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LATERAL PHASE SEPARATIONS IN *ESCHERICHIA COLI* MEMBRANES

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SUMMARY

Membranes from unsaturated fatty acid auxotrophs of *Escherichia coli* were studied by spin labeling and freeze-fracturing. From measurements of the partition of the spin label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) between the aqueous phase and fluid lipids in isolated membranes, temperatures corresponding to the onset and completion of a lateral phase separation of the membrane phospholipids were determined. By freeze-fracture electron microscopy a change in the distribution of particles in the membrane was observed around the temperature of the onset of the lateral phase separation. When cells were frozen from above that temperature a netlike distribution of particles in the plasma membrane was observed for unfixed preparations. When frozen after fixing with glutaraldehyde the particle distribution was random. In membranes of cells frozen with or without fixing from a temperature below the onset of the phase separation, the particles were aggregated and large areas void of particles were present. This behavior can be understood in terms of the freezing rate with the aid of phase diagrams.

INTRODUCTION

In recent work a study has been made of lateral phase separations and sugar transport in a β-oxidation-deficient fatty acid auxotroph of *Escherichia coli* supplied with various exogenous unsaturated fatty acids [1]. It was found that breaks in the Arrhenius plots of β-glucoside and β-galactoside transport corresponded closely to the temperatures representing the onset and completion of lateral phase separations of the membrane phospholipids [2–4].

The results were particularly interesting when this mutant was grown on elaidic acid, since the membrane phospholipids have a very simple fatty acid composition and the onset and completion of the phospholipid phase separation are easily discernible from sugar transport as well as from spin-label data.

The purpose of the present paper is to describe a freeze-fracture electron mi-

Abbreviations: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; t_h and t_l, "higher" and "lower" characteristic temperatures (°C) as revealed by spin labeling.

croscopy study of the inner membranes of this β-oxidation-deficient fatty acid auxotroph. From earlier freeze-fracture studies [5–11] of various cells grown on different fatty acids, it is clear that the distribution of particles found in the plasma membrane depends on the fatty acid composition, and that the particle distribution somehow depends on the solid ⇌ fluid phase equilibria of the phospholipids. The present study differs from this earlier work in that the fatty acid composition of the membrane is especially simple, and the temperatures corresponding to the onset and completion of the phase separations are known quite accurately.

MATERIALS AND METHODS

Growth of bacteria

The strain 3OEβox$^-$ used in these studies was a gift of Ms C. D. Linden and its isolation has been described by Linden et al. [1]. It is a β-oxidation-deficient unsaturated fatty acid auxotroph of *E. coli* K12. Cells were grown in medium A [12] supplemented with 1% casamino acids (Difco), 5 μg/ml of thiamine hydrochloride, 0.5% of the detergent Triton X-100 (Sigma) and 0.02% elaidic (trans-Δ^9-octadecenoic) acid (Analabs) or 0.01% oleic (cis-Δ^9-octadecenoic) acid (Sigma). Cultures of 1 l or 100 ml were grown at 37 °C with vigorous rotary agitation in 2-l or 250-ml flasks. Cells were grown overnight in oleic acid supplemented medium, sedimented, washed with elaidic acid supplemented medium and then grown in elaidic acid supplemented medium for approximately four generations. Cells were harvested by centrifugation at $3000 \times g$.

Inner membrane samples

The inner membranes of *E. coli* were isolated following the procedure of Fox et al. [13], Tsukagoshi and Fox [14] and Linden et al. [1]. The final pellet of inner membranes was washed several times in buffer containing 10 mM Tris–HCl (pH 7.5) and 2 mM EDTA. It was then suspended in 1 ml buffer and 0.2 ml of a 10 mM solution of the spin label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) was added. After approximately 20 min the membranes were centrifuged for 40 min at $110\,000 \times g$. The pellet was taken up in an EPR sample tube [2] and centrifuged to obtain a high concentration of membranes.

EPR measurements

The TEMPO spectral parameter f [2] was measured as a function of temperature. All experiments were carried out at X-band on a Varian E12 EPR spectrometer. The temperature was controlled with a variable temperature accessory and measured with a copper-constantan thermocouple. The logarithm of f was then plotted as a function of the reciprocal of the absolute temperature. The TEMPO spectral parameter is approximately equal to the fraction of molecules of TEMPO that is dissolved in the fluid hydrophobic region of the membrane.

