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The liquid condensed diffusional transition of dipalmitoylphosphoglycerocholine in monolayers

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The fine details of the phase transition of dipalmitoylphosphoglycerocholine (DPPC) monolayers at air/NaCl solution interfaces were investigated at $21 \pm 1°C$ by using the fluorescence after photobleaching technique employing 12-(9-anthroyloxy)stearic acid as fluorescent probe. The mode of compression of the monolayer (i.e., continuous compression or successive additions of the lipid at fixed area) together with the ionic strength of the subphase (0.1 or 1.0 M NaCl) were particularly studied. The photobleaching results show that the lateral diffusion coefficient of the probe molecules decreases drastically within the liquid-condensed phase, i.e., from the end of the liquid-expanded–liquid-condensed phase transition to the beginning of the solid-condensed phase. The molecular areas at which the phase transition occurs under the various experimental conditions, together with a parallel analysis of the hydration states and related molecular areas of the DPPC molecules in multilayers, strongly suggest that the steric hindrance associated with the hydration water of the polar head of DPPC molecules in the monolayer is responsible for the drastic decrease in diffusion coefficient in the liquid-condensed phase. Furthermore, the fluorescence characteristics of the probe molecules also show that, together with the aforementioned reorganization of the polar head, a structural reorganization of the aliphatic chains of the lipid molecules also takes place in the liquid-condensed phase. The liquid-condensed phase therefore appears as a transition region from liquid to solid phases in which the lipid molecules present a significant decrease in their lateral diffusion related to a structural reorganization of both their polar and aliphatic components.

1. Introduction

The importance of membrane phase transitions (mainly the gel-to-liquid crystal phase transition) in biology has been studied for a number of membranes and is particularly well documented for *Acholeplasma laidlawii* [1]. On the other hand, a number of studies using multilamellar vesicles (MLV) have also demonstrated the importance of phase transitions in numerous processes. For instance, it has been shown that the gel-to-liquid crystal phase transition implies: (i) changes in the transverse mobility of constituents such as Na^+ [2], K^+ [3], anilinonaphthalenesulfonate [4], glucose [5] or tempo-choline [6]; (ii) increased susceptibility of the lipids towards phospholipase A_2 [7] as well as to bee venom [8]; (iii) maximum incorporation of constituents such as chlorpromazine [9] and (iv) significant increase in the lateral diffusion coefficient at the transition temperature [10].

Inasmuch as monolayers are concerned, the region of the liquid-expanded (LE) to liquid-condensed (LC) phase transition shows similar behaviour. For example, during the transition: (i) the activity of phospholipase A_2 is maximum [11]; (ii) an increase in the extent of penetration by glucagon and melittin is observed [12]; (iii) in pre-

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formed dipalmitoylphosphoglycerocholine (DPPC) films, either chlorpromazine [13] or β-naphthol [14] are incorporated at their maximum concentrations; (iv) the lateral diffusion coefficient decreases dramatically [15]. In the last two cases, close examination of the results, of importance in the context of the present paper, shows that the changes observed do not occur during the LE-LC phase transition but rather at the very beginning of the pure LC phase.

In fact, the LE-LC phase transition in monolayers has been, and still remains, a controversial subject. For example, it is still unclear whether this phase transition is a true first-order process or a diffused phase transition. On the other hand, it has been clearly shown from fluorescence microscopy experiments [16–18] that the LE-LC transition is heterogeneous in nature. The importance of the phase transition as such and the interest it has aroused in several researchers have occulted to a large extent the study of the LC phase per se. In this context, we have been led to study the molecular dynamics of DPPC spread at air/solution interfaces as a function of both the mode of compression of the monolayer film and the ionic strength of the subphase. In the present paper, we show that, in fact, the diffusion coefficient, D_{lat}, obtained from our fluorescence experiments is drastically reduced only within molecular areas that are characteristic of the pure LC phase. In other words, the entire decrease in D_{lat} occurs at the end of the LE-LC transition, when the lipid enters the pure LC phase, and not before. We show, with the help of evidence gathered from the literature, on either monolayers or MLV, that this change is accompanied by structural reorganization of both the polar head and aliphatic chains of DPPC molecules.

