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ORIGINAL CONTRIBUTION

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Relationships between miscibility behavior and chemical structure of phospholipids in pseudobinary systems

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Abstract The influence of chain length differences of cephalines and the influence of the head group methylation on the miscibility behavior of N-methylated phosphatidylethanolamine (PE) mixtures in aqueous dispersions were tested. Nine different phase diagrams were studied by means of differential scanning calorimetry. The phase diagrams of the five pseudobinary cephaline/cephaline/water systems (fatty acid chain length: C_n, n = 12–18) showed that in the high temperature L_α phase all the homologous cephalines were completely miscible. In the low-temperature phase a distinct succession of the phase diagram types was observed according to increasing chain length differences of the PEs: complete miscibilty (C_{12}/C_{14}; C_{14}/C_{16}), peritectic mixing behavior (C_{12}/C_{16}; C_{14}/C_{18}), eutectic mixing behavior (C_{12}/C_{18}). Furthermore four phase diagrams of pseudobinary phospholipid systems consisting of N-methylated PEs with different numbers of methyl groups and a constant length of fatty acid chains were investigated and compared. These four phase diagrams showed phase separations in the low-temperatures phase (gel phase). The width and the concentration range of the miscibility gaps changed systematically with increasing degree of methylation of the head group of the mixing components and are connected with different possibilities of PEs to form hydrogen bridges between the mixture components.

Key words Phase diagrams · Pseudobinary systems · Miscibility · Calorimetry · Phospholipids

Introduction

The relationship between structure and function of biomembranes has generally become a subject of interest from a biophysical and biotechnological point of view [1, 2]. The physical and functional properties of biomembranes depend essentially on the arrangement and the distribution of the membrane components within the bilayer [3, 4]. In particular, variations in the chemical structures of the lipids and the phospholipids, and also in the mixing state in the bilayer, are of fundamental importance to biomembrane functions.

A number of analytical tests [3, 4] have shown that biomembranes may differ considerably in their phospholipid composition. The essential substance classes of phospholipids are lecithins and cephalines, which differ in their head groups. In biomembranes, fatty acids of chain length C_n, n = 8–22 (n = number of carbon atoms per chain), with up to four double bonds have been found. Moreover, the chains of the fatty acids may be branched [5].

Within this biological background, the mixing behavior of phospholipids in the so-called water-saturated two-phase region [6] is of special interest. The goal of this paper is to systematize the mixing behavior of phospholipids, especially the homologous lecithins and cephalines, in order to establish a relationship between the chemical structure of the phospholipids and their miscibility behavior.

The aim of the first part is the analysis of the miscibility properties of five pseudobinary systems of homologous cephalines by means of differential scanning calorimetry (DSC). We intended to test the influence of chain length differences on their phase diagrams.

The second part is concerned with investigations of the mixing behavior of aqueous N-methylated phosphatidylethanolamine (PE) dispersions with constant length in the fatty acid group on the mixing properties of phospholipids in the water-saturated concentration range.

Experimental

Methods

Calorimetric measurements were analyzed in the plot of solidus and liquidus curves of the quasi-binary phase diagram referring to the phase-transition low-temperature phase (gel phase) and the high-temperature phase (L_α phase, L = lamellar, α = chains fluid).

The calorimetric measurements were performed with mixed phospholipid mixtures (dispersions) using a DSC 121 from Setaram (France). Samples were heated and cooled at different scanning rates. Onset temperatures were taken for sharp transition peaks as the point of intersection between the tangent to the leading edge of the endotherm or exotherm and the approximately extrapolated baseline.

For broad transitions the point of baseline departure was used. The temperatures were corrected for the finite widths of the transitions of the pure components and extrapolated to a theoretical scanning rate of zero [7]. The corrected onset temperatures, obtained from heating scans, were used for the solidus curves, and those from cooling scans were used for the liquidus curves.

Materials

The L-cephalines (PEs), di-(C_n:0)-PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine and lecithins di-(C_n:0)-phosphatidylcholine (PC) were obtained from Fluka (Buch, Switzerland) and were used without further purification. Weighed amounts of the corresponding components were dissolved in a chloroform/methanol mixture (85:15 v/v). The solvent was removed by rotary evaporation and the mixtures were dried over P_2O_5 in a vacuum (1.33×10^{-2} Pa) at 351 K for 2 h above the main transition. The dried samples were mixed with 50 wt% distilled water, deposited in DSC pans and annealed for 2 h above the main transition temperature of the higher-melting-temperature component.