Samples for freeze-etching

Cultures of 100 ml were grown at 37 °C and harvested by centrifugation at approximately 30°C. The cells were washed in distilled water and the final pellet was dispersed by agitation with a Vortex mixer. The tube was then equilibrated at

the desired temperature in a water bath. Small samples were pipetted onto copper discs on a metal block at the same temperature as the bath and equilibrated for one minute. They were then quickly frozen in partially solidified Freon-22 (du Pont), cooled by liquid nitrogen, and stored under liquid nitrogen until use. Fracturing and etching were carried out in a Balzers BAF301 Freeze-Etching Device, modified for manual operation of the microtome arm advance and cutting. Generally samples were fractured at -100 °C (with a pressure of 10^{-6} torr) and etched for 5–30 s. Control experiments were carried out at -115 °C with no etching. For comparison, some preparations were frozen without washing or after treatment with 20% glycerin.

Fixed preparations

For fixing, the cell pellet was resuspended in half the desired volume of distilled water and equilibrated at the desired temperature. An equal volume of 1% glutaraldehyde (Aldrich) was equilibrated at the same temperature and the two solutions were then quickly mixed. After 30 min the fixed cells were centrifuged at 30 °C and the pellet was equilibrated, frozen and fractured like the other samples.

Electron microscopy

Replicas were floated off the specimen disc in distilled water and transferred to a solution of commercial sodium hypochlorite (Chlorox). They were left overnight at room temperature, then washed in distilled water and picked up on uncovered 400-mesh copper grids. Electron micrographs were taken on 35-mm film with a Philips EM 200 instrument operated at 80 kV.

RESULTS

Spin-labeling experiments

Measurements of the TEMPO spectral parameter f were made in order to

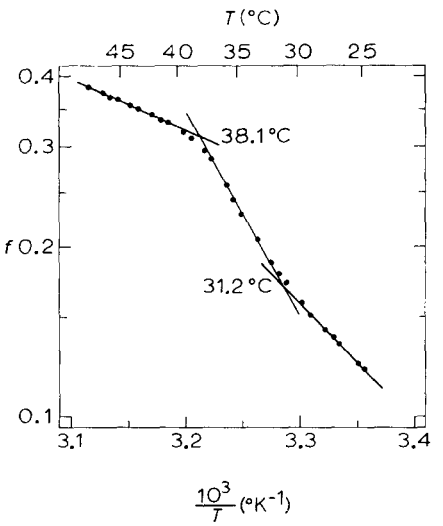


Fig. 1. The TEMPO spectral parameter f as a function of the reciprocal of the absolute temperature ($1/T$). The membrane pellet in the EPR sample tube was concentrated by centrifugation in a clinical centrifuge followed by centrifugation for 2 h at 32 000 × g.

confirm the identity of the cells used here with those used by Linden et al. [1]. The plots of f versus $1/T$ obtained when cooling the samples showed two characteristic temperatures: $t_h = 36.8$–38.1 °C and $t_l = 30.0$–31.2 °C. As an example Fig. 1 shows the curve obtained in one of the experiments. The agreement with results published earlier [1] is good.

The experiments generally were carried out starting at the high temperature and then slowly cooling the system. Reversing the process, i.e. starting at the low temperature, revealed some hysteresis in the measured curves. Thus, cooling in one experiment gave $t_h = 36.6$ and $t_l = 30.0$ °C whereas heating the same sample afterwards led to $t_h = 38.6$ and $t_l = 32.5$ °C. Overath and Träuble [15, 16], using optical methods, observed a similar phenomenon when measuring the transition temperature T_t of phospholipids extracted from *E. coli* fatty acid auxotrophs grown on elaidic acid. Thus, on cooling they found $T_t = 37$ °C and on heating $T_t = 38$ °C for measurements carried out in buffer. They did not observe a lower transition temperature, which may be due to the limited temperature range investigated or due to insensitivity of the method to the second "transition".

