Journal of Thermal Analysis and Calorimetry, Vol. 82 (2005) 457–462

EFFECT OF SULFADIAZINE ON BIOLOGICAL MODEL MEMBRANES

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The effect of sulfadiazine on dipalmitoylphosphatidylethanolamin-dipalmitoylphosphatidylglycerol-water (DPPE-DPPG/water, 20 mass/mass%, with 0.2 DPPG/DPPE+DPPG molar ratio) vesicles considered as a model system of the cytoplasmic bacterial membranes was studied using DSC and freeze-fracture methods. The sulfadiazine/lipid molar ratio was varied from 10^{-3} up to 1. It was found that the DPPE-DPPG/water system is drastically affected by the sulfadiazine, but there is no concentration effect in a wide range of sulfadiazine/lipid molar ratios from 10^{-2} up to $2 \cdot 10^{-1}$. The DSC and freeze-fracture methods reveal that a homogeneous incorporation of the sulfadiazine molecules occurs in the liquid crystalline phase while in the gel phase separation appears. The different local structures can be classified into two different types: vesicle-like and block-type. Although the surface morphology of the domains of both types shows lamellar arrangement, the blocks are constituted from closely packed long units.

Keywords: *DPPE-DPPG vesicles, DSC, freeze-fracture, phase separation, sulfadiazine*

Introduction

Antibiotics are used in the treatment and prevention of microbial diseases, not only as human and veterinary medicines but some of them also as feed additives [1, 2]. These substances affect the microbes of the host organisms and passing through the target organism they cause environmental risks [3]. Their harmful effects can appear in all organisms present in the environment. Among the antibiotics, sulfadiazine is one of those most frequently used for treating domestic animals and farmed fish.

When antibiotics come into contact with the cell walls and cell membranes, even small changes in the membrane composition can influence membrane properties [4]. In spite of the fact that the biological membranes do not consist of a very large number of kinds of chemical components, they are rather complex systems. Therefore, the elucidation of the effect of guest molecules on their behaviour still remains rather ambiguous [5–7]. That is why vesicles (liposomes) as model membranes are studied in the first approximation to obtain information about the effect of different foreign molecules generally, and the membrane-active antibiotics on membrane properties [8–11].

Dipalmitoylphosphatidylethanolamine (DPPE) is the main lipid component of the bacterial membranes therefore this phospholipid is generally used to mimic the structural and thermotropic behaviour of the biological membranes. In our work, dipalmitoylphosphatidylglycerol (DPPG) was added to DPPE in 0.2 molar lipid ratio (DPPG/DPPE+DPPG) to approach the phospholipid composition of the cytoplasmic membranes. The thermotropic behaviour of the DPPE-DPPG/water system was thoroughly studied [12, 13]. If the lipid mixture contains DPPG in less than 0.1 lipid molar ratios the system is not homogeneous and two types of domains exist. One type mostly consists of DPPE molecules, while the other type has both lipid molecules. According to this phase separation, a complex phase transition behaviour appears. The domains rich in DPPE exhibit a slightly higher transition temperature than those consisting of both lipid molecules. In this study we show that sulfadiazine (SD) significantly influences the DPPE-DPPG vesicle system. Besides calorimetrical investigations freeze-fracture technique proved to be a valuable method of revealing characteristic changes in the system [14]. Especially the latter method always showed the complex morphological changes of the multilamellar systems indicating a high variety of the SD-induced structural formations.

Experimental

Materials and methods

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phospho-ethanol-amine (DPPE, purity>99%) and 1,2-dipalmitoyl--*sn*-glycero-3-[phospho-rac-1-glycerol] (DPPG, Na salt, purity>99%) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Ala, USA). The SD (benzene-

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1388–6150/$20.00

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sulfonamide, 4-amino-N-2-pyrimidinyl, purity>99%) was obtained from Sigma-Aldrich (Steinheim, Germany). Substances were used without further purification.

