Novel Observation of Nucleation and Growth of Insulin Crystals via Liquid Droplets Generated by Liquid–Liquid Phase Separation

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Formation behavior of liquid droplets in aqueous solutions of insulin was studied by varying pH values and Zn^{2+} concentrations of the mother solutions. The nucleation and growth of insulin crystals that preferentially occurred on the droplet surface were first observed.

Insulin, one of the most important hormone proteins, plays an essential role in controlling the amount of sugar in the blood. Not only the molecular insuline and its oligomer but also the suspended particles in solutions are utilized as a medicine for diabetes. For instance, the solution containing amorphous particles of zinc insulin shows a slightly slower therapeutic effect and that of its crystallites indicates rather retarded effect in comparison with the regular insulin solution, which are known as lente and ultralente insulins, respectively.[1] Therefore, the control of the aggregation and crystallization of insulin in aqueous solutions is very important to improve a quality of the medicine.

Recently, ten Wolde and Frenkel[2] theoretically simulated a crystal nucleation process in colloidal solutions with a metastable liquid–liquid phase separation, and found that the appearance of the liquid–liquid phase separation drastically changes the generation pathway of a crystal nucleus and that the crystal nucleation rate increases with steps. Then, the two-step process has newly been proposed as a nucleation mechanism; at the first step small liquid droplets with a high protein concentration are generated from the mother solution, and next the nucleation of crystals occurs inside the droplets.[2,3] The generation of these droplets makes us expect to promote and/or control the crystallization of proteins.[2–5]

To manipulate such liquid droplets, we should have to prepare them and study a relationship between formations of droplets and crystals. The liquid–liquid phase separation or liquid droplet formation from protein solutions have been studied in protein solutions, such as lysozyme,[6] lens protein,[7,8] phosphoglucomutase,[9] intact immunoglobulin,[10] and appoferritin.[11] However, to our knowledge, there are no reports on those for insulin. Among the above studies,[6–11] the liquid droplet formation and the successive crystal nucleation have clearly been observed for phosphoglucomutase[9] and intact immunoglobulin.[10] The nucleation and growth of the crystals are not detected inside the droplets but on the surfaces. These facts are inconsistent with the two-step mechanism. Thus, it is very interested to study how the droplets of insulin take part in the crystal nucleation process. We succeeded in obtaining the liquid droplets in aqueous solutions of insulin by changing the pH values and Zn^{2+} concentrations of the mother solutions. In this paper, the behaviors of nucleation and growth of insulin crystals generated on the surface of the droplets are reported for the first time.

Bovine insulin was purchased from Wako Pure Chemical Industries. Other reagents were of the highest purity grade available. The appropriate amount of the powder insulin was dissolved into 10 mL of 0.10 M HCl solution with Zn^{2+} ion, which promotes crystallization of Zn–insulin. Precipitation of insulin was induced by mixing of the 0.10 M sodium citrate solution (1.0 mL) into the 0.10 M HCl solution (1.0 mL) in a glass bottle cell. The concentrations of insulin and Zn^{2+} ion were estimated by UV absorption band using the molar absorption coefficient of $1.05\,M^{-1}\,cm^{-1}$ at 276 nm[12] and by the ordinary EDTA titration method,[13] respectively. After the preliminary examinations, the concentration of insulin was profitably set to 0.7 mg/mL and that of Zn^{2+} ion was changed from 0.1 to 5.0 mg/mL. The pH values of the mixed solutions were adjusted by an addition of small amount of 1.0 M HCl or NaOH solution into the sodium citrate solution in advance. The cell was sealed and kept at 20 °C. No significant change of the pH of the solutions was detected during the experiments.

The pH regions, where the formation of visible liquid droplets was observed under an optical microscopy, were dependent upon the Zn^{2+} ion concentration. Interestingly, the liquid droplets were generated in restriction near the acidic end of the crystallization region of insulin, within a narrow pH range about ±0.5. In the range of 0.1–2.5 mg/mL of Zn^{2+} ion, the pH, in which the formation of droplets is well observed, decreased from about pH 4.5 to 4.0 with increase of the Zn^{2+} ion concentration. Whereas the value oppositely increased to about pH 4.5, when the concentration increased from 2.5 to 5.0 mg/mL. Here, the representative phenomena observed in solutions of the Zn^{2+} ion concentration of 3.5 mg/mL are described.

In such acidic regions, the mixed solutions became cloudy at first, which is due to the rapid formation of random aggregates occurred after mixing. Small bright spots, which were subsequently identified as clear micrometer-sized liquid droplets, began noticeable in the cloudy solutions in an optical microscopy (Figure 1a). At pH 4.2, many clear droplets with a hundred μm in diameter generated within a few days with a high contrast image under a microscopy (Figure 1b), indicating that the droplets of insulin should be in a high concentration. According to the development of the droplets, the random aggregates slightly dissolved. After generation of the droplets, some crystals successively nucleated on the surfaces of droplets, not inside of them (Figure 1c). The nucleation process belongs to the so-called heterogeneous nucleation mechanism occurred by using the surface of an external substance. The important feature of the droplets on crystallization of insulin should be not its high internal protein concentration but the structure and physical properties of the surface, such as the molecular arrangement and charge distribution. High viscosity of droplets must cause the suppression of nucleation inside them.

