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Salt-induced protein phase transitions in drying drops

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Abstract

Protein phase transitions in drying sessile drops of protein–salt–water colloidal systems were studied by means of optical and atom-force microscopy. The following sequence of events was observed during drop drying: attachment of a drop to a glass support; redistribution of colloidal phase due to hydrodynamic centrifugal stream; protein ring formation around the edge; formation of protein spatial structures inside a protein ring that pass into gel in the middle of the drop; salt crystallization in the shrinking gel. It was assumed that rapid drying of a protein ring over the circle of high colloidal volume fraction and low strength of interparticle attraction leads to formation of colloidal glass, whereas gel forms only in the middle of the drop at very low protein volume fraction and strong attraction between the particles. Before gelation, colloidal particles form fractal clusters. In dried drops of salt-free protein solutions, no visual protein structures were observed. Structural evolution of protein in sessile drying drops of protein–salt aqueous colloidal solutions is discussed on the basis of experimental data.

Keywords: Protein phase transitions; Drying sessile drops; Colloidal stability; Spatial protein structures

1. Introduction

In this paper we used drying sessile drops as model objects for studying salt-induced protein phase perturbations in protein–salt aqueous solutions. Desiccated sessile drops of colloidal suspensions are favorite objects for studying liquid instabilities and nonequilibrium pattern formation [1–6]. The main principles of deposit ring formation over a circle ("coffee drop deposit"), as well as of pattern formation depending on particle size, concentration, ionic strength, and surfactant presence, were discovered in drying drops of model colloidal suspensions of polystyrene microspheres in deionized water [1,2] (see also [6] for details). A number of papers concerned with protein–salt–water colloidal systems aimed at studying drop-drying phenomenon [3,7–9]. The evaporating, gelling, and cracking behavior of a deposited drop of BSA–salt solution were investigated in [3]. The authors showed that the initial stage of drop drying is characterized by the appearance of regularly spaced cracks at the edge of the gelling drop. Then a transition from this evaporation-controlled behavior to purely elastic behavior occurs, where the change of the crack spacing is a consequence of the shrinkage stress evolution in time. Thus, the authors pointed out different mechanical properties of the drop circle and the central part of the drop. In [7,8] the drop is considered to be a homogeneous liquid medium during drying, with different diffusion coefficients for protein and salt. The authors of [7,8] assumed that salt has no effect on solution density because of very low concentration. They meant that the density of the solution is a linear function of the albumen concentration. These ideas are in disagreement with our preliminary observations [10], which showed strong influence of salt on the protein phase state during drop drying.

There are quite a number of works by Russian physicians in which they found a relationship between morphological structure of dried biological liquids and state of health [11–15]. It was shown that pattern formation in drying droplets of serum would differ between normal samples and those containing monoclonal proteins [16]. Although these findings have multiple descriptions in the literature, there is no correct explanation of the cause–effect chain between morphology of dried biological liquids and definite pathology. In our earlier work [17], we have shown phenomenologically that dynamics of pattern formation in drying sessile drops of biological liquids can be used

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as an informative parameter for medical diagnostics. But, at that time, we did not understand in detail what processes really took place there.

Recently protein phase transitions have been actively studied in bulk solutions by means of static and dynamic light scattering and small-angle neutron and X-ray scattering [18–22]. Critical conditions leading to a decrease of protein phase stability are of particular importance for medicine, as well as for separation processes in biotechnology. It is well known that protein aggregation is responsible for a variety of serious diseases, including eye cataracts, sickle-cell anemia, and Alzheimer's disease. Better understanding of the phase behavior of aqueous proteins may help to prevent and correct such diseases. Phase separation is achieved by addition of precipitation agents—inorganic salts [19,20,24] and polyelectrolytes and organic solvents [18,19,24,25]—as well as by changing concentration, temperature, and pH level [19,20,22,23]. Experimental studies show that protein precipitation by salts requires electrolyte concentration in the 1–10 molar range [26].

This paper is concerned with finding the main mechanisms responsible for protein structure formation in drying sessile drops of albumen–salt aqueous solutions at physiological concentrations. A new point of view on the cause–effect chain of protein phase transitions is proposed.

