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Molecular volumes of DOPC and DOPS in mixed bilayers of multilamellar vesicles

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The mixtures of 1,2-dioleoyl-*sn*-*glycero*-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-*glycero*-3-phospho-L-serine (DOPS) in bilayers of multilamellar vesicles were studied by method of densitometry. In the range of DOPS molar fraction 0–100%, specific volumes of mixtures of lipids coincide with theoretical values in the case of ideal mixing of lipids. The coefficient of thermal volume expansivity was evaluated for different DOPS molar fractions; it has values in the range (71.1–73.6) $\times$ 10^{-5} K^{-1}. Molecular volumes for pure DOPC and DOPS were evaluated for temperature range 15–45 °C. At 30 °C, molecular volumes are 1304 Å^3 and 1254 Å^3 for DOPC and DOPS, respectively. The estimated volume of head group of DOPS at 30 °C is 275 Å^3. Time-dependent density scans revealed that the dispersion of DOPC vesicle sedimentation during measurements induces an observed increasing density of dispersion in agreement with recently published observations. The presence of charged DOPS in vesicles prevents them from sedimentation and values of density are stable over a prolonged time.

Introduction

All living cells, cell organelles and their compartments are separated from the environment by membranes. Lipids are one of the main components of biological membranes. Lipid composition of biomembranes widely varies in different types of cells and organelles.[1] This composition influences structure and dynamic properties of the membrane[2] and some cellular functions.[3] The main class of lipids composing biomembranes is that of phospholipids. The present paper concerns mixtures of phosphatidylcholine and phosphatidylserine in fluid bilayers. Zwitterionic phosphatidylcholine is the major representative of phospholipids and is frequently used in model membrane studies. Since its molecule has a nearly cylindrical shape, it forms bilayer structures and thus plays a central role in membrane structure. Phosphatidylserine is the most abundant anionic lipid in eukaryotic membranes.[1] It is preferentially found in the inner leaflet of plasma membrane and in endocytic membranes.[2,4] Phosphatidylserine asymmetry in membranes has a connection with apoptosis[5–8] and blood clotting.[9–11]

Mixing of two phospholipids A and B in bilayers can be ideal or non-ideal depending on pairwise A–A, B–B and A–B interaction energies and on the entropy of mixing. Non-ideal mixing provides the physical basis of domain formation and macroscopic phase separation in bilayers. Domain formation can have a large impact on a number of membrane physical properties such as transversal lipid asymmetry, membrane elasticity, lipid lateral diffusion, permeability, and binding properties for peptides and proteins. There are some evidences that mixtures of phosphatidylcholine and phosphatidylserine behave non-ideally. In papers,[12,13] their mixing in fluid bilayers was studied by measuring the binding of aqueous calcium ions. Measured calcium ion concentration was used to derive the activity coefficient for PS, γ_{PS}, in the lipid mixture. For POPS in binary mixtures with either POPC, DMoPC, or DOPC, $\gamma_{PS} > 1$; *i.e.*, mixing was found to be non-ideal, with PS and PC clustered rather than randomly distributed in bilayers, despite the expected electrostatic repulsion between PS head groups. The non-ideality of PC and PS mixing was also observed in SOPC and SOPS monolayers by measuring compressibility (isotherms, bulk modulus, and excess area per molecule) and surface potential.[14] From their analysis, it is clear that for monolayers containing 25 mol% of SOPS, excess area per molecule is positive, and for mixtures containing 75 mol% of SOPS, excess area per molecule is negative. This means that at low concentrations of SOPS, monolayers are in a more expanded state than in the case of ideal mixing, and at high concentration of SOPS, monolayers are in a more compressed state. The non-ideal lateral mixing of POPC and POPS in fluid bilayers was observed also using fluorescent probes.[15] The non-ideal mixing could be caused by acyl chain composition of the PC–PS mixtures studied; in all PCs and PSs described above, one chain was saturated and one was unsaturated. In DOPC/DOPS bilayers at 1 : 1 molar ratio, the formation of microdomains was not observed by atomic force microscopy.[16]