Electron microscopy

E. coli 3OEβox$^-$ cells grown at 37 °C on elaidic acid were frozen from various temperatures. After fracturing, the preparations were etched in order to aid in the interpretation of the micrographs. The interpretation of a given fracture face is based on the work of Fiil and Branton [17] ,van Gool and Nanninga [18] and Gilleland et al. [19]. The fracture plane which reveals the plasma membrane passes through the hydrophobic region of the membrane and splits it [20,21]. Apart from the convex cytoplasmic membrane surface, glycerin cryoprotected cells showed three other indentifiable fracture faces, namely the concave cytoplasmic membrane surface, the convex cell wall layer and the concave cell wall layer. The general appearance was similar to that reported for *E. coli* [17, 18] and for *Pseudomonas aeruginosa* [19]. Cells which were frozen without glycerin showed flagella on the outside of the cell wall as reported by other investigators [18, 22]. The cell wall layer fracture faces were not commonly observed when the cells were not treated with glycerin.

Our main interest centered on the plasma membrane. It appeared, in agreement with other investigations [17, 19] that in the fracture process most of the particles in the membrane adhered to the convex fracture face, i.e. that part of the membrane bilayer on the cytoplasmic side. Samples were frozen from room temperature (22 °C) and in approximately one degree intervals between 31 and 45 °C. The distribution of the particles, presumably protein, revealed two major patterns. Above approximately 40 °C, a netlike arrangement was seen (Fig. 2) as reported earlier for *E. coli* [17, 18, 23], for *P. aeruginosa* [19] and for other bacteria [24]; for all temperatures below 37 °C the particles appeared to be pushed together into larger, continuous areas, leaving other parts of the membrane devoid of particles (Fig. 3). In the intermediate temperature range, between 37 and 40 °C, examples of both the low and high temperature appearance of the plasma membrane were found and also hybrids, i.e. membranes showing netlike particle arrangement and areas void of particles at the same time. Cells maintained at a high temperature for 1 h or more exhibited the low temperature appearance of the particle distribution in the membrane if they were maintained at 31 °C for 10 min before freezing. The reverse was true also, i.e.

Fig. 2. High temperature appearance. Cells were washed once in distilled water and quenched from 43 °C. Fracturing was carried out at −100 °C followed by etching for 10 s. The plasma membrane shows a netlike distribution of particles. Magnification, 83 000×.

Fig. 3. Low temperature appearance. Cells were washed once in distilled water and quenched from 37 °C. Fracturing and etching for 30 s were carried out at −100 °C. Areas of particle aggregation and areas void of particles can be seen in the plasma membrane. Magnification, 88 000×.

cells frozen from 43 °C had the high temperature appearance even after being kept at a low temperature for prolonged periods. (Cells grown entirely on medium supplemented with oleic acid showed the netlike arrangement of particles if frozen from 22 °C and the aggregation of particles if frozen from 5 °C).

Cells grown at 37 °C on elaidic acid supplemented medium were fixed for one hour with 0.5% glutaraldehyde at 44, 41, 37 and 23 °C, equilibrated at these temperatures after centrifugation and frozen. Those cells fixed at 44 or 41 °C had particles distributed all over the plasma membrane in an apparently random fashion

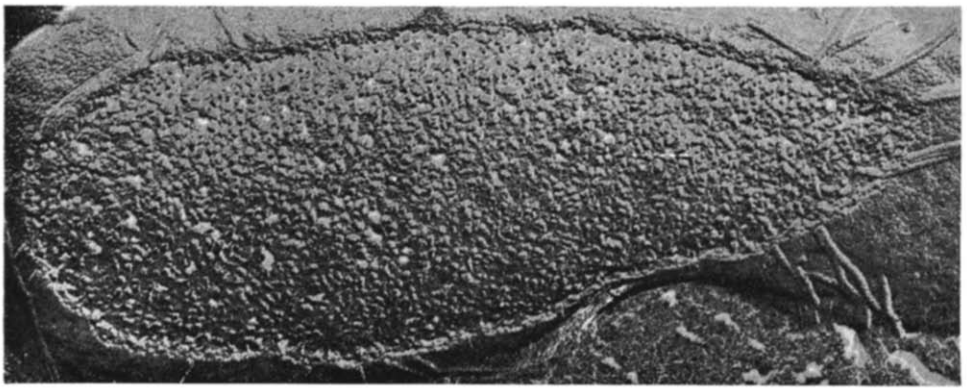


Fig. 4. Fixed preparation. Cells were fixed in glutaraldehyde at 44 °C and quenched from the same temperature. Particles are randomly distributed throughout the plasma membrane. Magnification, 88 000 ×.