2. Materials and methods

We used 7% w bovine serum albumin solution (BSA, 68 kDa, Sigma, USA) in distilled water or in physiological salt solution (0.15 M NaCl, chemically pure, "Reactiv, Inc.," Russia). Also, a 10% w food gelatin solution in distilled water or in physiological salt solution was used. All solutions were prepared without buffering, a day prior to experimentation, refrigerated overnight, and allowed to come to room temperature before testing. The samples under study were placed using a micropipette onto clean glasses in the form of drops 3 μl in volume (6–8 drops for each sample) and left to dry under room conditions. Morphological observations were carried out during drying, and 2–3 days after drops were placed on the glass, using LUMAM-I-3 microscope and a video camera–computer setup. Dried drops were also investigated by means of the atom force microscope (AFM) "Smena" NT-MDT, Russia, using a CSG11 sensor.

3. Results and discussion

The principal requirement for initiation of self-organizing processes in drying sessile drops is attachment of a drop to a substrate [1]. Attachment leads to the appearance of a hydrodynamic centrifugal flow that carries the colloidal phase to the drop periphery. A drop of suspension of polystyrene microspheres has the attachment line over the circle (pinning) [1,2], whereas a drop of protein solution attaches to a substrate by forming an adsorption layer covering the whole area between the drop and the substrate. Fig. 1 shows the main stages of the drop drying process of a protein–salt solution as observed in real time. Due to the centrifugal flow, a protein ring forms

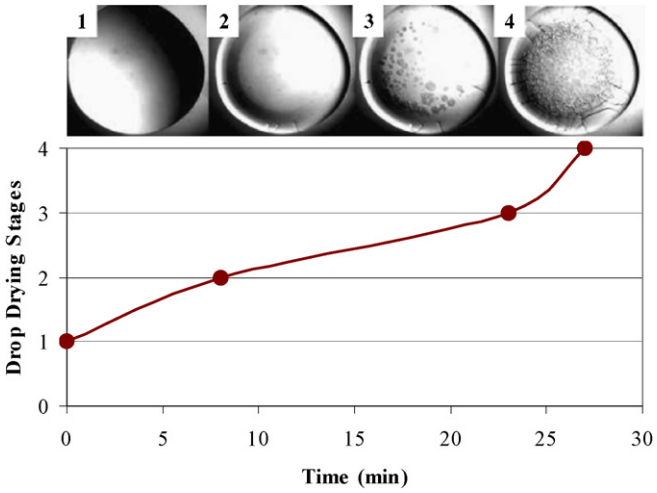

Fig. 1. The main stages of the drop-drying process of 7% w BSA in 0.9% w NaCl solution on a glass support, observed in real time. Drop volume is 3 μl. (1) Beginning of drying; (2) protein solid ring formation around the edge, and protein gelation in the middle of the drop; (3) beginning of salt crystallization in a semi-liquid protein gel; (4) end of salt crystallization.

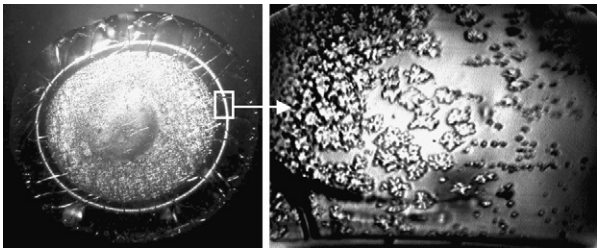

Fig. 2. Dried drop of protein–salt aqueous solution (left) ×10. Light-diffusing circle is the site of protein structure formation. Right picture shows protein structure evolution from separate precipitates (right) to protein clusters that transform into gel (left). ×280.

over the drop edge. It becomes solid while the core of the drop is a semiliquid gel. Some time later, salt crystallization begins in this gel matrix [9]. Time intervals may vary depending on temperature and humidity, but the sequence of events, as well as the final morphological picture, is quite stable and well reproducible.

After 2–3 days of drying up (film water evaporation), an additional light-diffusing circle arises over the middle part of the drop (Fig. 2). This circle consists of spatial protein structures comprising precipitates and clusters. The following concentric zones can be observed from the drop edge to the drop center: (1) zone of homogeneous protein; (2) zone of micrometer-size single protein precipitates; (3) zone of fractal protein clusters; (4) gel; and (5) zone of salt crystals in shrinking protein gel (Figs. 2 and 3). The same picture can also be observed in a dried drop of gelatin–salt solution. In salt-free solutions, dried drops of both BSA and gelatin were transparent and did not contain optically visible protein structures. Thus, this cascade of protein structures was springing up only in the presence of salt.

We did not find in the literature any description of these protein structures. That is why we tried to understand their origin in terms of salt-induced protein phase transitions in bulk solution.