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The specific volume is one of the thermodynamic parameters whose value can indicate the non-ideal volume mixing.[17] For the case of ideal mixing, the specific volume of a two-component lipid system ν_{mix} is obtained as

$$\nu_{mix} = \frac{V_A^m \cdot f_A + V_B^m \cdot (1 - f_A)}{M_A \cdot f_A + M_B \cdot (1 - f_A)}, \quad (1)$$

where V_A^m and V_B^m are the molar volumes of pure lipids A and B, respectively, M_A and M_B their molar masses, and f_A and $f_B = 1 - f_A$ their molar fractions. In the case when lipids mix non-ideally, the value of ν_{mix} deviates from the one calculated by eqn (1). There are examples where non-ideal mixing has been observed by densitometry. Aagaard *et al.*[18] studied the mixing of four alkanes (*c*-hexane, *n*-octane, *n*-decane, *n*-dodecane) and a homologous series of ten alcohols (C3–C12) with DMPC in fluid bilayers. They found that the volume change of transferring these compounds from their pure states into the membrane was positive for short (C4–C6) alkanols, while it was negative for longer alkanols and all alkanes. Small positive excess volume was observed also for fatty acid concentrations below 10% in mixtures of oleic or stearic acids with fluid DMPC bilayers.[19] Deviations from ideal mixing were found for mixed phospholipid–cholesterol bilayers.[20]

In the present work, we study mixtures of DOPC and DOPS in multilamellar vesicles using densitometry. Our aim is not only to check the ideality or non-ideality of their mixing, but also to obtain their molecular volumes. This information is crucially important for the calculation of electron densities and neutron scattering length densities of lipids in bilayers, which are used for the interpretation of diffraction and small angle scattering experiments (see ref. 21 and references therein). The next aim of our work is to check the reliability of densitometric results. Earlier it was shown that the densities of vesicle dispersions (and consequently molecular volumes of lipids) of SMPC, DPPC and DMPC change upon cycling temperature scans measured on a vibrating densitometer.[22] The authors suggested the changing of the packing of the acyl chains between the different cycles. However, Hallinen *et al.*[23] have found that the changing molecular volume of DPPC during repeated thermal cycling is an artifact connected with the non-homogeneous redistribution of multilamellar vesicles within the U-tube of the densitometer during a prolonged measurement. We check this result by measuring vesicles from unsaturated phospholipid DOPC and DOPS at temperatures well above their gel–fluid phase transition temperatures.

Experimental

Sample preparation

Synthetic DOPC and DOPS were purchased from Avanti Polar Lipids (Alabaster, USA). Organic solvents of spectral purity were obtained from Slavus (Bratislava, Slovakia). The stock solution of DOPS was prepared in a methanol–chloroform mixture at 1 : 2 volume ratio. DOPC was weighed in glass vials, and the required amount of DOPS organic solution was added into these vials with DOPC. After mixing, the solvent was evaporated under a stream of gaseous nitrogen, and its traces were removed by oil vacuum pump evacuation. The amounts of lipids in vials were gravimetrically controlled. The freshly prepared MilliQ water (18.2 MΩcm at 25 °C) was added to the samples 1–4 hours before densitometric measurements. The amounts of water added were gravimetrically controlled. The final concentration of lipids was 2.5–3 wt%. The mixtures were homogenized by vortexing and a brief soft sonication (2–5 min) in a K-5LE bath sonicator (Kraintek, Podhájska, Slovakia). After that, the samples were degassed by stirring under a low pressure to prevent bubble formation in the U-tube of the densitometer. Weight loss after degassing was negligible (~0.03 wt%). Selected samples were intentionally not degassed to check results obtained with degassed samples. The accuracy of weight of lipids and water was ±0.0005 g. After densitometric measurements, the samples were stored in a freezer at −20 °C. For checking the stability of densitometric results, these frozen samples were used.

Measurements and analysis

Densitometric measurements were performed on the vibrational densitometer DMA4500M (Anton Paar, Graz, Austria) in the temperature range 15–55 °C. The principle of vibrational densitometry is extensively described by Kratky *et al.*[24] The accuracy of the measured density was ±0.00005 g cm^{-3} and of the temperature was ±0.03 °C. The densitometer calibration was frequently checked by measuring the densities of air and water.