Results and discussion

Influence of chain length differences on the mixing behavior of cephalines in aqueous dispersions

Phase diagrams of the di-(C_{12}:0)-PE/di-(C_{14}:0)-PE/H_2O and di-(C_{14}:0)-PE/di-(C_{16}:0)-PE/H_2O *pseudobinary systems*

The chain lengths of the components differ in two CH_2 units per chain in both systems. The DSC curves are characterized by homogeneous peaks. The phase diagrams obtained from the heating and the cooling curves are plotted in Figs. 1 and 2. Both systems were miscible in the high-temperature and the low-temperature phases. The high- temperature phase corresponds to the L_α phase and the low-temperature phase corresponds to the L_β phase (gel phase).

The dotted lines in Figs. 1 and 2 represent the solidus and liquidus lines in the concentration range between 0 and 10 mol% of the lower-melting-temperature component. The onset temperatures were below the main transition temperature of the lower-melting-temperature component.

Phase diagrams of the pseudobinary systems di-(C_{12}:0)-PE/di-(C_{16}:0)-PE/H_2O and di-(C_{14}:0)-PE/di-(C_{18}:0)-PE/H_2O

The chain length difference of the mixing components is four CH_2 units per chain. The shape of the DSC curves (Fig. 3) depends characteristically on the mole fraction of the mixtures. Near the borders of the phase diagrams we again find homogeneous DSC peaks, but in the middle of the concentration range two peaks appear. The first peak is sharper and a change in mole fraction does not affect its place on the temperature scale. The second peak shifts towards higher temperatures with increasing concentration of the component with the higher main transition temperature.

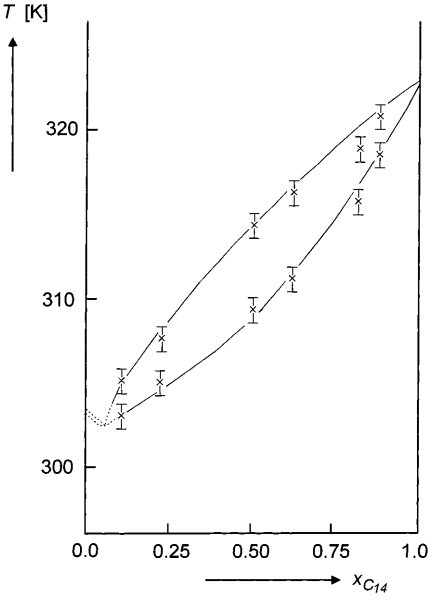

Fig. 1 Phase diagram of the pseudobinary system di-(C_{12}:0)-PE/di-(C_{14}:0)-PE/H_2O (50 wt % water). The mole fraction is related to cephaline di-(C_{14}:0)-PE

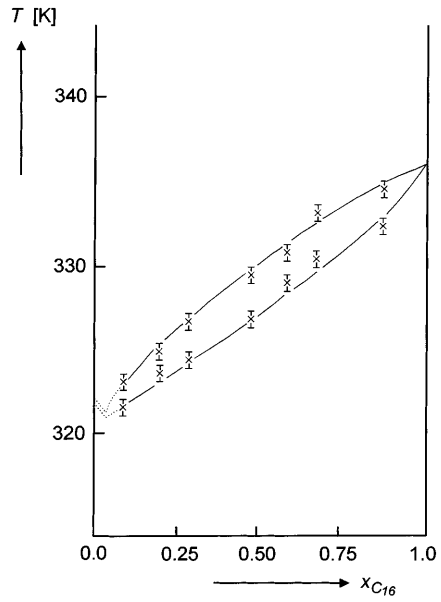

Fig. 2 Phase diagram of the pseudobinary system di-(C_{14}:0)-PE/di-(C_{16}:0)-PE/H_2O (50 wt % water). The mole fraction is related to cephaline di-(C_{16}:0)-PE