(Fig. 4), cells fixed at 23 °C exhibited the familiar aggregation pattern. Fixing at 37 °C produced plasma membranes with particles all over the membrane in some cases, but some large areas void of particles have also been observed in other cases. When cells fixed at 37 °C were slowly cooled at 30 °C prior to freezing, they retained the largely random distribution of particles in the membrane. *E. coli* K12-1100 thiamine$^-$, a gift of Dr Robert D. Simoni, were grown under the same conditions as the 3OEβox$^-$, except that the fatty acid supplement and the detergent were omitted. These wild type like cells were then treated like the fatty acid auxotrophs and the replicas were examined. Over the temperature range from 3.5 to 42 °C all plasma membranes were totally and randomly covered with particles. No netlike arrangement or aggregation was seen.

DISCUSSION

The results reported here are interpreted as follows. The particle distribution in the plasma membrane of *E. coli* K12-3OEβox$^-$ is dependent on temperature and on the fatty acid supplement in the growth medium. The beginning of a phase separation of the membrane lipids [1] lies in the temperature range in which the particle distribution changes from a netlike one (or random in the case of glutaraldehyde fixed cells) to one of nearly complete particle aggregation. In the course of the phase separation the particles (protein molecules) are initially excluded from the growing solid patches and collected into aggregates, as indicated for certain freezing processes for *Tetrahymena* [10]. Some of the membrane particles may even be excluded from the membrane, as suggested by the relatively small number of particles observed in many cases. This could be due to a motion of the particles normal to the plane of the membrane as discussed by Speth and Wunderlich [10]. The smallness of the number of particles could also be due to preferential fracture of bilayer areas which do not contain any protein.

Above the temperature at which the phase separation begins, the particles are randomly distributed over the entire membrane. The round patches void of particles, which lead to the netlike appearance, are patches of lipid which froze and excluded

the protein during quenching of the sample for the freeze-fracture experiments, indicating that the freezing rate was not high enough to preserve the particles in their random distribution. There was, however, not sufficient time for the patches to join and drive the protein into larger aggregates. This explanation is further supported by the observation that the round patches void of particles are smaller at the edge of the replica than in the middle. At the edge the rate of freezing is expected to be higher and thus the solid patches should be smaller. Fixation with glutaraldehyde immobilized the protein molecules and probably also the lipid molecules because the majority of the lipid is phosphatidylethanolamine. The particles were then randomly distributed throughout the plasma membrane, if the cells were fixed at a temperature above the onset of the phase separation.

When cells were frozen from below t_h the particles were found to be aggregated in the membrane. According to our interpretation, i.e. if this protein–lipid system can be described by a phase diagram of the type shown in Fig. 6 (see Appendix), the extent of aggregation should increase as the cells are cooled from t_h to t_l and then remain unchanged. A quantitative assessment of this process is not possible because the fracturing process is not understood well enough. If the cells were to fracture truly randomly a statistical evaluation might be possible. However, large areas void of particles and other areas with closely packed particles are present in the membrane. This is likely to influence the direction of the fracture plane and may explain why no change of appearance in the freeze-fracture pictures associated with t_l can be detected. A knowledge of both t_h and t_l in these studies is helpful because only then is it possible to know at which of the "transition temperatures" one is working.

Much of this explanation at the present time cannot be extended to membranes of a more complex lipid composition. Although a random distribution of particles in wild type cells was observed in these experiments, other authors in many cases found round patches void of particles and a netlike appearance [17, 18, 23]. Even the 3OEβox$^-$ cells grown on oleic acid cannot be included in the scheme without further study because a netlike appearance was observed for cells frozen from 22 °C and the EPR determined characteristic temperatures are 31.1 and 15.8 °C [1]. The appearance of the membrane in freeze-fracture experiments depends on the temperature at which freezing starts, the rate of freezing, possibly the growth temperature and medium (c.f. Speth and Wunderlich [10]) and the lipid composition. The explanation for the freezing behavior of the cells grown on elaidic acid rests on our interpretation of the lipids as a binary system. This is outlined more fully in the Appendix. For more complex membranes an attempt at interpretation has to await a better understanding of the freezing behavior of more complex lipid mixtures.