After introducing the sample into the U-tube of the densitometer, temperature equilibration from room temperature to 15 °C took about 5 minutes. In temperature scans, the scan rate was 1 K min^{-1}. The specific volume of lipid was calculated as:

$$\nu_L = \frac{\nu_s - (1 - w_L) \cdot \nu_{H_2O}}{w_L}, \quad (2)$$

where ν_{H_2O} is the specific volume of water obtained from its density and w_L is the mass fraction of lipid in the sample.[25] Specific volumes were used for the calculation of molar V_L^m and mean molecular V_L volumes of lipids:

$$V_L^m = \nu_L \cdot M, \quad (3)$$

$$V_L = \frac{\nu_L \cdot M}{N_A}, \quad (4)$$

where M is the molar mass of the lipid and N_A is the Avogadro constant.

Results

Stability of densitometry results

We checked the stability of the results of densitometric measurements for two types of sample dispersions: DOPC vesicles and DOPC–DOPS vesicles with 50% molar fraction of DOPS. Before checking the stability, samples were stored in a freezer. Before the experiment, they were thawed, vortexed and briefly sonicated in the K-5LE bath sonicator. The DOPC sample was degassed, while the DOPC–DOPS sample for which the results are shown below was intentionally not degassed. The time-dependence of DOPC dispersion density was measured at 20 °C, close to the

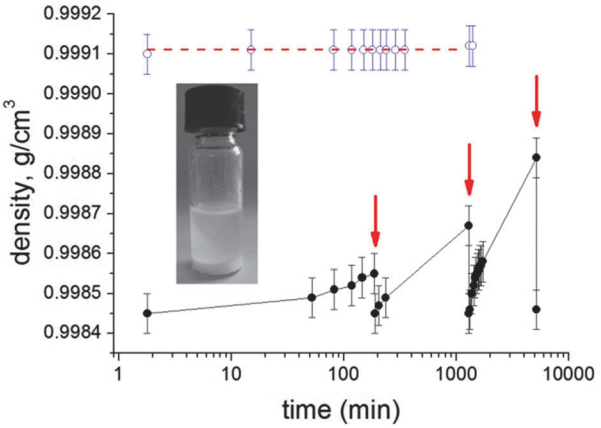

Fig. 1 Density of degassed dispersion of multilamellar vesicles formed by DOPC at concentration 23.94 mg ml^{-1} (full circles) and of non-degassed dispersion of DOPC and DOPS mixture with molar fraction of DOPS 50% at total lipid concentration 20.64 mg ml^{-1} (open circles). Arrows indicate reintroducing the DOPC sample. Dashed line presents linear fit with zero slope for DOPC–DOPS mixture. Error bars are the errors of density readings of the instrument. Inset, photo of the DOPC sample 2400 minutes after preparation.

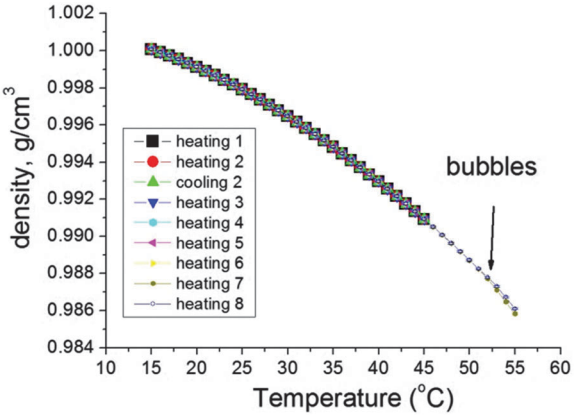

Fig. 2 Density of vesicle dispersion formed by equimolar mixture of DOPC and DOPS at total lipid concentration 20.64 mg ml^{-1}. Errors are smaller than symbols. Inset, the description of symbols. The arrow indicates appearance of bubbles in the sample.