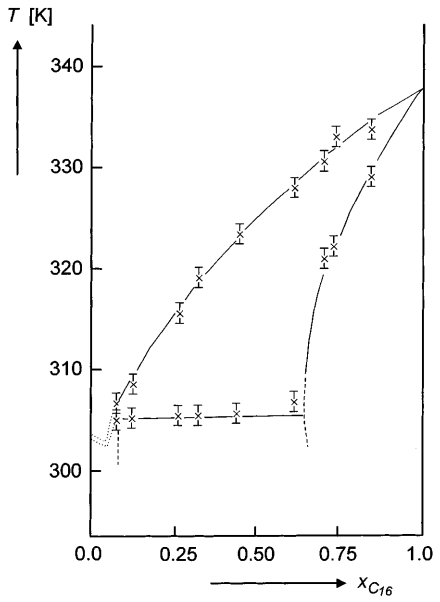

Fig. 4 Phase diagram of the pseudobinary system di-(C_{12}:0)-PE/di-(C_{16}:0)-PE/H_2O (50 wt % water). The mole fraction is related to cephaline di-(C_{16}:0)-PE

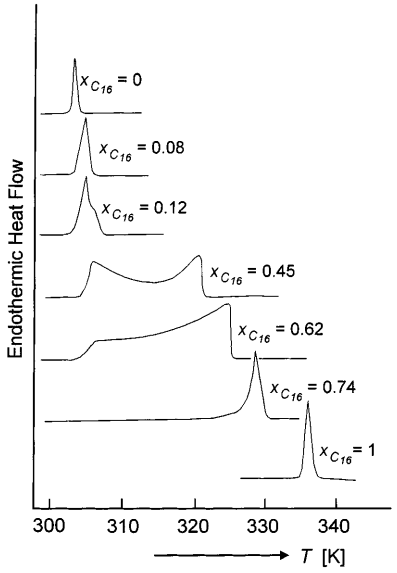

Fig. 3 Selected differential scanning calorimetry (*DSC*) heating scans at a rate of 2 K min^{-1} of the pseudobinary system di-(C_{12}:0)-PE/di-(C_{16}:0)-PE/H_2O (50 wt % water)

Simultaneously the relative areas of the two peaks change. Such DSC curves are typical for the existence of a miscibility gap in the low-temperature phase [10]. The corresponding phase diagrams are shown in Figs. 4 and 5. Within the concentration range of the gel phase miscibility gap, the corrected onset temperatures lie on a horizontal three-phase line which is distinctly placed above the main transition temperature of the components with shorter chains. This indicates a peritectic mixing behavior.

Phase diagrams of the pseudobinary system di-(C_{12}:0)-PE/di-(C_{18}:0)-PE/H_2O

The difference in the chain length of the mixing components is six CH_2 units per chain. The DSC curves show a homogeneous peak in the concentration range between $x_{C_{18}} = 0.1$ and 0.9. These curves are characterized by double peaks similar to the DSC curves in Fig. 3. The three-phase line is clearly situated below the main transition temperature of the di-(C_{12}:0)-PE/di-(C_{18}:0)-PE/H_2O system.

Summarizing we can conclude that the phase diagrams of the di-(C_{12}:0)-PE/di-(C_{14}:0)-PE/H_2O, di-(C_{14}:0)-PE/di-(C_{16}:0)-PE/H_2O, di-(C_{12}:0)-PE/di-(C_{16}:0)-PE/H_2O, di-(C_{14}:0)-PE/di-(C_{18}:0)-PE/H_2O and di-(C_{12}:0)-PE/di-(C_{18}:0)-PE/H_2O systems show that in the concentration range around 5 mol% of the lower-melting-temperature component, a mixture with a minimum transition temperature is formed. Our interpretation is supported by the fact that in the concentration range between 0 and 10 mol% of the lower-melting-temperature component, the corrected onset temperatures are below the main transition temperature of the lower-melting-temperature component.