The crystals nucleated on the droplets gradually grew for a few weeks (Figure 1d). According to the progress of the crystal growth, the random aggregates gradually dissolved, which is

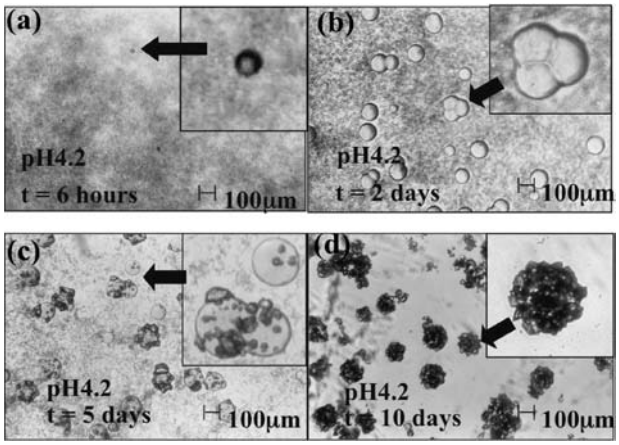

Figure 1. Development of liquid droplets of insulin and the successive nucleation and growth of the crystals at pH 4.2. (a) Cloudy random aggregates with tiny liquid droplets, (b) clear liquid droplets generated in the cloudy solution, (c) droplets with crystals nucleated on their surfaces, and (d) crystals with a spherical assembly developed from the droplets.

prominent around the growing crystals. The random aggregates act as a solute reservoir for growth of insulin crystals. The lower solubility phase is ascribed to be in an amorphous state.

Formation of droplets similarly occurred in the mother solutions, from which the random precipitates were filtrated out by 0.1 μm membrane filter (Figure 2). Thus, the droplets obtained here should be generated from the solution phase directly by a liquid–liquid phase separation, not through the amorphous phase. In Figure 2, the number of the crystals nucleated on the surface of one droplet seems to be less than that which coexisted as the random precipitates. Sometimes, only one crystal grew and one droplet was covered. The random precipitates should also play a role of buffer to keep the concentration of the mother liquid to be in a high level where the nucleation on the surface of the droplets can continuously take place. According to growing up of crystals nucleated on the droplets, some of droplets gradually became obscure and invisible before the nucleation of crystals on their surfaces, as shown in the inset of Figure 2b. The phenomena indicate that the crystallizing components in the concentrated droplets are transportable to the bulk solution through the droplet surface. Thus, liquid droplets can also act as a solute reservoir at pH 4.2.

Generation behavior of liquid droplets, however, is sensitively dependent upon pH values of the mother solutions. At

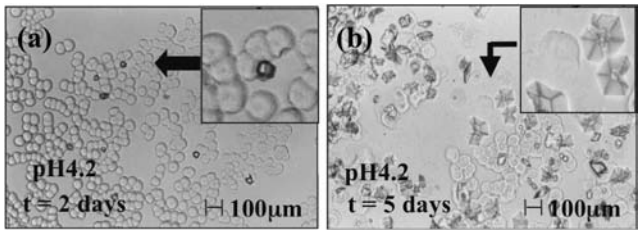

Figure 2. Generations of liquid droplets and crystals in the solution at pH 4.2 without random aggregates. In (a), several dark spots are crystals nucleated on the droplets. (b) The droplets are covered by a small number of crystals, and the outline of the droplet is partially obscure (in the inset).

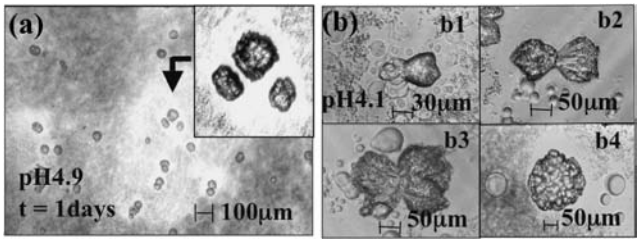

Figure 3. (a) Typical developments of the liquid droplets and crystals at pH 4.9. (b) The sequential images of the formation of bow tie-like and spherical crystalline aggregates were occasionally observed at pH 4.1, which grow from the burst droplets.

higher pH than 4.9, the droplets were covered by many tiny crystallites within a day before growing up enough, without exceptions (Figure 3a). On the other hand, at slightly lower pH than 4.1, some droplets occasionally burst and the spherically-massive crystalline aggregates developed from the burst concentrated droplets through a dumbbell-like morphology as shown in Figure 3b. Thus, surprisingly, the droplets formed in the solutions were clearly distinguished into two patterns; one is the formation of clear droplets kept over several months without any generation of crystals on the surface and another is that of spherical crystals completely converted by the droplets (Figure 3(b4)). Such a novel pH-dependent nucleation and growth of insulin crystals via liquid droplets would also be applicable to control the nucleation of crystals and their micro assembly.

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