It is very difficult to fix component concentration in different parts of the drop during drying, because a drop-drying process means not only water evaporation, but also protein redistribution in the drop. For rough estimation of the component ratio in the liquid (central) part of a drying drop by the time of protein spatial structure formation, we took photos of dried drops and made their 1 to 10 plasticine models, bearing in mind that the density of dried protein is the same in different parts of the drop. In our calculations we ignored salt mass because of its insignificance. In this way we obtained relative protein conversion to the solid ring, as a percentage of the whole drop mass. Thus, according to our estimates, about 70% of BSA (of the original 7% w BSA in 0.9% w NaCl solution) escapes the liquid part of the drop by the moment of protein spatial structure formation. We cannot observe protein structure formation in real time using our technique, but it is clear that it arises between the second and third stages of the drop-drying process (Fig. 1). Thus, we supposed that approximately 50% of water evaporated by this moment. Salt mass in the liquid part of the drop does not change until salt is crystallized. So the concentrations of the components should be approximately 2.5% w BSA in 1.8% w NaCl. Watching the drying drop of this solution, we found that the protein ring did not form, whereas protein structures were formed just over the drop's edge (Fig. 4). To our mind, this indicates that small-size protein precipitates were present in the initial solution. Thus, our assumption seems to be correct.

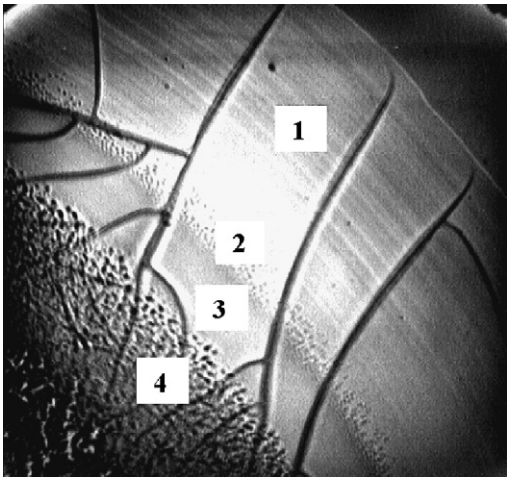

Fig. 3. Zones in dried drop of BSA–salt solution: (1) homogeneous protein film (colloidal glass); (2) zone of protein precipitates, from single ones to their clusters; (3) gel; (4) zone of salt structures in shrinking protein gel. ×70.

It was interesting to estimate the ratio of the components in the liquid part of a drop of 7% w BSA in 0.9% w NaCl solution for the principal critical points during drying. For simplification of our calculations, let us imagine a huge drop with a mass of 100 g. Then we can write the initial concentration of the components in grams (Table 1). The second set of points—decrease of mass—was obtained in experiment. When protein gel forms in the middle of the drop, the protein concentration in the liquid part becomes zero. We estimated water content in the gel in accordance with experimental data [2], which showed monotonic temporal change of the drop mass during drying. It is easy to calculate water content for saturation of saline solution before crystallization at room temperature. Our experiments [9] show that after the end of salt crystallization in drying drops, free water continues to evaporate. This means that the salt crystallization process occurs ahead of free water evaporation. Thus, there is a short period when water is the only component of a liquid part of a drop. This point is reflected in the last column of Table 1. If every column in Table 1 is set to be equal to 100%,