Appropriate amounts of the lipids (DPPE and DPPG) were mixed and dissolved in chloroform containing 30 vol% methanol, then the solution was evaporated at 70°C. The resulting lipid film was kept in vacuum to remove the residual traces of the solvent. Sodium phosphate buffer prepared from triple-quartz-distilled water (10 mM, pH 7.4) was added to the dried lipid and the mixture of the dried lipid and the appropriate amount of SD powder (see below) to yield a lipid concentration of 20 mass/mass%. The sample was heated above the phase transition (65°C) for 15 min, then cooled to 5°C, reheated once again up to 65°C and vortexed intensively. This treatment was repeated 20 times to achieve homogeneous dispersions. The concentration of SD was varied in a wide range: the molar ratio of SD relative to the lipids were (SD/lipids): 10^{-3}, 10^{-2}, $2\cdot10^{-2}$, 10^{-1}, $2\cdot10^{-1}$ and 1.

The DSC measurements were performed with a DSC 2920 instrument (TA Instrument, US) operated at a heating rate of 1°C min^{-1} in the temperature range of 25 to 70°C. The DSC curves were recorded in the heating direction. The reference pan was empty. The calibration of the calorimeter was carried out by using a pure indium sample (T_{onset}=156.6°C). The transition points were characterized by the temperature at which the heat flow curve exhibited a minimal value.

For freeze-fracture the sample and also the gold specimen holders were incubated at the respective temperatures. Droplets of 1–2 μL of the sample were pipetted onto gold holders (also incubated at the desired temperatures) and frozen by plunging them immediately into partially solidified Freon for 20 s and stored in liquid nitrogen. Fracturing was performed at –100°C in a Balzers freeze-fracture device (Balzers AG, Vaduz, Liechtenstein). The replicas of the fractured faces etched at –110°C were made by platinum-carbon shadowing then cleaned with a water solution of surfactant and washed with distilled water. The replicas were placed on 200 mesh copper grids and examined in a JEOL JEM-100 CX II electron microscope (Japan).

Results and discussion

Differential scanning calorimetry

The (DPPE-DPPG)/water system is drastically influenced by SD, as can be seen in Fig. 1. The 'pure' lipid (DPPE-DPPG/water) system exhibits a single broad endothermic peak around 60°C as a consequence of the phase transition between the gel and liquid crystalline phases. By adding SD to DPPE-DPPG two

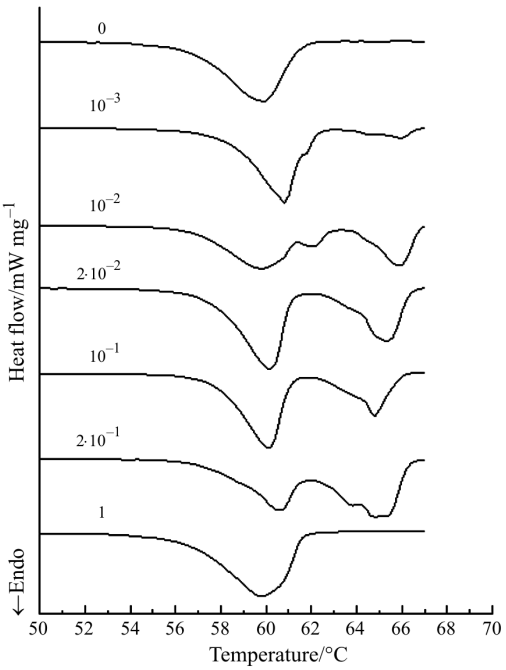

Fig. 1 DSC curves of the DPPE-DPPG/water systems having different SD/lipid ratios marked on each curve

phase transitions can be clearly observed in a wide concentration range of SD, whereby the enthalpy of these transitions as well as the occurrence of additional less resolved peaks varied with the amount of SD. At the smallest investigated sulfadiazine concentration (at 10^{-3} SD/lipids molar ratio) the profile of the first complex peak is modified as the small peak (located at about 61.7°C) appears in its right-hand side. Moreover, a second endothermic peak is detectable about at 65°C. The change in enthalpy of this latter is low, but its appearance was always reproducible in all measurements. By increasing the SD/lipid molar ratio up to 10^{-2} the changes in the DSC signal observed at 10^{-3} SD/lipid ratio are more clearly expressed; the small shoulder on the first peak became higher and the second peak (positioned at about 66°C) more significant. At $2\cdot10^{-2}$, 10^{-1} and $2\cdot10^{-1}$ SD/lipid molar ratios two characteristic changes were observed: the shoulder on the first peak disappeared and the second peak became more intensive relative to the first one. At $2\cdot10^{-1}$ SD/lipid molar ratio the two intensive peaks began to overlap. At 1 SD/lipid only a single peak appeared. Nevertheless, the two complex transition peaks and at the 1 SD/lipid ratio the single one can be considered as the sums of several peaks. It must be mentioned that the samples obtained after three independent preparation processes did not show exactly the same DSC characteristics. There are some small alterations in the characteristic temperatures and in the enthalpies, but they exhibit the same tendency as described above and presented in Fig. 1.