laboratory temperature. Temperature equilibration of the sample took 2–3 minutes after introducing it into the U-tube of the densitometer. After the first introduction into the U-tube, the density of DOPC dispersion was ρ = (0.99845 ± 0.00005) g cm^{-3}. Over time, the density increased (Fig. 1, black circles) and in 190 min, it was ρ = (0.99855 ± 0.00005) g cm^{-3}. After that, the sample was sucked into a syringe, shaken and reintroduced into the U-tube. The density after this operation was ρ = (0.99845 ± 0.00005) g cm^{-3} (first arrow in Fig. 1). Over 1100 minutes it increased up to ρ = (0.99867 ± 0.00005) g cm^{-3}. The sample was again sucked into a syringe, shaken and reintroduced into the U-tube (second arrow in Fig. 1). The density was ρ = (0.99845 ± 0.00005) g cm^{-3}. Over 3900 minutes, it increased up to ρ = (0.99884 ± 0.00005) g cm^{-3}. The rest of the DOPC dispersion stayed in a vial, and it had an observable sediment at the end of this experiment. A photo of the DOPC sample after 2400 minutes is shown in Fig. 1.

From this experiment, one can conclude that changes in the measured density are caused by a macroscopic redistribution (sedimentation) of multilamellar DOPC vesicles in the U-tube of the densitometer, as reported[22] for DPPC vesicles. Thus, density measurements for such samples should be performed shortly after their introduction into the U-tube of the densitometer and repeated several times after thorough mixing.

Such changes in density were not observed for the DOPC–DOPS mixture (50% molar fraction of DOPS) (Fig. 1, open circles). At the beginning of taking measurements, the density of lipid dispersion was ρ = (0.99910 ± 0.00005) g cm^{-3}, and in 1400 minutes, it was ρ = (0.99912 ± 0.00005) g cm^{-3}. Evidently, the presence of charged DOPS prevents vesicles of sedimentation. The vesicles could be smaller and/or paucilamellar and unilamellar.[26] One could test this hypothesis by performing dynamic light scattering experiments or another suitable scattering method.

As the DOPS + DOPC sample data were stable in time, we checked the stability of densitometric results in the case of cycling temperature scans. Most of the scans were performed in the range 20–45 °C, and the last two were performed at 20–55 °C. Between scans the sample was located in the densitometer U-tube, and it was not disturbed (no reintroducing and shaking). The results from eight sequential scans coincide (Fig. 2); also, coinciding are the density values for heating and cooling. At temperatures higher than 50 °C, bubbles were observed in the U-tube that caused instability in results for these high temperatures. These bubbles are caused by air dissolved in sample; the sample was not intentionally degassed. In the degassed samples, bubbles were not seen (not shown). To summarize, our results are in accordance with those of a previous study[23] stating that the instabilities in densitometric experiments with multilamellar phospholipid vesicles are caused by the creation of macroscopic inhomogeneities in the U-tube of the densitometer during prolonged measurements.

Specific volumes and molar volumes of pure DOPC and DOPS

Since the data for volumes of lipids can be useful in the analysis of diffraction and small angle scattering data, we present the densitometric results for pure aqueous DOPC and DOPS dispersions. The specific volumes of lipids were calculated according to formula (2). Then, molar, V_L^m, and mean molecular, V_L, volumes were calculated according to eqn (3) and (4). When using these equations, it is supposed that the partial molar volume of water in vesicle dispersions is equal to its molar volume in pure water.[27] When preparing the sample, we used sodium salt of DOPS. We stress that the molar mass of DOPS used in these calculations was M_{DOPS} = 810.1 g mol^{-1} as shown in Table 1, *i.e.*, we suppose in agreement with a previous study[28] that the Na^+ ions are not dissociated and are located in the DOPS bilayers. Obtained values at different temperatures are given in Table 1. Values of V_{DOPC} are in a good agreement with previously published results.[20,28–32] We are acquainted with only one publication with information about the volume of pure DOPS.[28] Our value at 30 °C, V_{DOPS} = 1254 Å^3, is higher than V_{DOPS} = 1228 Å^3,

