ac field enhanced protein crystallization

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(Received 14 April 2008; accepted 14 May 2008; published online 3 June 2008)

Uniform and nonuniform ac electric fields sustained by internal microfabricated electrodes are shown to sensitively affect protein crystallization by reducing the number of nucleation sites and enhancing the quality of the crystals formed. Under conditions when massive spontaneous nucleation occurs, a properly tuned ac field can desolvate the protein molecules to form a gel matrix with only a few nucleation sites. A dielectrophoretic force generated by the nonuniform ac field further consolidates these crystallites. Upon field removal, the gel transforms into crystals around these nucleation sites to form high-quality large crystals. © *2008 American Institute of Physics*. [DOI: 10.1063/1.2938887]

Protein crystals and their structures are of importance for structure-guided drug design and controlled drug delivery.[1] In solution, the solvated molecules are surrounded by a hydration cage.[2] This hydration cage hinders interactions between the protein molecules by acting as a dielectric and shielding the electrostatic attraction between adjacent molecules.[3] Crystallization can only occur upon the removal of these hydration cages in supersaturated protein solutions. If this cage is removed too quickly by evaporation or an external field, however, the molecules will not reach their native conformation and the solution will form a gel instead.[4] Alternatively, the solution can enter the spinodal decomposition region where spontaneous nucleation occurs and a massive number of crystals are produced. A delicate balance between gelation and spontaneous crystallization is hence necessary. Current methods for growing high-quality crystals rely on protein desolvation without triggering gel formation or massive nucleation. The diffusion coefficient for lysozyme during desolvation is estimated to be a low 10^{-6} cm^2/s.[5] Existing methods such as vapor diffusion, seeding, microfluidics, electric fields, and magnetic fields hence aim to accelerate this slow diffusion and increase protein saturation in the solution to enhance crystal nucleation and growth.[6–10]

In particular, dc electric fields have been used to orient the molecular dipoles and desolvate the protein molecules. However, when a dc field is applied to internal electrodes in a highly conductive solution, Faradaic reactions occur on the electrodes. To limit such contamination, the applied fields are limited to low voltages and low currents, no more than 1 V and 20 μA.[11] External electrodes are not limited by the Faradaic reaction but require higher fields of up to 6 kV/cm.[12]

As is true of many ac electrokinetic devices,[13] an ac electric field at high frequencies can minimize Faradaic reactions on the internal electrodes, allowing for higher applied voltages. Surprisingly, ac field effects on protein crystallization have not been investigated and are the topics of this letter. Besides orienting the molecule dipoles, it is shown in this letter that the frequency and voltage of the ac field can produce reversible gelation under conditions of spontaneous nucleation. Upon field removal, this ac field-induced gel phase transforms into a few high-quality crystals, thus drastically improving the quality and yield of lysozyme crystals. The ac field is also shown to consolidate the crystals to low field regions to form larger crystals by another ac electrokinetic force, dielectrophoresis (DEP). This mechanism is an important technique used for sorting and concentrating bioparticles, including proteins, in microfluidic systems.[13–16] It has also been found that the direction of DEP, toward regions of high or low field gradients, is dependent on the applied frequency and the particle size.[17,18] Therefore, these desirable effects suggest that the ac field at the right conditions and sustained by properly designed microelectrodes can profoundly improve protein crystallization.

The two electrode designs used are shown in Fig. 1. The parallel electrodes with no field gradient in Fig. 1(a) have a width of 50 μm and a gap width of 20 μm. The quadropole electrode in Fig. 1(b) with a large field gradient has four electrodes with a half angle of 40° such that the distance between the electrodes increases radially. The gap at the center of the quadropole measures 10 μm from the opposing tip and the electrodes are polarized alternatively, as shown in the figure. Both electrode designs were fabricated on glass slides using standard lithography techniques and are comprised of Ti/Au with a thickness of 5/25 nm.

A 0.1*M* sodium acetate-acetic acid buffer at a *p*H of 4.8 was used to crystallize lysozyme (Sigma) at room temperature.[6] Three function waveform generators were used to increase the range of applied frequencies and voltages: Agilent 33220A, Tektronix CFG253, and Hewlett Packard 3312A. The output and the current were measured using an oscilloscope (Tektronix TDS 2014) and multimeter probe (Radioshack Digital Multimeter). Images were captured us-

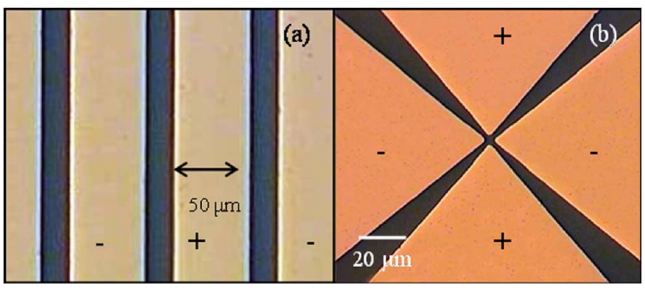

FIG. 1. (Color online) (a) Straight electrodes with electrodes oppositely polarized. (b) Quadropole electrode with oppositely polarized electrodes.