The phase diagrams, derived from DSC data, show that in PE/PE mixtures, the increase in chain length differences between the components results in an

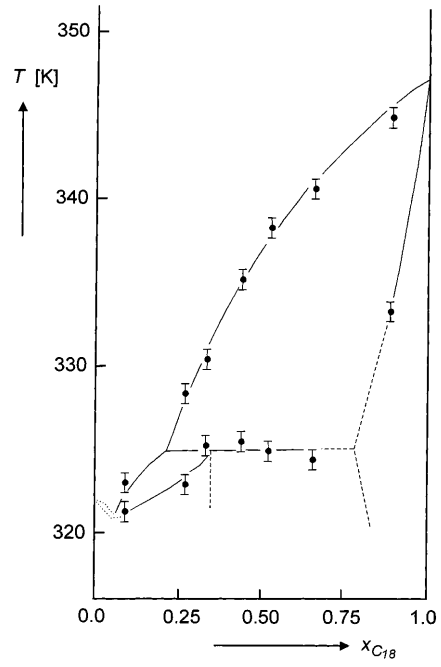

Fig. 5 Phase diagram of the pseudobinary system di-(C_{14}:0)-PE/di-(C_{18}:0)-PE/H_2O (50 wt % water). The mole fraction is related to cephaline di-(C_{18}:0)-PE

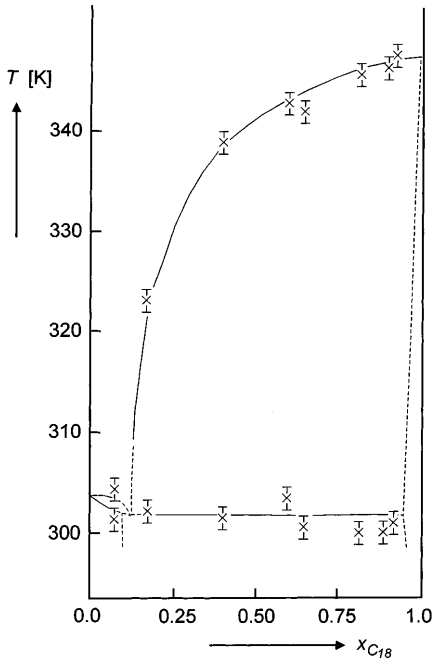

Fig. 6 Phase diagram of the pseudobinary system di-(C_{12}:0)-PE/di-(C_{18}:0)-PE/H_2O (50 wt % water). The mole fraction is related to cephaline di-(C_{18}:0)-PE

azeotropic (Figs. 1, 2) to semiazeotropic (Figs. 4, 5) to eutectic change (Fig. 6). In the liquid-crystalline L_α phase the PEs are miscible over the whole concentration range.

Influence of the head group methylation on the miscibility behavior of N-methylated PE mixtures in aqueous dispersions

Phase diagram of the pseudobinary system di-(C_{16}:0)-MMPE/di-(C_{16}:0)-PC/H_2O

Both mixing components differ by one CH_3 group (M) of the head group. In Fig. 7, the DSC curves show phase transitions which systematically change as a function of the composition of the mixtures. The pretransition peak can only be identified for the binary di-(C_{16}:0)-PC/H_2O system. For the other DSC curves of the pseudobinary mixtures, pretransition peaks of relatively broadened transitions are typical, which, however, cannot be analyzed quantitatively.

The main transition peak of the DSC curves in the concentration range x_{MMPE} = 0.25–0.46 was split; thus, the existence of a miscibility gap is indicated. The other mixtures of composition $x_{MMPE} > 0.46$ or $x_{MMPE} < 0.25$ had only one transition peak. This means complete miscibility of both components in these concentration ranges.

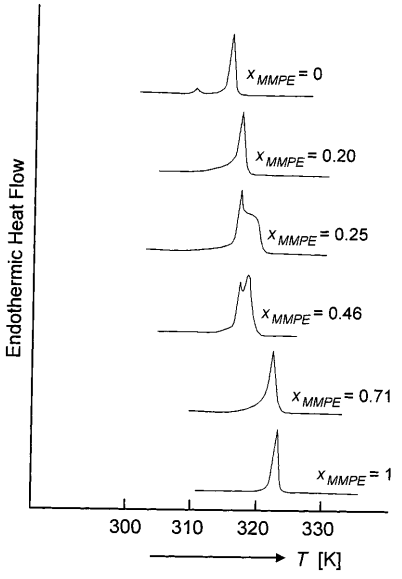

Fig. 7 Selected DSC heating scans of the mixtures of the pseudobinary system di-(C_{16}:0)-MMPE/di-(C_{16}:0)-PC/H_2O (50 wt% water) at a rate of 1.25 K min^{-1}. The mole fraction is related to di-(C_{16}:0)-MMPE