Table 1 Molar volume V^m and mean molecular volume V for DOPC and DOPS. The values were obtained from densitometric measurements of aqueous dispersions of pure DOPC and DOPS. Molar weights of DOPC and DOPS equal M_{DOPC} = 786.1 g mol^{-1} and M_{DOPS} = 810.1 g mol^{-1}, respectively. Values were calculated from densitometric data assuming that not all Na^+ ions are dissociated

T (°C)	V^m_{DOPC} (cm^3 mol^{-1})	V_{DOPC} (Å^3)	V^m_{DOPS} (cm^3 mol^{-1})	V_{DOPS} (Å^3)
15	775.8 ± 2.9	1289 ± 5	746 ± 3	1240 ± 5
20	778.9 ± 2.9	1294 ± 5	749 ± 3	1245 ± 5
25	781.9 ± 2.9	1299 ± 5	752 ± 3	1249 ± 5
30	785.0 ± 2.9	1304 ± 5	755 ± 3	1254 ± 5
35	787.9 ± 2.9	1309 ± 5	758 ± 3	1258 ± 5
40	790.2 ± 2.9	1313 ± 5	760 ± 3	1263 ± 5
45	792.6 ± 2.9	1317 ± 5	763 ± 3	1267 ± 5

obtained in this paper. The partial volume of DOPS in a mixture DOPC–DOPS (4 wt% of DOPS) was estimated in a previous study,[25] and it equals 1189.8 Å^3 at 20 °C, which is less than what was obtained in the present work, V_{DOPS} = 1245 Å^3.

From X-ray diffraction on gel lamellar phases, the head group volume of DPPC at 24 °C is V_H = 319 Å^3 ref. 33 and of DMPC at 10 °C V_H = 331 Å^3.[34] The error for these data is ±6 Å^3; thus, they overlap at the average value V_H = 325 Å^3. From the combination of densitometric data for the homologous series of monounsaturated 1,2-diacylphosphatidylcholines, the volume of the head group including the glycerol and acyl carbons V_H = 323.4–329.5 Å^3 was obtained.[31] The volume of PC head group was found to be practically temperature-independent compared to that of the PC hydrocarbon part.[31,35,36] Therefore, one can assume the value for the volume of phosphatidylcholine head group V^H_{DOPC} = 325 Å^3. The volume of the hydrocarbon part of DOPC at 30 °C is given by $V^{DOPC}_c = V_{DOPC} - V^{DOPC}_H$ = 979 Å^3. Let us assume that DOPC and DOPS hydrocarbon chains in the fluid state have the same volume. Then, the head group volume of DOPS at 30 °C is V^H_{DOPS} = 1254 − 979 = 275 Å^3. This is significantly higher than 244 Å^3 obtained in a previous study.[28]

We observed an increase in the lipid volume at rising temperature. The coefficient of isobaric thermal volume expansivity can be calculated as:

$$\beta = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_p = \frac{1}{\nu}\left(\frac{\partial \nu}{\partial T}\right)_p = \left(\frac{\partial \ln \nu}{\partial T}\right)_p, \quad (5)$$

where ν is the specific volume of a lipid. The value of coefficient β equals the slope of dependence $\ln \nu$ *vs.* T. Plots of $\ln \nu$ *vs.* T for DOPC and DOPS are presented in Fig. 3. The values of the coefficient β for pure DOPC and DOPS are similar and equal to $\beta_{DOPC} = (72.2 \pm 1.9) \times 10^{-5}$ K^{-1} and $\beta_{DOPS} = (72.7 \pm 1.5) \times 10^{-5}$ K^{-1}. These values are close to previously published values.[29,31,32]

When preparing the DOPS sample in the aqueous phase, the Na^+ ions can dissociate into water bulk. From the densitometric results alone, one cannot conclude if the DOPS in vesicles is dissociated or not. In previous papers,[25,28] it has been supposed that the Na^+ ions are not dissociated. *A priori*, one cannot exclude any of these alternatives; one cannot exclude even a partial dissociation of Na^+ ions. Therefore, we estimated the values of specific volume and molar volume of DOPS also