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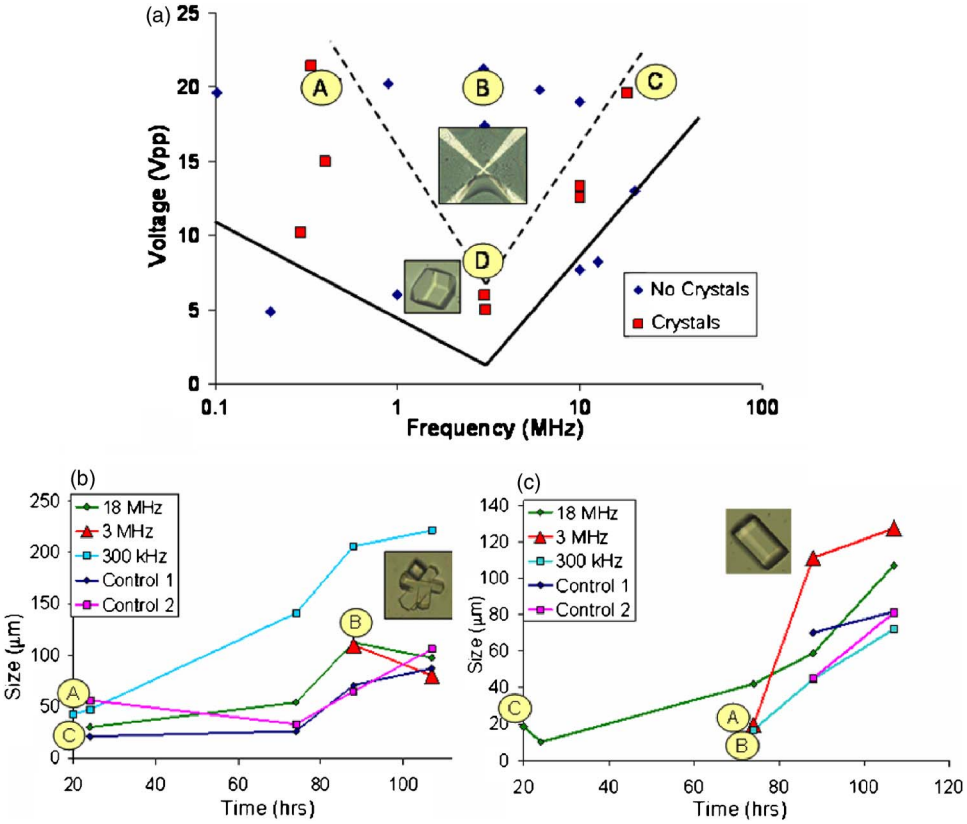

FIG. 2. (Color online) (a) Lysozyme solutions under different ac fields that were monitored for 24 h for indications of nucleation. Conditions where crystals were seen are marked by the red squares and conditions where no nucleation sites were seen are marked with a blue diamonds. A, B, C, and D represent solutions for (b) and (c) and Figs. 3(a) and 3(b). Irreversible gelation occurs above the window after the field is removed. (b) The average size of the fused and defected crystals over 110 h with the field activated only for the first 24 h. (c) The average size of single crystals over 110 h with an applied field in the first 24 h.

ing an Olympus 1X71 microscope and an I-Speed CDU camera system (Olympus America).

Silicon isolation wells (Grace BioLabs) with an open surface interface and an adhesive backing (9 mm in diameter) were used as loading wells with a holding volume of 130 μl. The wells were sealed to the glass slide with the electrodes centered in the wells. After the addition of the protein solution, the wells were sealed with Parafilm, a coverslip, and silicon grease to limit the rate of evaporation. X-ray diffraction (XRD) analysis was completed using a Bruker SMART APEX diffractometer.

Various frequencies and voltages were applied to lysozyme solutions by the parallel electrodes to scrutinize the voltage and frequency effects of the ac field. In each well, the ac field was applied for 24 h and then examined for nucleation sites, as indicated in Fig. 2(a). The solutions that contained crystals are represented by the red squares and solutions that contained no crystals are represented by the blue diamonds. No crystals were observed below the solid line in Fig. 2(a) in 24 h. However, upon continued exposure to the field, crystals formed in these solutions at rates and sizes comparable to the control crystals formed without electric fields. This suggests that the ac field had very little effect on crystallization at the voltages and corresponding frequencies below the solid curve. Crystals were observed within 24 h in the window between the solid line and the dotted line. An optimal frequency with the smallest voltage required for crystallization in 24 h exists at 3 MHz, corresponding to the minimum of the window in Fig. 2(a). This "resonant" frequency suggests that desolvation rate of the hydration cage surrounding the molecules is sensitive to the frequency of the applied field. The 3 MHz value is consistent with molecular simulations and NMR analyses that suggest that the rotation of the water molecules about the hydrogen bond on lysozyme has a characteristic time of roughly 10^{-7} s corresponding to 10 MHz.[19,20] In the region above the window, no crystals were observed in 24 h and with continued exposure to the field, irreversible gelation occurs. The window hence represents a competition between gel and crystal formation at different voltages and frequencies, with gelation dominating at high voltages above the window.