The DSC curves are shown as a binary phase diagram in Fig. 8. The system shows peritectic melting behavior. Taking into account the error intervals of the calorimetric measurements, the solidus line runs horizontally in the region of the miscibility gap. The miscibility gap

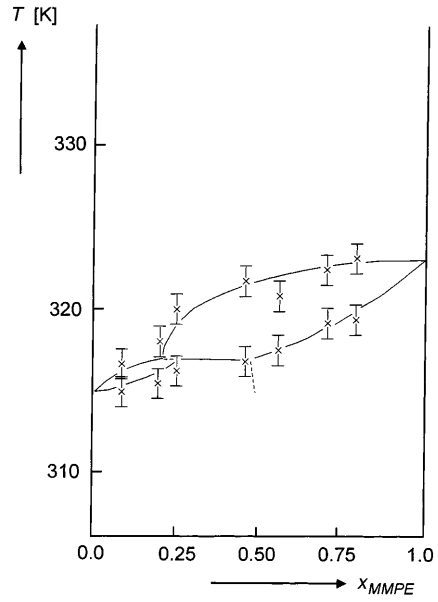

Fig. 8 Phase diagram of the pseudobinary system di-(C_{16}:0)-MMPE/di-(C_{16}:0)-PC/H_2O (50 wt% water). The mole fraction is related to di-(C_{16}:0)-MMPE

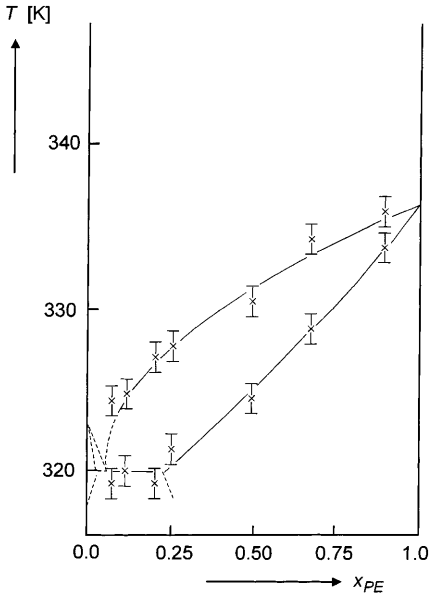

Fig. 9 Phase diagram of the pseudobinary system di-(C_{16}:0)-PE/di-(C_{16}:0)-MMPE/H_2O (50 wt% water). The mole fraction is related to di-(C_{16}:0)-PE. Hypothetical phase regions (- - - - - - -)

is fixed in the concentration range of about x_{MMPE} = 0.25–0.50.

Phase diagram of the pseudobinary system di-(C_{16}:0)-PE/di-(C_{16}:0)-MMPE/H_2O

Both components differ by two CH_3 groups of the head group. The DSC curves in the concentration range x_{PE} = 0.12–0.21 show double peaks, which indicate phase separation in the low-temperature phase, similar to the DSC curves in Fig. 3. The DSC curves of the other mixtures form only a comparatively wide transition peak, from which the complete miscibility of both components in this concentration range is inferred.

By evaluating all the DSC curves, a phase diagram with eutectic melting behaviour results (Fig. 9). In the range where double peaks were registered, the solidus line runs horizontally. This means that a miscibility gap is present in this concentration range of the phase diagram in the low-temperature-phase.

The investigations of the mixtures in the concentration range of $x_{PE} < 0.12$ did not result in quantitative findings, due to the insensitiveness of the DSC method. The definite width of the miscibility gap on the side of the lower-melting-temperature component as well as the exact position of the eutectic point could not be fixed unequivocally; however, in this part of the phase diagram (dashed lines in Fig. 9), a small miscibility range exists.

Phase diagrams of the pseudobinary systems di-(C_{16}:0)-PC/di-(C_{16}:0)-PE/H_2O and di-(C_{16}:0)-PC/di-(C_{16}:0)-MPE/H_2O

The phase diagrams of both systems are summarized in Figs. 10 and 11. The solidus curve and the liquidus curves show that in the high-temperature phase both components are completely miscible, but in the low-temperature phase a miscibility gap occurs in both systems. Where the di-(C_{16}:0)-PC/di-(C_{16}:0)-MPE/H_2O system is concerned, the mixing and phase-separation ranges in the low-temperature phase were identified by the ripple structure of the P_{β}' phase by means of electron microscopy [8, 9].

