1}) was used for all experiments.

2.2. Fabrication of the microfluidic chip

The protocol used for fabrication was adapted from Yamada et al. [19]. The two-layers device, sketched in Fig. 1, is fabricated in poly(dimethylsiloxane) (PDMS) using soft-lithographic techniques [19,21]. For the bottom layer that contains the channel and the microchambers, PDMS (RTV 615, 10:1 ratio, GE Silicones Co.) was molded on a master fabricated on a silicon wafer using a negative photoresist (SU-8 3050; MicroChem). Microchannels of 50 μm height were patterned on the photoresist by photolithography and hard-baking, following the process from the manufacturer. Then, liquid PDMS was poured onto the mold, degassed and cured at 75 °C for more than 2 h to allow the reticulation to take place. The bottom layer incorporates an array of 70 circular microchambers connected to the main channel by a small neck as shown in Fig. 1. For these experiments, the diameter of the chambers is set to 100 μm and the neck has a rectangular cross section of $15 \times 20\ \mu m^2$. To fabricate the upper part of the chip, that will ultimately serve as an osmotic reservoir, a ca. 5 mm thick PDMS layer was degassed and cured in a plastic petri dish prior to punching a 6 mm diameter hole into it. The two layers of the chip are cut and glued together with uncured PDMS so that the hole of the upper layer covers all of the micro-chambers in the lower layer. This two-layer PDMS block is then baked on a hot plate at 95 °C for 5 min to cure the PDMS glue. The inlet and outlet holes are punched and the PDMS device is bonded to a glass coverslip using an oxygen plasma. Finally, the assembled device is baked on a hot plate at 150 °C for 1 h to make the PDMS wall of the channel recover their hydrophobicity lost during the plasma process [19].

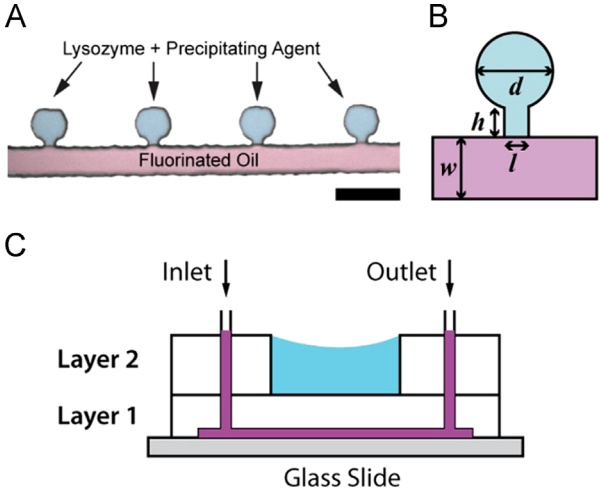

Fig. 1. (A) The chip is made from 70 microchambers, each containing a 35 mg mL^{-1} supersaturated solution of HEW lysozyme in an acetate buffer (pH=4.5) with 1 mol L^{-1} NaCl (blue), separated by a main channel filled with a FC-40 fluorinated oil (pink). Scalebar: 100 μm. (B) In our experiments, the chambers have a diameter $d=100$ μm, a neck length $h=15$ μm and a neck width $l=20$ μm. The main channel has a width equal to $w=80$ μm and the height of all the structures is equal to 50 μm. (C) The microdevice is made from two layers: the lower layer contains the microchambers and the upper layer is used as an osmotic reservoir filled with an acetate buffer solution with 1 mol L^{-1} NaCl. The channel and the reservoir are separated by a piece of PDMS through which the water can diffuse [22]to equilibrate their respective osmotic pressures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Microscopy and image analysis

We used a Zeiss Axio observer inverted microscope equipped with an EM-CCD camera, PhotonMAX (Princeton Instruments). All image analyses were done with the ImageJ software.

3. Results and discussion

The microfluidic chip is composed of two PDMS layers having different functions and structures, as sketched in Fig. 1C: the bottom layer incorporates an array of 70 circular crystallization microchambers connected to a main channel by a small neck, as shown in Fig. 1A. For these experiments, as shown in Fig. 1B, the diameter of the chambers is set to 100 μm, the neck has a rectangular cross section of $15 \times 20\ \mu m^2$, and the height of the structures is set to 50 μm.

The upper layer plays the role of an osmotic reservoir that will be detailed further. The crystallization solution, depicted in blue in Fig. 1A, is introduced in the microdevice with the help of a syringe pump (Harvard Apparatus) at a 30 μL min^{-1} flow rate until all the chambers are filled with it, as shown in Fig. 2. Since PDMS is permeable to gas [22] and the outlet is closed, the air in the chambers is gradually pushed out thanks to the pressure of the solution, and totally replaced by the crystallization solution in a short time.

The sealing of the microchambers is done by injecting a fluorinated oil with the microsyringe pump at a flow rate of 25 μL min^{-1}. This flow rate has been optimized to avoid the pressure-driven deformation of the channels and to allow the oil/water interface to be positioned at the neck of the chambers, as shown in Fig. 1A. At the end of the injection, the inlet and outlet tubes are disconnected from the microchip so that the pressure equilibrium is able to avoid any oil movement in the microchannel. According to the manufacturer (3M Company), water in oil and oil in water share the same very low solubility of about 5 mg kg^{-1} which is lower than any of the oil used in previous microbatch studies [7]. The device is thus an array of identical and independent microchambers.

Lysozyme is a 14.7 kDa, 129 aminoacid residues enzyme, present in the mucosal secretion such as saliva and tears and also in chicken egg-white. The catalytic activity is non-specifically