2. Materials and methods

2.1. Chemicals

DPPC and 12-(9-anthroyloxy)stearic acid (12-9-AS) were purchased from Sigma (St. Louis, MO). Their purity was checked by thin-layer chromatography and found to be satisfactory. They were used without further purification. The sodium chloride used for the subphases (0.1 and 1.0 M NaCl) was analytical grade. Ultrapure water (specific resistivity greater than 17×10^6 Ω cm) for the semiconductor industry was obtained from Motorola (Toulouse, France) and was used throughout the experiment.

2.2. Monolayer and fluorescence measurements

The interface fluorometer used in the present study as well as the experimental and theoretical details of our techniques have been described elsewhere [19–21]. In addition, Denicourt et al. [15] have presented a comparison of our methodology with alternatives published in the literature.

3. Results

The surface pressure isotherms of DPPC incorporating 2% (mol/mol) 12-9-AS as fluorescent probe are shown in fig. 1. The subphase used was 0.1 M NaCl. The isotherms shown are obtained either by continuous compression (unbroken line) or by successive additions of the lipid at a fixed area of the trough (dotted line). For the two types of compression, the isotherms are identical to

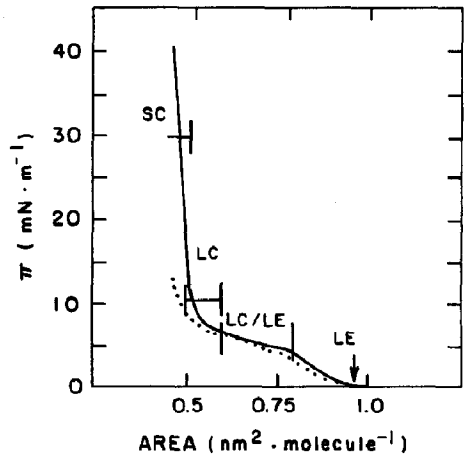

Fig. 1. Surface pressure isotherm of DPPC+2% (mol/mol) 12-9-AS at the air/water interface. $T = 21 \pm 1°$ C. Subphase: 0.1 M NaCl. (———) Continuous compression; (·····) successive additions of lipid.

those recorded when no probe molecules are present. This behaviour is consistent with the results obtained by Cadenhead et al. [22] who have shown that the surface pressure isotherms are not affected by the presence of probe molecules at the concentrations used here.

The surface pressure isotherm of DPPC obtained for both modes of compression shows phase behaviour similar to that reported by various authors [23–26]. The various two-dimensional phases in fig. 1 have been assigned following the terminology of Adam [27]. The border between the various phases is more or less established arbitrarily. In particular, the pure LC phase is usually taken as the beginning of the curvature in the isotherm at the end of the LE-LC phase transition. The lateral diffusion measurements described herein allow us to determine more precisely the beginning of the pure LC phase. Hence, these measurements show that for the DPPC surface pressure isotherm obtained by continuous compression, the pure LC phase is found for molecular areas ranging from 58 to 48 Å^2/molecule. On the other hand, when the isotherm is recorded by successive additions of the lipid, the pure LC phase is now observed for molecular areas ranging from 52 to 48 Å^2/molecule. The surface pressure isotherm obtained by successive additions of the lipid therefore shows an increase, in terms of molecular areas, of the plateau region characterizing the LE-LC transition, together with a corresponding decrease (6 Å^2/molecule) of the molecular areas within which the pure LC phase is found.

Fig. 2 shows the diffusion isotherms (i.e., log D_{lat} vs. molecular areas) of the DPPC film under the two compression modes (a, continuous compression; b, successive additions of lipid). For the two types of compression, the lateral diffusion coefficient decreases by about three orders of magnitude on going from the fluid to the more condensed phases (i.e., LC and SC), ranging from 10^{-5} cm^2 s^{-1} in the fluid region to 10^{-8} cm^2 s^{-1} in the condensed region. The change in D_{lat} during the diffusional transition occurs continuously over a range of 10 Å^2/molecule when the monolayer is compressed continuously (fig. 2a) and 4 Å^2/molecule for successive additions at fixed area (fig. 2b).