We are able to summarize the phase diagrams as follows: It definitely follows from the summarizing graphic representation in Fig. 12 that the width and the concentration ranges of the miscibility gaps in mixed systems containing dipalmitoylphosphatidylcholine or dipalmitoylphosphatidylethanolamine and the N-methylated PEs change systematically. With increasing degree of methylation of the head group, the width of the miscibilty gap of the solidus line in the phase diagram increases. In addition, it is obvious from Fig. 12 that on the left side of the graphic representations, a wide mixing range always occurs on the more protonated component, for example, on the side of the PE or of the N-methylated compounds in the phase diagram.

The pseudobinary systems di-(C_{16}:0)-PC/di-(C_{16}:0)-PE/H_2O and di-(C_{16}:0)-PC/di-(C_{16}:0)-MPE/H_2O exhibit the behavior described previously. The phase separation occurs on the side of the PC, i.e. of the less protonated

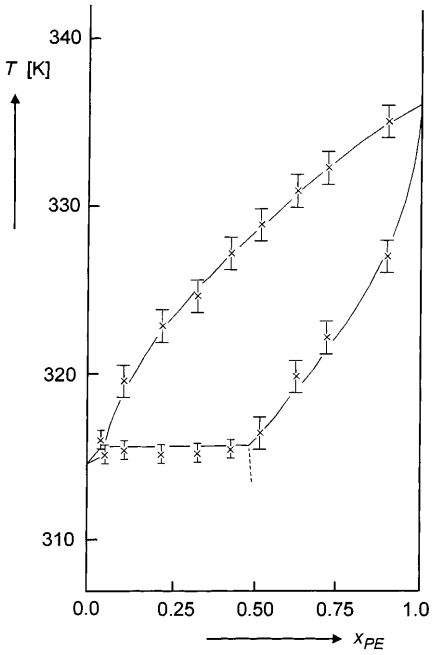

Fig. 10 Phase diagram of the pseudobinary system di-(C_{16}:0)-PC/di-(C_{16}:0)-PE/H_2O (50 wt% water). The mole fraction is related to di-(C_{16}:0)-PE

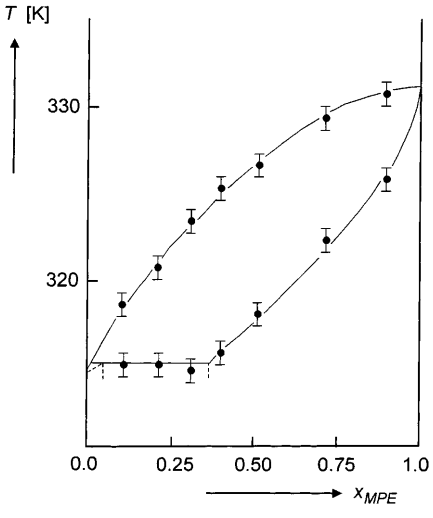

Fig. 11 Phase diagram of the pseudobinary system di-(C_{16}:0)-PC/di-(C_{16}:0)-MPE/H_2O (50 wt% water). The mole fraction is related to di-(C_{16}:0)-MPE

component. With increasing degree of methylation of the second mixing component, the width of the miscibility gap decreases. The only exception to this behavior is the di-(C_{16}:0)-PC/di-(C_{16}:0)-MPE/H_2O system. In this system, the comparatively wide mixture range is situated on the less-protonated-component side of the phase diagram. Hence, it can be concluded that the head group interaction in the PC/water systems is of the same