Table 1 The characteristic values of the DSC curves of the DPPE-DPPG/water systems with sulfadiazine (the sulfadiazine/lipid ratios are given)

SD/lipid	T_1/°C	ΔH_1/kJ mol^{-1}	T_2/°C±0.5	ΔH_2/kJ mol^{-1}	ΔH/kJ mol^{-1}
0	60.3±0.2	41.1	–	–	41.1
10^{-3}	60.8±0.5	36.6	–	–	36.6
10^{-2}	60.0±0.5	28.1	66.0	12.8	39.3
$2\cdot10^{-2}$	60.3±0.5	30.0	65.7	20.3	47.2
10^{-1}	60.2±0.5	28.4	64.9	16.9	45.2
$2\cdot10^{-1}$	60.7±0.5	27.8	65.2	24.6	49.1
1	59.8±0.5	26.1	–	–	26.1

In Table 1 the characteristic values of the DSC curves are summarized. The temperatures and the changes in enthalpy of each complex transition are given. Surprisingly, the characteristic temperatures do not tend to change with increasing SD concentration. Only the sum of the changes in enthalpy of the two consecutive melting processes increases up to $2\cdot10^{-1}$ SD/lipid ratio and decreases drastically at the investigated highest SD/lipid ratio.

Freeze-fracture morphologies

The gel and liquid crystalline phases of the fully hydrated DPPE-DPPG/water vesicles are multilamellar systems but they do not exhibit regular spherical forms as can be observed in the electron micrographs taken on the freeze-fractured surfaces. The pictures of both phases in Figs 2 and 3 show substantial similarities. They consist of stacks with closely and regularly packed layers embedded in nearly spherical giant liposomes. A number of the liposomes are deformed. Their outer crumpled layers reach into the spaces between the surrounding liposomes. In the gel phase (at 50°C) the size of the liposomes is extremely heterodispersive and all kinds of structural defects appear as can be observed in Figs 2a and b. In the liquid crystalline state (Figs 3a and b) the spherical form is less typical; there are layered domains with peculiar and in many cases with polygon prints on their surfaces as shown in Fig. 3b. These pictures suggest that

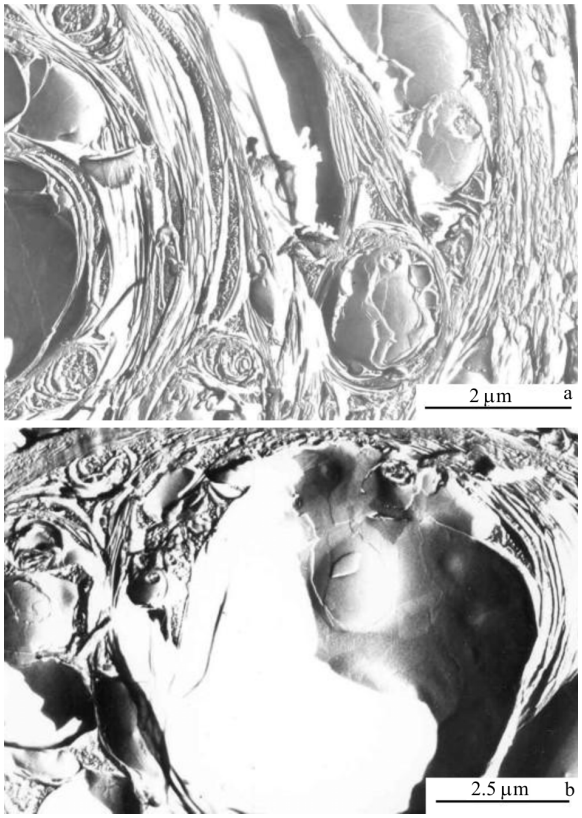

Fig. 2 Surface morphologies of the DPPE-DPPG/water vesicles in the gel phase at 50°C