It is clear that in the elaidic acid grown cells the growth temperature is close to the temperature corresponding to the onset of the lateral phase separation (t_h) and probably slightly below this latter temperature. The variation in t_h reported here and by Linden et al. [1] allows no conclusion as to whether t_h is slightly above the growth temperature or slightly below. If, as suggested previously [1], lateral compressibility is important for the insertion of protein into membranes it should be advantageous for the cell to have t_h above the temperature of growth. Then patches of solid lipid are present in the membrane to which other lipid molecules can add if the area occupied by the lipid has to decrease so that a new protein molecule or protein–lipid complex [25] can be inserted.

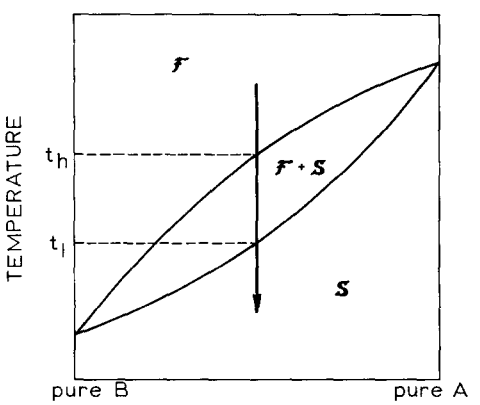
For *E. coli* 3OEβox$^-$ cells grown on oleic acid the temperature of the onset of the phase separation (t_h) as measured by the spin-label technique [1] is much lower than the growth temperature. However, the lipid composition for these cells is more complex [1] than for elaidic acid grown cells. Small domains of solid lipid may be present, just not large enough to be detected by any of the techniques employed.

APPENDIX

Lipid–lipid and lipid–protein lateral phase separations

In trying to interpret freeze-fracture electron microscopy photographs of the type shown in the present paper, one must consider at least two types of lateral phase separations. Lipid–lipid phase separations arise for example, when a two-dimensional homogeneous solution of a binary mixture of lipids is cooled below the temperature t_h. Fig. 5 illustrates a solid solution–liquid solution phase diagram, and the temperatures t_h and t_l for a 50 : 50 mixture of the two lipid components. Solid solution–liquid solution phase diagrams of this type are usually obtained when the two lipid components have very similar physical properties (e.g. dimyristoyl phosphatidylcholine–dipalmitoyl phosphatidylcholine) [2]. In cases where the physical properties of the two components of a binary mixture are quite different (e.g. dipalmitoyl phosphatidylcholine–dipalmitoyl phosphatidylethanolamine, or dipalmitoyl phosphatidylcholine–cholesterol) [2, 3] one typically obtains a triangular phase diagram of the type illustrated in Fig. 6. The horizontal line indicates a region of solid phase immiscibility, so that at low temperatures, lipid domains of different composition co-exist under conditions of equilibrium.

Although no phase diagrams of binary lipid–protein mixtures have yet been determined, it is quite possible that phase diagrams similar to those in Figs 5 and 6



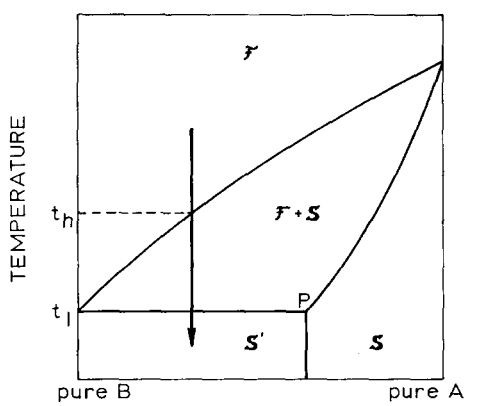


Fig. 5. Schematic phase diagram for a binary lipid mixture exhibiting solid and liquid miscibility over the entire range of compositions. In the *F* phase the lipids form a liquid solution, in the *S* phase they form a solid solution. The arrow indicates a possible temperature drop experiment, the temperatures t_h and t_l correspond to the onset and the completion of a lateral phase separation.