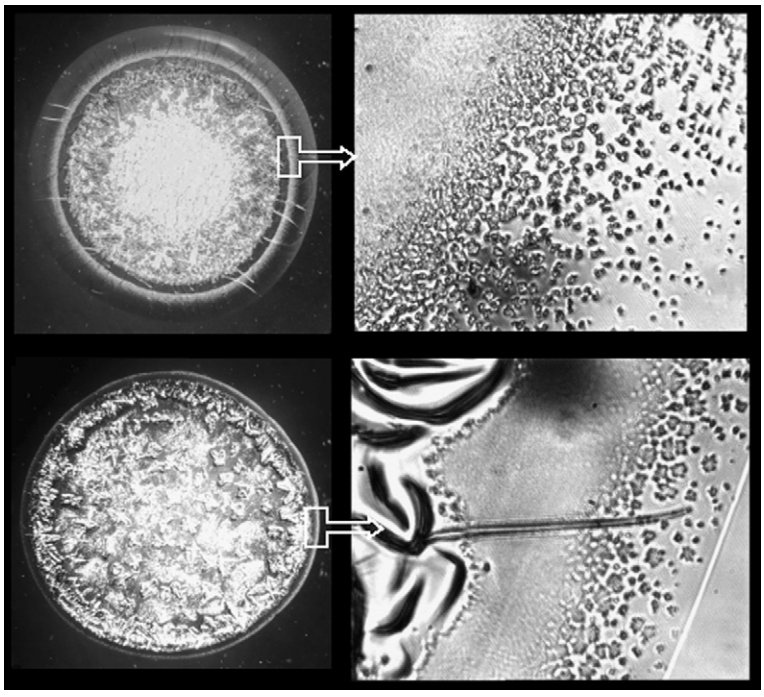

Fig. 4. Dried drops of protein–salt solutions: 7% BSA in 0.9% NaCl (above) and 2.5% BSA in 1.8% NaCl (bottom). Light-diffusing ring of protein structures has different positions (see the text). Magnification: left—×10; right—×70.

Table 1
Decrease of mass (in grams) of the components in the fluid part of a drying drop of 7% w BSA in 0.9% w solution in some critical points during drying

Components	Initial	Protein spatial structures formation	Gelation	Beginning of salt crystallization	End of salt crystallization
H_2O	92.1	46.1	20.0	2.5	~1.0
BSA	7.0	2.1	0.0	0.0	0.0
NaCl	0.9	0.9	0.9	0.9	0.0

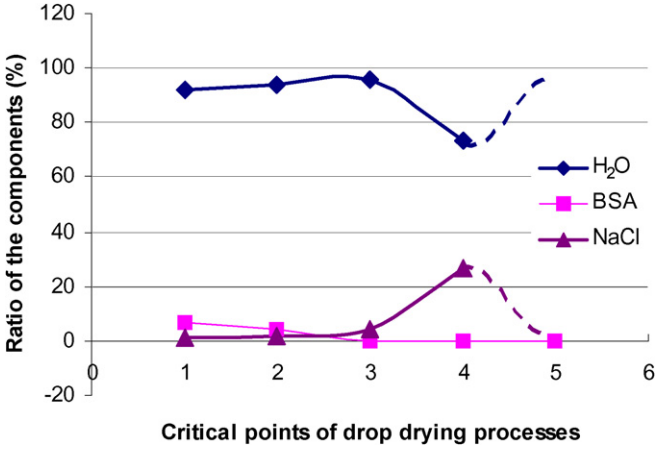

Fig. 5. Ratio of the components in the liquid part of a drying drop of 7% w BSA in 0.9% w NaCl solution at principal critical points of the process: (1) initial conditions; (2) formation of protein spatial structures; (3) gelation; (4) beginning of salt crystallization; (5) end of salt crystallization (admittedly).

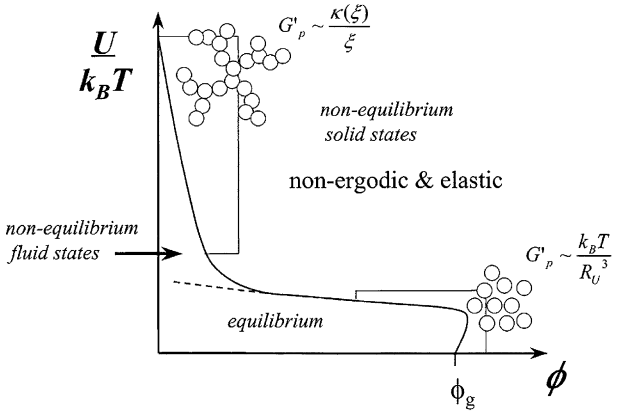

Fig. 6. Schematic state diagram of colloidal particles with short-range potentials, after Trappe and Sandkuhler [27]. ϕ is the volume fraction of colloidal phase; U is the strength of the interparticle attraction.

we can obtain a real ratio of the components in the liquid part of the drop for the principal critical points of the drop-drying process (Fig. 5). We can see the paradoxical fact of increasing water abundance in the liquid part of the drop during drying up to gelation, as well as during salt crystallization. These data are in disagreement with the ideas and mathematical descriptions of the drop-drying process made in [7,8], because the authors ignore loss of significant mass of protein and subsequent cascade of protein phase transitions in the liquid part of the drop.