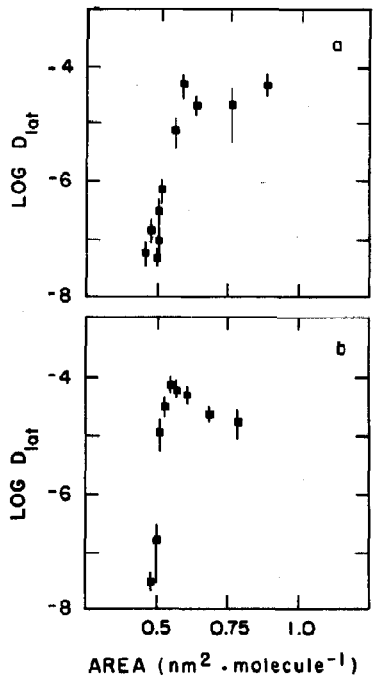

Fig. 2. Lateral diffusion coefficient (D_{lat} in cm^2 s^{-1}) as a function of the molecular area of DPPC. Subphase: 0.1 M NaCl. (a) Continuous compression; (b) successive additions of lipid.

The diffusion isotherms in fig. 2 also demonstrate that, for both modes of compression, the end of the diffusional transition is found at 48 Å^2/molecule. However, the beginning of the diffusional transition is modulated according to the manner in which the monolayer is compressed. Indeed, when the monolayer is compressed by continuous reduction of the area, the beginning of the diffusional transition is observed at 58 Å^2/molecule, whereas it is shifted to 52 Å^2/molecule in the case of successive additions of lipid. For the two modes of compression it is interesting to note that the molecular areas at which the beginning of the diffusional transition is observed coincide almost exactly with those that would be established for the pure LC phase by assuming that the LC phase corresponds to the beginning of the curvature in the isotherm at the end of the LE-LC transition, as referred to above.

Fig. 3 shows the surface pressure isotherm (panel a) and diffusion isotherm (panel b) of

DPPC + 2% (mol/mol) 12-9-AS at the air/subphase interface, the subphase in this case being 1.0 M NaCl. The isotherm was obtained by continuous compression of the monolayer. The behaviour observed here is qualitatively the same as that observed with 0.1 M NaCl, i.e., the diffusion coefficient decreases abruptly on going from fluid to condensed phases. Furthermore, this decrease again takes place when the film reaches the pure LC phase. However, for 1.0 M NaCl the isotherm is shifted towards greater molecular areas than in the case of 0.1 M NaCl. Therefore, the diffusional transition now occurs between 64 and 56 Å^2/molecule as compared to 58 to 48 Å^2/molecule when using 0.1 M NaCl. In addition, the diffusion coefficient in the fluid phase is reduced by one order of magnitude with 1.0 M NaCl, being now 10^{-6} cm^2 s^{-1} as compared to 10^{-5} cm^2 s^{-1} for 0.1 M NaCl.

Fig. 4 presents the dimerization rate constant, K_d, for DPPC + 2% (mol/mol) 12-9-AS under the following experimental conditions: continuous compression using 1.0 M NaCl (curve a) or 0.1 M NaCl (curve b) and successive additions of lipid with 0.1 M NaCl (curve c). For the various experimental conditions, one observes that K_d decreases within the region of molecular areas associated with the LE-LC phase transition to reach a minimum value corresponding exactly to entry of the film into the pure LC phase, i.e., 64, 58 and 52 Å^2/molecule for curves a–c, respectively. Therefore, the minimum of K_d in fig. 4 corresponds to the beginning of the diffusional transition observed in figs. 2 and 3. K_d then increases to reach a maximum value at the end of the pure LC phase and remains constant when the molecular area is reduced further. In addition, we observed (results not shown) that the fluorescence intensity of the probe, I_f, remains almost constant over the entire isotherm.

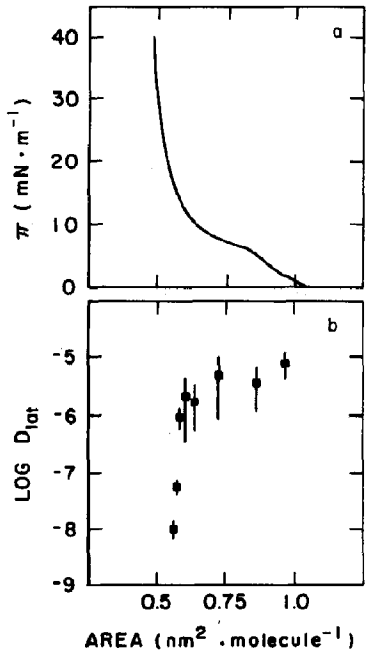

Fig. 3. (a) Surface pressure isotherm of DPPC + 2% (mol/mol) 12-9-AS at the air/water interface. Compression mode: continuous. $T = 21 \pm 1$° C. Subphase: 1.0 M NaCl. (b) Lateral diffusion coefficient (D_{lat} in cm^2 s^{-1}) as a function of area of DPPC. Same conditions as a.

