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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · MARCH 2008

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Shape-Control of Protein Crystals in Patterned Microwells

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The development of low volume, high throughput platforms for screening and manipulating protein crystallization has been driven, in part, by the importance of understanding protein structure−function relations. Recently, significant attention has been directed to design protein crystals as higher efficiency catalytic materials[1] and to use crystalline proteins for controlled protein/drug delivery.[1,2] In the latter case, micrometer or submicrometer crystals with uniform shapes are attractive for improving bioavailability and providing alternative release routes. Controlling the growth of protein crystals with small sizes, however, is a challenge for conventional vapor-diffusion methods and robotics techniques because the smallest volumes are limited to 0.1 μL using standard pipettes. Alternative strategies, such as microfluidics, can confine the volumes for crystallization down to nanoliters, but the crystals produced are ca. one hundred micrometers or larger.[3−5]

This Communication reports how patterned microwells can be used as crystallization vessels to grow protein crystals. This surface-patterned approach for crystallizing proteins requires only small volumes (fL in individual wells) and enables the growth of submicrometer crystals with well-controlled shapes and uniform sizes. Previously, patterned surfaces have been used to crystallize organic molecules such as glycine[6−8] and organic semiconductors including pentacene and C_{60}.[9] The patterned substrates for glycine were composed of 25−725-μm square gold islands, which produced different polymorphs of organic crystals ranging from 10 to 350 μm in size. Here we introduce a method based on smaller, patterned microwells ($\leq$10 μm in diameter) that can grow uniform protein crystals with sizes from 3 μm down to 600 nm. These wells consisted of a transparent glass bottom (which allowed the growth process to be monitored using fluorescence microscopy) surrounded by gold sidewalls (which enabled the inside and outer areas of the wells to have opposite chemical functionalities). Three proteins, lysozyme, thaumatin, and glucose isomerase, were crystallized as model systems to demonstrate the generality of this technique. We found that different polymorphs could be formed simply by controlling the evaporation conditions.

Microwells for protein crystallization were generated using microfabrication techniques and then selectively functionalized using thiol chemistry so that the crystallization solution could be preferentially loaded into the wells (Figure 1a and Supporting Information). The diameters and shapes of the wells were defined by the features on the chromium mask used in photolithography and are referred to as d-μm wells, where $d = 2-10$. The depth of the wells was controlled by the amount of gold deposited and was kept fixed at 20 nm; the resulting well-volumes ranged from 0.08 to 2 fL. Protein crystallization in the microwells was carried out using a modified sitting-drop method (Figure 1b), where patterned substrates filled with the crystallization solution were carefully placed into a glass beaker and set over a bulk reservoir solution.

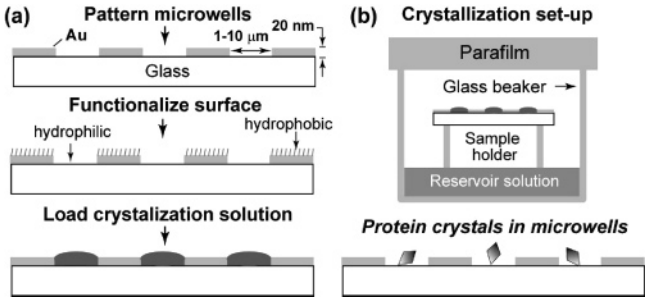

Figure 1. Scheme for crystallizing proteins in patterned microwells.

To grow lysozyme crystals, the crystallization solution was a mixture of 1:1 (by volume) protein solution (100 mg/mL in H_2O) and reservoir solution (50 mM sodium acetate buffer, pH 4.6, 1 M NaCl) as well as 2% rhodamine 6G (10 mM in H_2O). In a sealed environment, the difference in initial concentrations of the NaCl precipitant between the crystallization solution in the microwells and the reservoir solution caused water to evaporate from the wells until supersaturation was reached. Then, nucleation was initiated, and crystals formed. The presence of the dye did not cause any appreciable change in the morphology of the protein crystals (Figure S1). The dye was added to the crystallization solution (analogous to the "Izit" test[10,11]) to (1) monitor the protein crystallization process and (2) verify that the crystals formed in the wells were protein and not inorganic salts (Figure S2).

When the crystallization was performed in 10-μm wells, environmental scanning electron microscopy (SEM) revealed that lysozyme crystals were ca. 1−3 μm in size and had tetragonal shapes (Figure 2a), similar to those grown under bulk hanging drop conditions (Figure S1). As we reduced the diameter of the wells to 3 μm, two different crystal morphologies with well-defined crystalline facets were found in a single well: tetragonal and plate-like (Figure 2b). Interestingly, as we decreased the size of the wells to less than 2 μm (or even as small as 250 nm), only single crystals were formed in individual wells (Figure 2c), and the morphology of these protein crystals had plate-like shapes.

Although many factors can influence crystal morphology, including the precipitant, solvent evaporation rates, and crystal growth rates, the polymorphism of lysozyme in microwells most likely resulted from a combination of different water evaporation rates and supersaturation conditions as the volume of the well changed. We tested this hypothesis by controlling the water evaporation rates in 3-μm wells. To increase the evaporation rate, the concentration of NaCl was increased from 1 to 2 M, which decreased the solubility of protein in the crystallization solution and established a larger concentration gradient between the solution in the microwells and the reservoir. Lysozyme crystals were formed in 1 day instead of 2 and exhibited only plate-like shapes (Figure 3a) whose heights were around 600 nm (Figure S3). To decrease the evaporation rate and reach supersaturation conditions more

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10.1021/ja077956v CCC: $40.75