Thus, it was shown that due to protein redistribution during drop drying, protein deposits on the drop edge and protein in the middle part of the drop are in different conditions, and form materials with different properties. The authors [27] argue that colloidal particles can form different structures: from colloidal glasses with very high volume fractions and low strength of interparticle attraction to colloidal gels with very low volume fractions and strong attraction between the particles (Fig. 6). Before gelation, colloidal particles form fractal clusters, which turn into space-filling networks. We think that a drying drop of protein–salt aqueous solution is an excellent illustration of this dynamics. Taking into account hydrodynamic motion of the colloidal phase to the drop periphery and its rapid consolidation there, we suppose that this solid phase really represents the protein glass transition: it is transparent and extremely fragile. In contrast to the drop periphery, low protein volume fraction and high ionic strength in liquid residuals in the middle part of a drying drop stimulate liquid–liquid separation and further cascade of protein phase transitions leading to gel formation. Thus, protein gel probably forms only inside the protein ring of a drying drop.

It is known [28] that increasing electrolyte concentration causes the diffuse double layer over colloid particles to shrink closer to the particle, so that the electrostatic potential falls off more quickly with distance ("double layer compression," in accordance with the Debye–Hückel theory of strong electrolytes). Continued decreasing of repulsive forces between particles leads to their coagulation (liquid–liquid separation) when the coagulation threshold was achieved. Against a background of further increase of salt concentration, protein-rich droplets become harder and form precipitates that sediment onto a homogeneous protein film. Then fractal clusters are formed from these single precipitates.

We tried to find some morphological evidence of initial stages of protein phase transitions by means of AFM. It seamed reasonable to find such initial structures formed at the beginning of drying in the bottom adsorption layer of a dried drop. So we removed the upper dry film with a scalpel (it was removed easily because of cracks), and investigated the uninjured bottom protein layer (Fig. 7). We observed separate aggregates of round subunits, which might be micelles formed as a result of liquid–liquid separation (Fig. 8). According to our idea, the dynamics of protein structure formation is the following (Fig. 9): structures of the first generation are represented by micelles; structures of the second generation are micrometer-size precipitates of the micelles that become harder under the influence of salt; structures of the third generation are fractal protein clusters that consist of structures of the second generation passing into gel (structure of the fourth generation).

On the other hand, these findings are close to the "morphodrom" of Tanaka et al. [23], who studied complex pattern evolution in bulk solution as a result of the relationships between the solid–liquid phase separation and the liquid—liquid

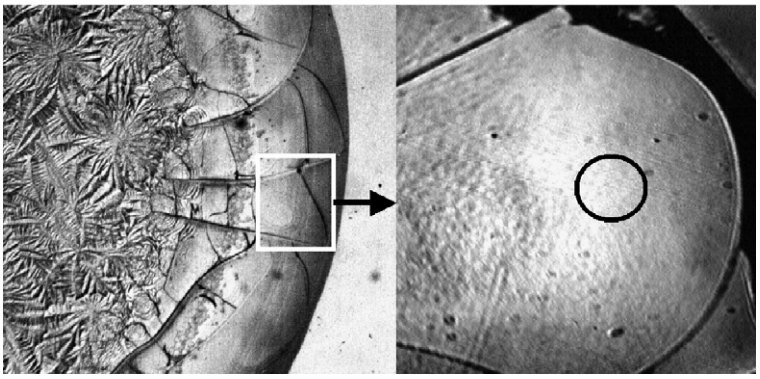

Fig. 7. Fragments of dried drops of 7% BSA in 0.9% NaCl aqueous solution. Left—zone of protein ring (in a white rectangle) before removal; right—the same zone after removal of the upper film. The black circle shows the area of bottom protein adsorption layer used for AFM investigation.

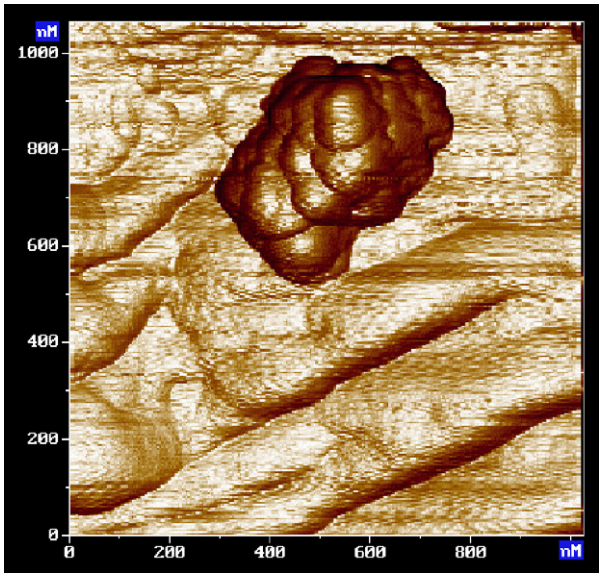

Fig. 8. AFM data: single protein precipitate lying on the protein film in dried drop of BSA–salt aqueous solution. It consists of some subunits, which admittedly represent consolidated micelles.