How the initial state after 24 h affects subsequent crystallization dynamics was examined for three lysozyme solutions by applying fields at 21.4 V_{pp}, 334 kHz, 21.2 V_{pp}, 2.9 MHz, and 19.6 V_{pp}, 18 MHz for 24 h and then examining the crystals for the next 110 h without an applied field. The voltage/field conditions in the first 24 h correspond to positions A, B, and C in Fig. 2(a), where conditions A and C are within the window for crystallization in 24 h whereas gelation occurs for condition B. The wells were monitored for $4\frac{1}{2}$ days and were compared to two control wells where no electric field was initially applied. The average crystal size for each well was plotted in Figs. 2(b) and 2(c) and was sorted by whether the crystal was a single crystal with no defects or a defected crystal. The average size of the defected crystals was plotted against time, as seen in Fig. 2(b). The defected and single crystals that form in solutions A and C in the first 24 h continue to grow monotonically over 100 h. Gelation was not observed for the entire 100 h experiment. Solution B at 3 MHz, on the other hand, did not form any crystal, single or defected, until over 60 h. Gelation occurs first but gives way to a small number of crystals after 60 h. Condition B with the gel-reduced nucleation density also produced relatively few defected crystals compared to conditions A and C, with most of them arising from cracked single crystals. Condition B in fact produces the largest single crystals and contained the highest yield of single crystals. When the field at condition B is left on for 72 h instead of 24, fewer and larger crystals are formed but the time for

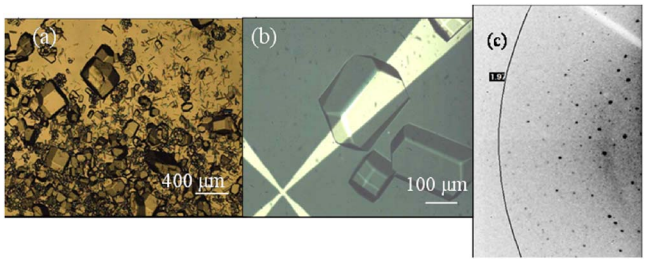

FIG. 3. (Color online) (a) Uncontrollable nucleation of lysozyme with a wide size distribution in the absence of an electric field. (b) Lysozyme crystals under an ac field of 8 V_{pp} and 3 MHz [point D in Fig. 2(a)] for 72 h. (c) Diffraction pattern of the lysozyme crystals grown under an ac field.

them to form is also longer. Consequently, at the optimal frequency of 3 MHz, an intermediate and reversible gel state delays and suppresses the density of nucleation to produce large single crystals. Instead of spontaneous nucleation, nucleation in the gel matrix is apparently activated and is hence slower with smaller nucleation sites. Far above the window or prolonged exposure to the field yielded irreversible gelation, suggesting that the activation barrier between gel and crystal increases with voltage and exposure time. Optimal intermediate gel formation is estimated to occur just above the window in Fig. 2(a) at point D (3 MHz) with an exposure time between 24 and 72 h.

The effect of this gel-delayed nucleation phenomenon under an ac field is further amplified by a field gradient. The contrast can be seen in Figs. 3(a) and 3(b) where a control and a solution exposed to an ac field of 8.4 V_{pp}, 2.9 MHz, point D in Fig. 2(a), for 72 h using the quadropole electrode design are compared after 10 days. It is apparent that in the control well, Fig. 3(a), massive uncontrolled nucleation occurred and the majority of the crystals formed contained defects. In comparison, the well under the ac field in Fig. 3(b) produced only ten crystals and all the crystals were single crystals that were at least 100 μm in size. At a lower voltage than D, the difference in crystal number and quality was not as apparent because crystals would have appeared within the first 24 h.

How the field gradient plays a role is evident from the crystal locations in Fig. 3(b). In the quadropole design, the high field regions lie at the tips and edges of the electrodes while the low field regions lie on the lines of symmetry of the electrode and the gaps. It is apparent that the crystals formed at the lines of symmetry which are loci of field minima, indicating that the dielectrophoretic force concentrated the crystals toward these locations.

The structure of the lysozyme crystals were compared to those grown by standard conditions using XRD analysis. The diffraction pattern, as shown in Fig. 3(c), and the measurements of the tetragonal lysozyme structure are consistent with those found in the Protein Data Bank.[21]

The proper frequency, voltage, and exposure time for other proteins would need to be determined from a phase diagram such as Fig. 2(a). Electrode designs that produce higher field gradients than the quadropole electrode may further improve the crystal size and quality. It is important, however, to employ a nonuniform field just above the voltage-frequency window at point D where the intermediate gel state can evolve into the largest single crystals.

The authors thank Tiffany Carney and Dr. Bruce Noll for their contributions with the XRD analysis.

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