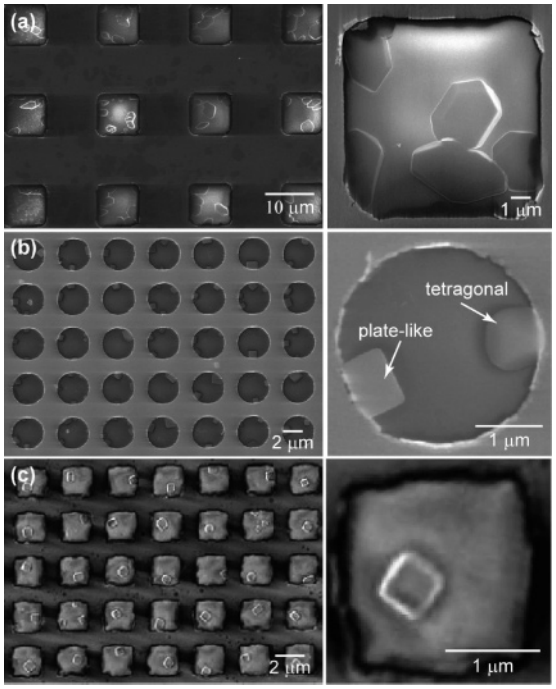

Figure 2. SEM images of lysozyme crystals grown in (a) 10-μm wells, (b) 3-μm wells, and (c) 2-μm wells.

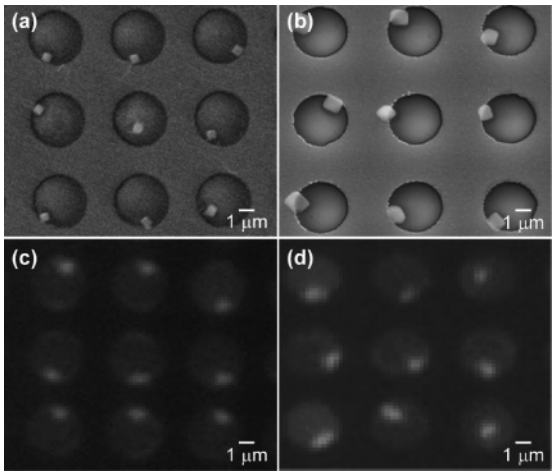

Figure 3. Lysozyme crystals formed under high and low evaporation rates in 3-μm wells. SEM images of (a) plate-like and (b) tetragonal crystals and fluorescence images of (c) plate-like and (d) tetragonal crystals.

slowly, a layer of paraffin oil was placed on the top surface of the reservoir solution.[12] We observed that lysozyme crystals formed after 3 days instead of 2, and the crystals had a tetragonal morphology (Figure 3b). Epifluorescence microscopy was also used to monitor the crystallization process to show that the crystals in adjacent wells had nearly the same shapes (Figure 3c,d), and the intensity profile indicated that the sizes were fairly uniform since similar quantities of dye were incorporated into the crystals (Figure S4).

Thus, we found that slower evaporation rates and larger well-volumes facilitated the growth of tetragonal crystals, which is the morphology in bulk solutions, while fast evaporation rates favored the formation of plate-like shapes. These results are consistent with those reported for the confined crystallization of glycine, where fast crystallization conditions tended to modify the shape of the crystal from granular to needle-like.[7] In addition, for bulk vapor diffusion methods, variations in evaporation rates and volumes[12] produced only larger sizes and fewer numbers of protein crystals,

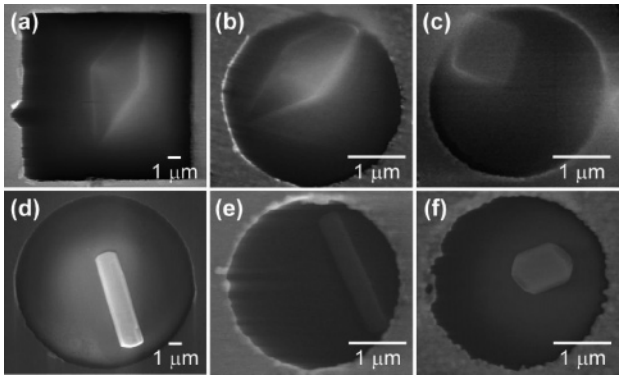

Figure 4. SEM images of (a−c) thaumatin and (d−f) glucose isomerase crystals grown under (a,b and d,e) slow and (c,f) fast evaporation rates.

while surface-patterned platforms with very low volumes allowed high-energy polymorphs to be crystallized.[6] Moreover, we selectively controlled the shape of thaumatin protein crystals by manipulating the sizes of the wells and water evaporation rates. In 10-μm wells, thaumatin crystals with tetragonal shapes were formed (Figure 4a), which were the same morphologies as those grown in a bulk hanging drop (Figure S5). Different shapes of crystals could also be selectively grown in 3-μm wells under different water evaporation rates (Figure 4b,c). Also, glucose isomerase crystals were grown using the same strategy, and different polymorphs were formed under controlled evaporation conditions (Figure 4e,f and Figure S6).

In summary, we demonstrated that microscale wells provide a flexible platform for controlling the crystallization of protein crystals with sizes as small as 600 nm using industry-standard crystallization conditions. Although the proof-of-concept studies presented here focused on three simple proteins, this approach is general and could be expanded to more complex proteins, organic molecules, and anticancer drugs. Furthermore, this surface-patterned method to generate protein crystals may provide an alternative approach to produce drug crystals with well-defined sizes and shapes that could be useful in delivery applications.

Acknowledgment. This work was supported by Baxter and the Institute for BioNanotechnology in Medicine (IBNAM) at Northwestern and the David and Lucile Packard Foundation.

Supporting Information Available: Protein crystallization details and other optical and fluorescence microscopy images. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA077956V