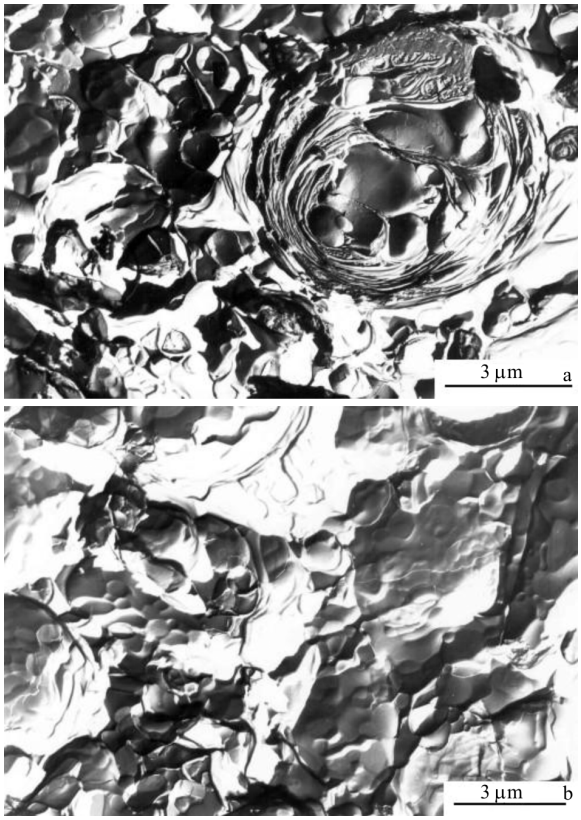

Fig. 3 Surface morphologies of the DPPE-DPPG/water vesicles in the liquid crystalline phase at 62°C

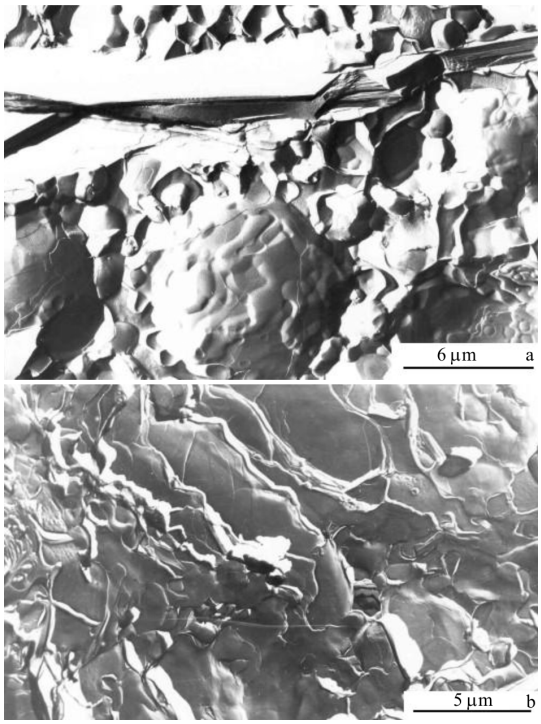

Fig. 4 Surface morphologies of the DPPE-DPPG/water system having SD in 10^{-2} SD/lipid molar ratio; a – with blocks embedded between the vesicles and b – destroyed outer layers of giant vesicles. The system was quenched from 50°C

the broadened shape of the small angle X-ray scattering patterns of DPPE-DPPG liposomes presented in the literature [12] is a consequence of the destruction and the loss of correlation between the layers.