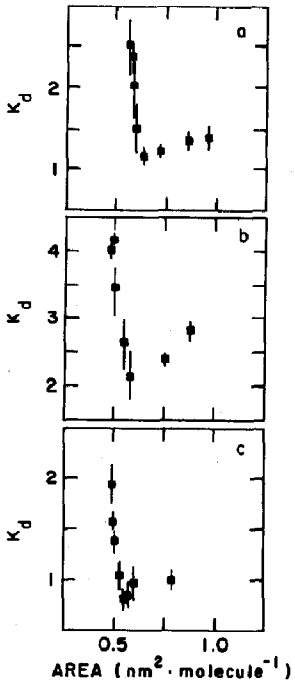

Fig. 4. Dimerization constant of the probe, K_d (cm^2 mol^{-1} min^{-1}), as a function of the molecular area of DPPC. (a) Continuous compression of the film, 1.0 M NaCl; (b) continuous compression of the film, 0.1 M NaCl; (c) successive additions of lipid, 0.1 M NaCl.

4. Discussion

The diffusion isotherms presented in fig. 2 show that the end of the diffusional transition is reached at 48 Å^2/molecule. This molecular area corresponds to the inflexion point in the surface pressure isotherms also found at 48 Å^2/molecule, which is associated with the appearance of the solid-condensed (SC) phase. Albrecht et al. [24] have attributed this point to a second-order phase transition between a crystalline tilted phase (i.e., the LC phase) and a crystalline non-tilted phase (i.e., the SC phase).

Fig. 2 also clearly demonstrates that, depending upon the mode of compression of the DPPC film, the diffusional transition does not begin at the same value of the molecular area. When the DPPC film is compressed continuously, the diffusional transition commences at 58 Å^2/molecule, corresponding to the entry of the film into the pure LC phase (fig. 1). These results are in agreement with those reported by Lösche et al. [16] who have shown that the decrease in D_{lat} using the same mode of compression takes place between 55–60 and 48 Å^2/molecule at 20 °C. However, the decrease they observe is only two orders of magnitude as compared to three in our case, which can probably be explained by their indirect measurement of D_{lat} from convection movements at the interface. Our results are also comparable to those of Peters and Beck [17] who have shown that the diffusional transition takes place between 8 and 15 mN m^{-1} as compared to 7 and 15 mN m^{-1} in our case. It is not possible to compare the actual molecular areas involved as those authors did not present the surface pressure isotherm of their DPPC. On the other hand, they also observe that the diffusion coefficient decreases by a factor of three on going from fluid to more condensed phases. However, their diffusion coefficients differ from ours by about two orders of magnitude. The reader is referred to our earlier work [15] for a detailed discussion of the possible cause of this discrepancy.

When the DPPC film is compressed by successive additions of lipid, the end of the diffusion transition also occurs around 48 Å^2/molecule, this value bearing some uncertainty due to the fact that the limit of resolution of our instrumentation had been reached. However, under this condition of compression, the beginning of the diffusion transition was observed at 52 Å^2/molecule which again corresponds to the entry of lipid into the pure LC phase (fig. 1, dotted line). This value is comparable to that reported in the work of Pallas and Pethica [28], namely, 52–54 Å^2/molecule, for the same mode of compression. Under the two different modes of compression used here, it is therefore observed that the diffusional transition takes place within the range of molecular areas characterizing the pure LC phase. As mentioned above, the LC phase is usually arbitrarily taken as the beginning of the strong curvature at the end of the LE-LC phase transition. Lateral diffusion measurements therefore provide a means of precisely positioning the frontiers of this phase.