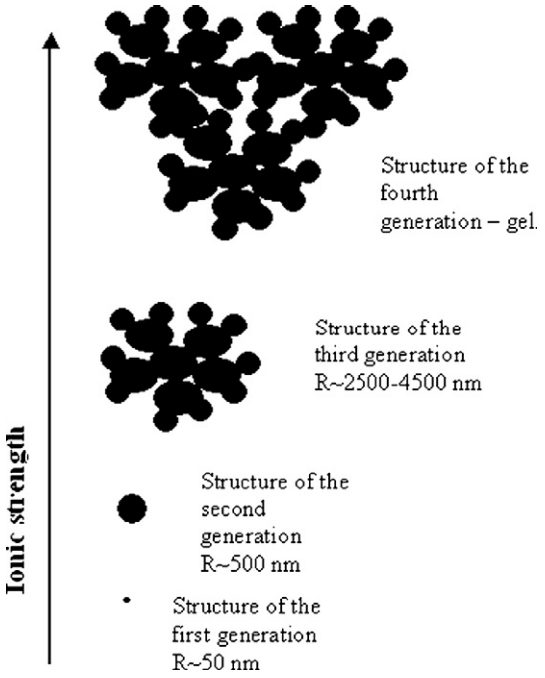

Fig. 9. Protein phase transitions in liquid part of a sessile drying drop of protein–salt–water solution. *R* is the radius of the structure.

phase separation in lysozyme–salt–water systems depending on temperature and concentrations of the components. A characteristic feature of our research is that processes in drying drops of protein–salt solutions are driven not only by the above factors, but also by drop attachment to a substrate, evaporation, and protein redistribution in a drop due to centrifugal flow. All these protein perturbations are fully reversible: addition of water to dried drops transfers them to a colloidal solution, and further drying leads to creation of the same morphological images, as it was before the dilution.

We are ready to subscribe to the opinion of Deegan [2, p. 484] that "A drying drop is a new, rich, and unexplored example of a pattern forming system." It is a complex working mechanism, driving the system to a nonequilibrium state. Earlier we gave a phenomenological description of such protein structures in human blood serum [29], but could not give a correct explanation of their origin at that time. Protein phase transitions in human biological liquids used to be a cause of some diseases, such as sickle-cell anemia [30], cataracts [31–35], rheumatoid arthritis, and cryoprecipitation of immunoglobulins [36,37]. Perhaps this list might be extended due to the nonspecific nature of such protein changes against diseases. Any disbalance of the components in the plasma of patients can lead to decreasing protein phase stability and sometimes to phase separation. It was shown that the dynamics of the mechanical properties of drying protein–salt solutions is closely related to protein and salt concentration in the initial solutions, as well as to protein and cation type and surfactant presence and concentration [38]. Our attempt to use this dynamics in medical diagnostics gave promising results [17].

4. Conclusions

Thus, in this paper experimental data on the dynamics of protein phase transitions in drying sessile drops of protein–salt aqueous solutions were presented. The nonlinear interplay of the components during drying was shown. Water abundance in the liquid part of a drop increases by the time of formation of

spatial protein structures and gelation. It can be assumed that water abundance also increases during salt crystallization.

The following sequence of self-organizing processes was found:

1. attachment of a drop to a substrate by means of protein adsorption layer;
2. redistribution of the colloidal phase in a drop due to centrifugal flow;
3. creation of different conditions for protein phase transitions on the drop edge and in the middle part of the drop;
4. protein glass transition over the drop edge;
5. cascade of protein phase transitions—from micelles to gel—in the liquid part of the drop;
6. salt crystallization in the gel matrix.

We hope that these data will be useful for further investigation of self-organizing processes in drying liquids. We believe that the most important application of the drying drop model is for further understanding of the pathogenesis of some sicknesses and of their possible prevention and correction. The drying drop technique can be applied also to forming colloidal structures with ordered properties (from nanoscale to micrometers) for technical needs.

Acknowledgments

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