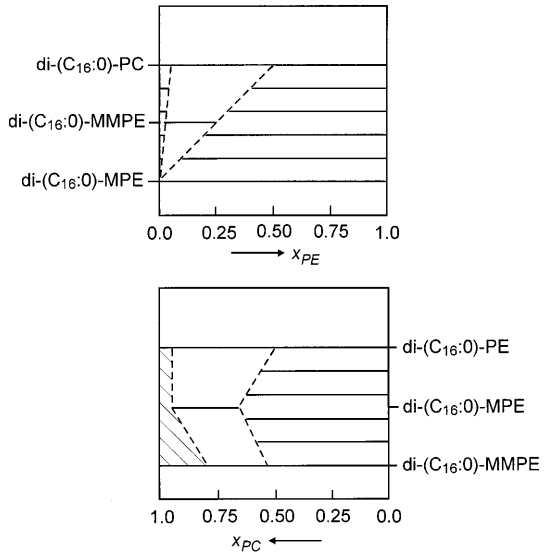

Fig. 12 Graphic representation of dependence of the concentration regions, x (x = mole fraction), of the mixing gaps on the degree of methylation of the head group. Region of mixing gaps (- - - - -); regions of complete miscibility of the phospholipids in which the L_β phase exists (——) mixing regions in which the L_β' phase or the P_β' phase exists ■

magnitude as that of the head group interaction between the dimethylphosphatidylethanolamine groups.

Consequently, the substitution of the second proton on the nitrogen atom by the CH_3 group results in a strong decrease in the head group interaction between the mixtures of the PE molecules. With respect to the head-group interaction, di-(C_{16}:0)-MMPE shows similar mixing properties to di-(C_{16}:0)-PC. Hence, it can be concluded that two "structural protons" are of decisive importance for the interaction between the head groups of the PEs. This finding is substantiated by stereochemical considerations of the head-group interactions, which were proposed by Elder et al. [10]. From the steric arrangement of the PE molecules in a "monolayer" of the lamella, it follows that each molecule can only form two hydrogen bridges each over the nitrogen atom of the head group to the adjacent molecule of the same layer. The third proton juts out of the level. Consequently, the substitution of the hydrogen atoms on the nitrogen atom by CH_3 groups reduces the possibility to form hydrogen bridges.

As already discussed in Refs. [8, 9], the PE/water systems form a L_β phase and the nonbranched PC/water systems form a L_β' phase or a P_β' phase. It was proved by Mulukutla and Shipley [11] by means of X-ray diffraction investigations that the di-(C_{16}:0)-MMPE/H_2O system and the di-(C_{16}:0)-MPE/H_2O system form a L_β' phase. This structure was also found for the di-(C_{16}:0)-PE/H_2O system. Thus, it can be concluded that in the mixtures of the systems di-(C_{16}:0)-PC/di-(C_{16}:0)-PE/H_2O, di-(C_{16}:0)-PC/di-(C_{16}:0)-MMPE/H_2O and di-

(C_{16}:0)-PC/di-(C_{16}:0)-MPE/H_2O a mixture with such components is always selected, including a low-temperature phase in which the chains of the phospholipids are tilted or oriented vertically. To what extent it is possible that under these mixture conditions one component is able to "insert" or "incorporate" the other component in its own structure without phase separation or to form another structure is to be seen as an interesting aspect of the miscibility behavior of these pseudobinary phospholipid systems. Some new results have already been discussed by Laggner and coworkers [12, 13].

Comparing the position of the miscibility gaps in the phase diagram reveals that the L_β phase of the PE is considerably "better" able to take in the "strange component PC" to form a homogeneous mixing phase than vice versa.

With respect to two pseudobinary systems di-(C_{16}:0)-MPE/di-(C_{16}:0)-PE/H_2O and di-(C_{16}:0)-MMPE/di-(C_{16}:0)-PE/H_2O, both mixture components form the L_β phase in aqueous dispersion. Consequently, an important reason for the miscibility gap in the di-(C_{16}:0)-MMPE/di-(C_{16}:0)-PE/H_2O system is the different ability of mixture components to form hydrogen bridges and not in the differences in the macroscopic phase structure of both phospholipid/water systems.

The effect of lipid head groups on the structural and phase properties of bilayers is also discussed by Cevc [14, 15]. Phosphate derivatization or protonation, further changes that decrease the lipid capacity for hydrogen bonding and that lower the interfacial affinity for binding water, as well as any system alteration that diminishes the amount of interfacial water can all cause the lipid chain melting-phase-transition temperature, the persistence of the lamellar lipid phase and the colloidal stability of vesicle suspensions to change. Head group protonation, which results in partial lipid dehydration, also affects other bilayer properties, such as the rate of lipid vesicle association.

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