The results presented so far therefore lead to the conclusion that the LC phase is characterized by the disappearance of highly fluid regions within the film, the fluorescent probe we use being mainly solubilized in these fluid regions [29]. Upon compressing the monolayer, the diffusion coefficient begins to decrease within the less fluid (LC) phase to reach a minimum on entry of the film into the SC region. The diffusion coefficient then remains constant at around 10^{-8} cm^2 s^{-1}. In the LC region, the DPPC molecules are progressively restrained in their lateral movements as their molecular area is further reduced until the occurrence of the second-order LC-SC phase transition at 48 Å^2/molecule.

It is possible to gain some insights into the decrease of D_{lat} upon entering the LC phase by examining the hydration properties of the DPPC molecules. For example, it is clear that steric hindrance between the hydrated polar heads of the lipid molecules in a monomolecular film is particularly important for molecular areas below 58 Å^2/molecule i.e., at the onset of the pure LC phase. For instance, Cadenhead [14] has shown that the ease of entry of β-naphthol from the subphase to a preformed monolayer of DPPC begins to decrease from a surface pressure of 7 mN m^{-1} at 20 °C, which corresponds precisely to the onset of the pure LC phase. In the same kind of experiments, Beurer and Galla [13] have shown

that for surface pressures above 7 mN m^{-1}, a decrease in the entry of chlorpromazine is observed. These experiments show that in the LC phase, the packing of the lipid molecules provides an effective barrier to the penetration of substrate from the subphase into the monomolecular film. In fact, Ivkov [30] has shown that the intrinsic molecular area of a fully hydrated polar head of DPPC is 59 Å^2/molecule. This implies that at the beginning of the pure LC phase (which occurs at 58 Å^2/molecule) the packing of the hydrated DPPC molecules in the film is at its maximum and that the ease of penetration starts decreasing. For greater molecular areas (i.e., in the LE-LC transition), the film is biphasic, and the resulting two-dimensional defect structure seems to play a role in the ability of β-naphthol to penetrate the film [14]. These experiments clearly show that at 58 Å^2/molecule, the interaction between the hydrated polar heads of the lipid molecules dominates over the surface properties of the film.

In addition, the hydration state of DPPC molecules directly affects their lateral mobility. McCown et al. [31] have shown that the lateral diffusion coefficient of DPPC dispersed in MLV decreases as a function of the hydration state of the polar head between 40 and 5%. The decrease is particularly significant below 20% hydration. The decrease in lateral mobility under 20% hydration was associated with a decrease in intermolecular distance between the hydrated polar heads of the lipid, thereby increasing intermolecular steric hindrance. At 20% hydration, Chapman et al. [32] have shown that the area of the DPPC molecules amounts to 58 Å^2/molecule, corresponding exactly to the molecular area observed in the present work at the onset of the pure LC phase. For this area, White and King [33] have determined that about 10 water molecules are bound rather strongly to each lipid molecule, these water molecules being organized by the local electric field associated with the charged groups of DPPC [34,35].

In this context, therefore, it is possible to rationalize the drastic change of D_{lat} upon entering the LC phase in a monolayer. For molecular areas larger than 58 Å^2/molecule, the probe being preferably soluble in the fluid portions of the film, the diffusion coefficient is large (approx. 10^{-5} cm^2 s^{-1}), being characteristic of a fluid medium. On compressing the monolayer down to this molecular area, hydration of the DPPC polar head decreases from 40 to 20% with no change in D_{lat}. At 58 Å^2/molecule, the lipid molecules have about 10 water molecules tightly bound to their polar heads. A further reduction of the area results in significant steric hindrance between the adjacent hydrated polar heads, which gives rise to a decrease in the diffusion coefficient. At this point, the lipid is in the LC phase. D_{lat} continues to decrease (and the polar head continues to lose water molecules) upon compression until the SC phase is reached at 48 Å^2/molecule where the hydration is now under 5%.

On the other hand, we have noted above that when the monolayer is compressed by successive additions of lipid, the decrease in D_{lat} together with the onset of the pure LC phase are shifted to 52 Å^2/molecule as compared to 58 Å^2/molecule under continuous compression. The former value is consistent with the figure one can extract from the surface pressure isotherms of Pallas and Pethica [28] under the same mode of compression. In addition, Prats et al. [36], from H^+ conduction at the interface, also observed that conduction was lowered from 52 Å^2/molecule and inferred that from this surface pressure, structural reorganization of the water molecules commenced. The idea put forward above can be used to rationalize the facts that the decrease in D_{lat} and the onset of the LC phase are shifted to lower molecular areas on successive additions of lipid. It is indeed likely that when adding lipid solution to the monomolecular film to achieve compression, the solvent used, when spreading, would contribute to appreciable dehydration of the polar heads of the lipid molecules. However, it is well known, from hydration experiments on MLV, that the hydration process, especially in the gel state (and, most probably in the LC state of the monolayer), is particularly slow [37]. It therefore follows that the steric hindrance normally encountered at 58 Å^2/molecule would be greater, owing to the lower degree of hydration of the lipid molecules. The monolayer can then be further compressed until at 52 Å^2/molecule steric hindrance among the polar heads would start to prevail [38]. At this stage, the