By adding SD to the system, significant morphological changes can be observed in the electron micrographs. At 10^{-2} SD/lipid molar ratio, blocks consisting of densely packed layers appear, which exhibit entirely smooth surfaces and their fractured forms show sharp edges as can be observed in Fig. 4a. Beside the blocks the vesicles are still present. However, large and irregular layered forms with crumpled surfaces are also present as shown in Fig. 4b. Even at 10^{-1} SD/lipid ratio both types of morphological shapes can be observed; the blocks and the irregular vesicles with heterodispersive size distribution as demonstrated in Figs 5a and b, respectively. In fact, these shapes remain characteristic in the whole investigated SD/lipid ratio range.

In the transition range the morphological changes were investigated more closely in order to get information on the structural background of the two major transitions, which are best resolved at $2 \cdot 10^{-1}$ SD/lipid molar ratio. In the low temperature domain investigated at 50°C, the system also exhibits the main surface features described above. There are blocks and heterodispersive vesicles with well-expressed polygon

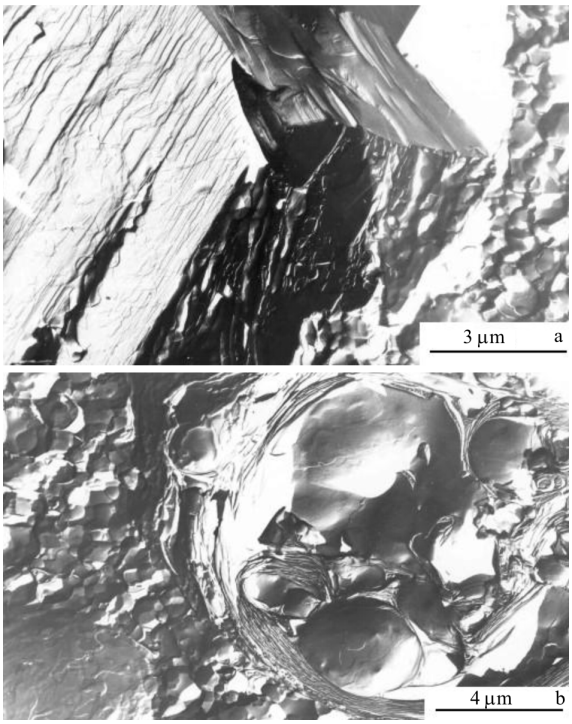

Fig. 5 Surface morphologies of the DPPE-DPPG/water system having SD in 10^{-1} SD/lipid molar ratio; a – with blocks extending between the vesicles and b – heterogeneous vesicles. The system was quenched from 50°C

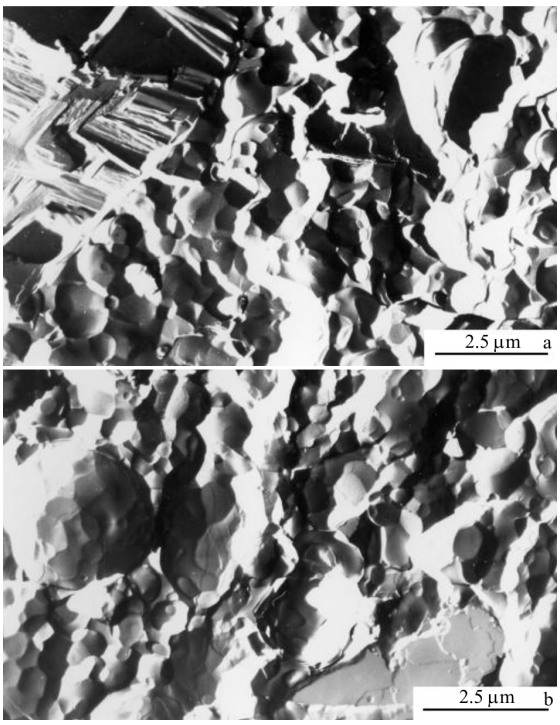

Fig. 6 Surface morphologies of the DPPE-DPPG/water system having SD in $2 \cdot 10^{-1}$ SD/lipid molar ratio; a – with blocks extending between the vesicles and b – heterogeneous vesicles. The vesicles with 'polygon' surface patterns appear. The system was quenched from 30°C