Fig. 6. Schematic phase diagram for a binary lipid mixture exhibiting only partial miscibility in the solid phase. In the *F* phase the lipids form a liquid solution. In the *S* phase a solid solution is formed for compositions to the right of point P. For compositions to the left of point P (phase *S'*) two solid phases coexist, one of pure B, the other one a solid solution corresponding to a composition of point P. The arrow indicates a possible temperature drop experiment.

will also be obtained. For example, Chen and Hubbell [26] have shown that when a binary mixture of dark-adapted rhodopsin and dimyristoyl phosphatidylcholine is slowly cooled below the transition temperature of dimyristoyl phosphatidylcholine, one observes two domains, one free of rhodopsin and having the typical band pattern of pure dimyristoyl phosphatidylcholine [7, 27], and the other enriched in rhodopsin. The corresponding phase diagram could then be similar to that in Fig. 6. On the other hand, when a binary mixture of dimyristoyl phosphatidylcholine and bleached rhodopsin is cooled below the transition temperature of dimyristoyl phosphatidylcholine, one observes a uniform distribution of membrane particles. The corresponding phase diagram may then be of the type illustrated in Fig. 5. A uniform distribution of particles in a slowly cooled binary mixture of glycophorin [28] and dipalmitoyl phosphatidylcholine has been observed by C. W. M. Grant, G. V. Bruno and H. M. McConnell (unpublished), again suggesting a phase diagram of the type shown in Fig. 5.

The vertical arrows in Fig. 5 and 6 illustrate two possible temperature drop experiments. In Fig. 5, the solid solution–liquid solution case, a slow temperature drop that maintains equilibrium conditions throughout, leads to a solid phase composition which is just the same as that which would be achieved by an instantaneous temperature quench. Thus, very rapid and very slow cooling from above t_h can lead to the same solid lipid compositions. This is not true for intermediate rates of cooling. A different situation prevails for the phase diagram in Fig. 6, where under equilibrium conditions two phases exist at the lower temperature, but very rapid quenching should produce a homogeneous mixture of the phospholipids.

We offer the following tentative interpretation of the freeze-fracture photographs of the 3OEβox$^-$ membranes grown on elaidic acid. The shape of the spin-label binding curve [2] reported here and earlier [1] for the inner membranes indicates that for this essentially binary lipid mixture of phosphatidylethanolamines [29], the appropriate phase diagram is either of the solid solution–liquid solution type (Fig. 5), or of the triangular type illustrated in Fig. 6, where the composition corresponds to a point well to the right of the point P in the diagram. Thus, rapid quenching from t_h or above to a low temperature can produce a solid solution without lateral motion of the lipids. The lipids "freeze" in a manner as if they were a pseudo single lipid component. The rapid freezing of this pseudo single lipid component brings about a lateral lipid–protein phase separation, giving rise to the netlike distributions seen in Fig. 2, where now the pseudo single lipid-protein composition is such that it is represented by some point to the left of P.

When the membrane is slowly cooled from above t_h one has domain patterns that are thermodynamically rather than kinetically determined.

The above interpretation should be distinguished from the following. If the lipids of the inner membrane were represented by a triangular binary phase diagram, with a composition well to the left of the point P, and if the quenching rate were slow relative to the rate of lateral phase separation of the lipids, then membrane particles (proteins) would have a choice of being concentrated in one lipid domain or the other on cooling. This would also give a non-uniform particle distribution in the freeze-fracture photograph. Although we suggest that this explanation may not be valid for the *E. coli* inner membranes, we have seen just this effect in preliminary experiments in which glycophorin is introduced in a dimyristoyl phosphatidylcholine–

distearoyl phosphatidylcholine mixture which does have a triangluar phase diagram. (C. W. M. Grant, G. V. Bruno and H. M. McConnell, unpublished.)

Freeze-fracture photographs of wild type *E. coli* K12 membranes show no evidence for lateral lipid–protein phase separation no matter what temperature the sample was quenched from. In terms of the present discussion, this could be due to one of two possible factors. The mixed lipids may be so complex that they produce a glass and thus provide no crystallization driving force for a lipid–protein phase separation. Alternatively, these lipid–protein systems may be represented by effective phase diagrams of the lipid–protein solid solution–liquid solution type, or the triangular type where the composition corresponds to a point to the right of P. In this latter case one expects a uniform particle distribution for either very fast or very slow cooling. If both conditions prevail, a complex mixture of lipids that only produces a glass (no lipid–lipid phase separation), and a stable lipid–protein solid solution, then one can expect a uniform particle distribution for all temperature quenching protocols.

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