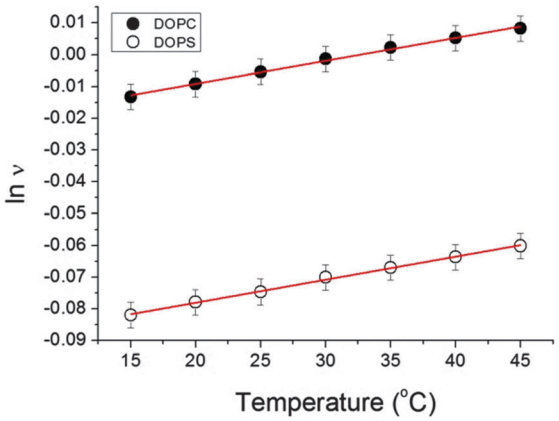

Fig. 3 Temperature dependence for natural logarithm of specific volume ν (in cm^3 g^{-1}) of DOPS and DOPC. Lines are the linear approximations of experimental points.

supposing that all Na^+ ions are dissociated into bulk water. Because of the well-known Na^+ electrostriction effect,[37] this should cause the change in water density, that is, in formula (2), ν_{H_2O} is changed to ν_{H_2O+Na}, whose value can be calculated as:

$$\nu_{H_2O+Na} = \frac{m_{H_2O} \cdot \nu_{H_2O} + V^m_{Na} \cdot \frac{m_{DOPS}}{M_{DOPS}}}{m_{H_2O} + M_{Na} \cdot \frac{m_{DOPS}}{M_{DOPS}}}, \quad (6)$$

where m_{H_2O} and m_{DOPS} are masses of water and DOPS in the measured samples (their values were gravimetrically evaluated during sample preparation), M_{DOPS} and M_{Na} are the molar masses of DOPS and sodium; ν_{H_2O}, the specific volume of water and V^m_{Na}, the molar volume of Na^+ ions. In accordance with previous studies,[38,39] the presence of Na^+ ions decreases the volume of water by 5.0–6.6 cm^3 mol^{-1} at 25 °C. Thus, V^m_{Na} equals −5.0 to −6.6 cm^3 mol^{-1}. The estimated values of specific volume and molar volumes of dissociated DOPS at 25 °C are given in Table 2. We stress that from the densitometric results alone, one cannot conclude if DOPS in vesicles is dissociated or not.

Ideality of DOPC–DOPS mixing

The values of specific volume $\nu_{PC/PS}$ of mixture of DOPC and DOPS were calculated according to formula (2) as:

$$\nu_{PC/PS} = \frac{\nu_s - (1 - w_{PC/PS}) \cdot \nu_{H_2O}}{w_{PC/PS}}, \quad (7)$$

where $w_{PC/PS}$ is the fraction of total mass DOPC and DOPS in the sample. The dependence of $\nu_{PC/PS}$ on DOPS molar fraction at 20 °C is presented in Fig. 4. The line indicates the $\nu_{PC/PS}$ for

Table 2 Specific volume ν, molar mass M and mean molecular volume V for dissociated DOPS. Values were calculated from densitometric data assuming that all Na^+ ions are dissociated into water

T (°C)	V^m_{Na} (cm^3 mol^{-1})	ν_{H2O+Na} (cm^3 g^{-1})	$\nu_{DOPSdiss}$ (cm^3 g^{-1})	$M_{DOPSdiss}$ (g mol^{-1})	$V_{DOPSdiss}$ (Å^3)
25	−5.036[38]	1.00226	0.962	787	1257
25	−6.6237[39]	1.00222	0.970	787	1260