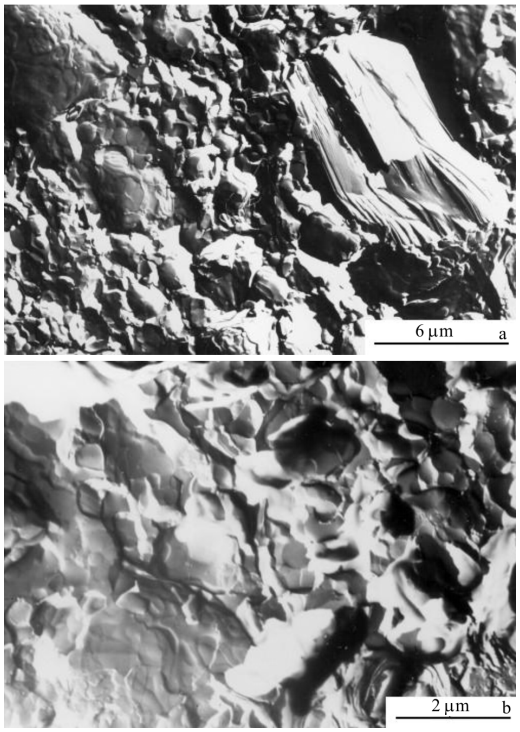

Fig. 7 Surface morphologies of the DPPE-DPPG/water system having SD in $2 \cdot 10^{-1}$ SD/lipid molar ratio; a – with blocks extending between vesicles and b – vesicles. The surface pattern of the vesicles have disappeared. The system was quenched from 62°C

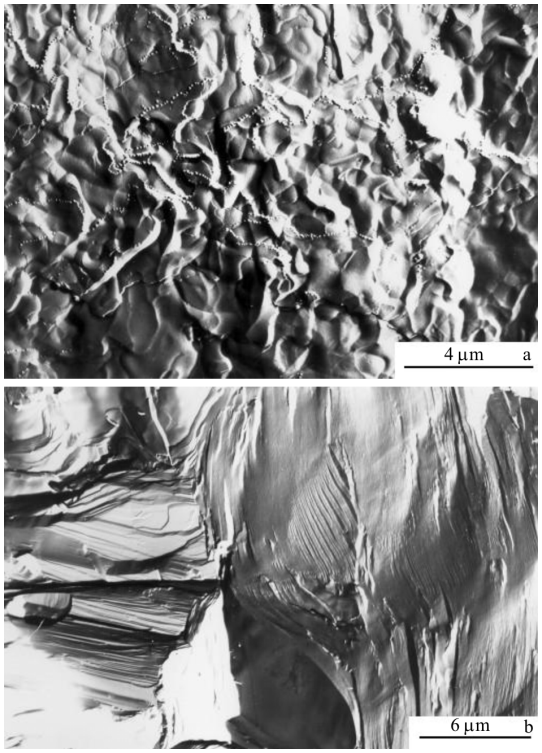

Fig. 8 Surface morphologies of the DPPE-DPPG/water system having SD in $2 \cdot 10^{-1}$ SD/lipid molar ratio; a – with crumpled layers and b – blocks. The system was quenched from 70°C

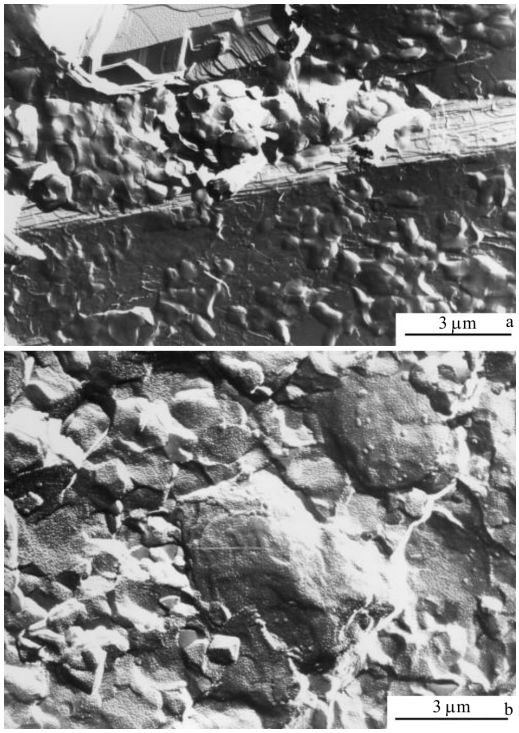

Fig. 9 Surface morphologies of the DPPE-DPPG/water system having SD in 1 SD/lipid molar ratio; a – with dominant blocks and b – vesicles. The system was quenched from 30°C