lipid enters into the LC phase and D_{lat} decreases abruptly, as observed in fig. 2b.

Some additional comments may help to give support to these seemingly speculative arguments. For example, Snik [39] has shown that significant hysteresis occurs when DPPC is continuously compressed to the point of collapse and then decompressed to larger molecular areas. The decompression isotherm is shifted toward the direction of lower areas. This is exactly the behaviour one would expect if the lipid molecules dehydrate appreciably upon compression and rehydrate only partially upon compression, due to the slow kinetics of the process. In addition, one would expect, from thermodynamic considerations, that for a given molecular area, the surface tension of the fully hydrated film would be lower (and hence the surface pressure π, higher) than the corresponding pressure for a less hydrated film, and that this effect is more important in the LC phase. This is indeed the situation found experimentally (fig. 1), the surface pressure for successive additions of lipids being lower than those for continuous compression of the monolayer.

Again, one can use the same line of ideas to rationalize the results of both surface pressure and diffusion isotherms of DPPC using 1.0 M NaCl (fig. 3). In this case, the diffusional transition (and hence, the LC phase) is now found between 64 and 56 Å^2/molecule as compared to 58 and 48 Å^2/molecule in the case of 0.1 M NaCl under continuous compression. This expansion effect with a subphase of higher ionic strength has been observed previously [40]. It has been shown by NMR studies [41] that hydration of the polar head of the DPPC molecules increases in high ionic strength media. As pointed out by Yeagle [42], as the salt concentration is increased the molecular interactions between head groups are weakened and more water molecules may bind. The volume of the polar head will thus increase as more water molecules are bound and one would expect steric hindrance to occur at higher molecular areas, as found experimentally. Again, from thermodynamic considerations, one would expect the surface tension to decrease in such circumstances and, indeed, this is found experimentally. For example, the LE-LC transition in the case of 1.0 M NaCl is clearly found at higher surface pressures than for 0.1 M NaCl (cf. isotherms of figs. 3 and 1).

Besides the structural reorganization of the polar head referred to so far, our fluorescence measurements also point to a structural reorganization of the aliphatic chains of the lipid molecules. Fig. 4 clearly shows that the dimerization constant of the probe reaches a minimum at precisely the onset of the pure LC phase under the various experimental conditions, i.e., at 64 Å^2/molecule using 1.0 M NaCl (curve a), 58 Å^2/molecule using 0.1 M NaCl (curve b) and 52 Å^2/molecule for successive additions of lipids using 0.1 M NaCl (curve c). In fact, according to the dimerization laws in homogeneous media, one would expect a direct relationship between the dimerization constant K_d and the diffusion coefficient. In contrast, one observes that at the onset of the LC phase (i.e., where D_{lat} starts to decrease), K_d begins increasing. In fact, this behaviour of K_d reflects the fact that the microdomains of the probe molecules are sensitive to the structure of the surrounding medium. In this case, since the fluorescence intensity does not depend upon the degree of compression of the film for all experimental conditions used, the changes of K_d are strictly parallel to those of the structural dimerization constant, K_{ds} [19]. In the fluid phase, K_d decreases until it reaches a minimum at the onset of the pure LC phase, reflecting the disorganization of the monolayer medium throughout the LE-LC transition, the film being there biphasic in nature. From the onset of the pure LC phase and for lower molecular areas, the microdomains are confined within a more rigid environment, the aliphatic chains in this case being mostly in an all-*trans* configuration instead of being more disordered (*gauche* conformers) as in the LE phase [43]. This more rigid environment would favour the occurrence of the dimerization reaction, the conformation of the anthroyl rings being in a configuration allowing them to dimerize with higher efficiency.

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