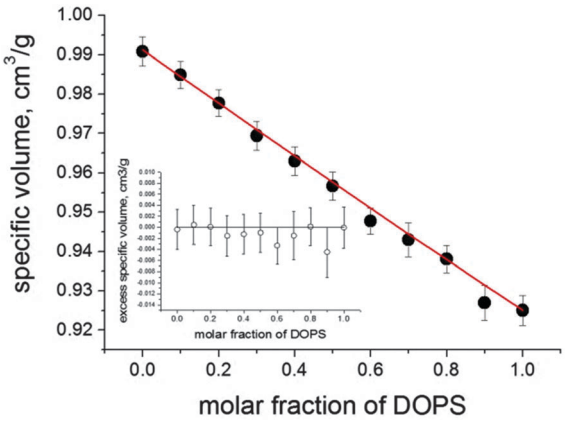

Fig. 4 Dependence of specific volume of mixture DOPC–DOPS on molar fraction of DOPS. The line presents theoretical values for specific volume in case of ideal mixing of DOPC and DOPS [calculated according to eqn (1)]. Inset shows excess specific volume. This is the difference between experimental and theoretical values for ideal mixing. Line in the inset presents zero value for excess volume.

ideal mixing of DOPC–DOPS according to eqn (1). Molar volumes V^m of pure DOPC and DOPS were evaluated as described above (for non-dissociated DOPS). The experimental points for $\nu_{PC/PS}$ coincide with theoretical values within error margins of experiment. This means that within the error limits of our experiment, the mixing of DOPC and DOPS is ideal at molar fractions of DOPS above 10%. Such behavior occurs at any temperature in the range of 15–45 °C.

The values $\nu_{PC/PS}$ were determined for several temperatures in the range 15–45 °C. From these data, the coefficient of thermal volume expansivity can be evaluated according to eqn (5). For different DOPS molar fractions, the coefficients β are similar and have values in the range (71.1–73.6) $\times$ 10^{-5} K^{-1}, the relative error is 2–3% (Fig. 5). Such behavior is expected since the coefficients β for pure DOPC and DOPS are similar.

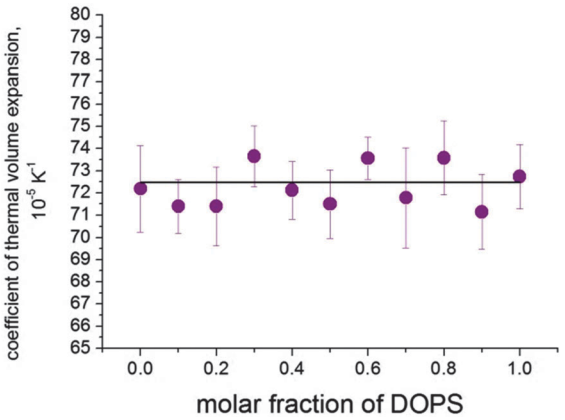

Fig. 5 Dependence of the coefficient of isobaric thermal volume expansivity for DOPC–DOPS mixtures on the DOPS molar fraction. The line presents mean value for thermal coefficient (72.5 ± 0.3) $\times$ 10^{-5} K^{-1}.

Conclusions

Within the experimental uncertainty of densitometry, DOPC and DOPS ideally mix in dispersions of multilamellar vesicles in the range of DOPS molar fraction 0–100%. The molar volumes of pure DOPC and DOPS were evaluated at different temperatures. The time-dependent scans of DOPC vesicles dispersion confirmed that changing density in time is an artifact connected with the sedimentation of vesicles that was earlier found for DPPC dispersions in a previous study.[23] Thus, density measurements for multilamellar dispersions of phospholipid vesicles should be performed shortly after their introduction into the U-tube of the densitometer and repeated several times. The presence of charged DOPS in vesicles prevents the dispersions from sedimentation for a prolonged time.

Abbreviations

DMPC	1,2-Dimyristoyl-*sn-glycero*-3-phosphocholine
DMoPC	1,2-Dimyristoleoyl-*sn-glycero*-3-phosphocholine
DOPC	1,2-Dioleoyl-*sn-glycero*-3-phosphocholine
DOPS	1,2-Dioleoyl-*sn-glycero*-3-phospho-L-serine
DPPC	1,2-Dipalmitoyl-*sn-glycero*-3-phosphocholine
POPC	1-Palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine
POPS	1-Palmitoyl-2-oleoyl-*sn-glycero*-3-phospho-L-serine
PS	Phosphatidylserine
PC	Phosphatidylcholine

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