forms in their outer surfaces as shown in Figs 6a and b. At 62°C, after the first phase transition there is only one significant change in the fractured surfaces. The polygonal patterns disappear from the surfaces of the heterodispersive vesicle, but the blocks with their sharp edges remain as can be observed in Figs 7a and b. By increasing the temperature up to 70°C (above the second transition), the vesicles as typical structural units disappear (Fig. 8a), while giant blocks still exist (Fig. 8b). The single phase transition (the DSC signal centred at 60°C) of the system having 1 SD/lipid ratio is accompanied by only one characteristic change in the freeze-fractured pictures. The surface features of two states are presented in Figs 9 and 10. At 30°C, the blocks with edges and the heterodispersive spherical vesicles with smooth surface are visible (Figs 9a and b). These are exactly the main characteristics which were observed in all SD/lipid systems in the low temperature domains. Heating up this system to 70°C, there is a substantial change; namely, the vesicles no longer exist. The layered stacks exhibit crumpled (Fig. 10a) or smooth (Fig. 10b) surfaces. The latter local formations show high surface similarity with the blocks observed in the lower temperature domain.

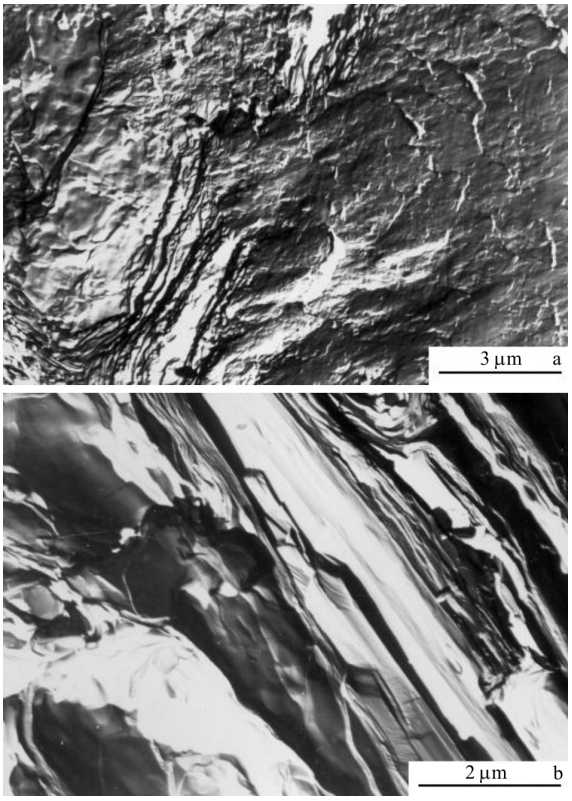

Fig. 10 Surface morphologies of the DPPE-DPPG/water system having SD in 1 SD/lipid molar rati;o a – with crumpled and b – with smooth layers. The system was quenched from 70°C

Conclusions

The complex structure of the DPPE-DPPG/water vesicles is drastically perturbed in the presence of SD molecules. The complex shapes of the DSC peaks indicate, and the freeze-fracture reveal unambiguously, that different macroscopic structures exist. The morphologies can be classified into two different types; one type is vesicle-like and the other one consists of blocks with layered arrangement. These two types are present in the whole investigated wide temperature domain (30–70°C) in the system which was a representative one for the two major transitions observed by DSC. Therefore a direct relation between the two characteristic morphologies and the two phase transitions can not be supposed. Presumably, beside the SD molecules the lipid components are located also inhomogeneously and the two DSC signals may be a consequence of the latter inhomogeneity.

Acknowledgements

This work was supported by the Hungarian Scientific Funds OTKA (Bota, T 43055) and a bilateral German–Hungarian Research Project. We thank Mrs. E. Tóth for the technical assistance at the DSC measurements and Mrs. T. Kiss for the technical assistance at the freeze-fracture apparatus.

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DOI: 10.1007/s10973-005-7247-x