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Influence of cytotoxic butyltin complexes with 2-sulfobenzoic acid on the thermotropic phase behavior of lipid model membranes

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Abstract In the previous study, we showed that butyltin complexes with 2-sulfobenzoic acid (commonly referred to as BTsbz) exhibit a very wide biological activity. The BTsbz in vitro are very active cytotoxic agents against tumor cells—more effective than cisplatin and carboplatin (traditional anticancer drugs). These complexes are antibacterial agents against gram-positive and gram-negative bacteria, as well. The aim of the present study was to investigate the influence of BTsbz on the thermotropic phase behavior of dipalmitoylphosphatidylcholine (DPPC) model membranes. The effect of this compound on the multilamellar liposomes was studied mainly by the means of differential scanning calorimetry and additionally by the steady-state fluorimetry and infrared spectroscopy. All investigated butyltin complexes with 2-sulfobenzoic acid change the thermotropic properties of lipid model membranes: The temperature of the main phase transition of DPPC is slightly decreased, and the transition's cooperativity of the peak is very much reduced. With increasing concentration, all investigated compounds abolished the pretransition. The results suggest that BTsbz are very active in the hydrophilic area of DPPC bilayer and at the same time have an effect on membrane fluidity.

Keywords Phase transition · DSC · Fluidity · Spectroscopy · DPPC · Butyltin complexes with 2-sulfobenzoic acid

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Introduction

Platinum compounds such as carboplatin and *cis*-platin are used in cancer chemotherapy to treat testicular, ovarian and bladder cancer. In addition to platinum compounds, derivatives of other transition metals are being investigated for their anti-tumor properties [1]. Many di- and triorganotin compounds have had been synthesized and tested for their anti-tumor activity against a variety of tumor. Some of organotin(IV) complexes were found to have much better activity in vitro than traditional anticancer drugs [1–9]. From this point of view, newly synthesized butyltin complexes with 2-sulfobenzoic acid (commonly referred to as BTsbz): $[Sn(C_4H_9)_2\{O_3SC_6H_4COO-2\}(H_2O)]\cdot(C_2H_5OH)$ (DBTsbz), $[Sn(C_4H_9)_3\{O_3SC_6H_4COOH-2\}]$ (TBTsbz) and $[Sn_2(C_4H_9)_6\{\mu\text{-}O_3SC_6H_4COO-2\}]$ (DTBTsbz) seem to be very promising. In the previous study, we showed that these complexes exhibit not only the antibacterial activity against gram-positive and gram-negative bacteria, but also are very active cytostatic agents against tumor cells line—more effective than *cis*-platin [7] and carboplatin [10]. We have analyzed cytotoxic activity of butyltin complexes against following tumor cell lines: Jurkat (T cell leukemia), CL-1 (T-lymphoblastoid cell line), GL-1 (B cell lymphoma cell line) and D-17 (canine osteosarcoma) and normal cell lines: J774.E (murine macrophages), D10.G4.1 (murine lymphoblasts) [10]. In comparison with carboplatin, all investigated compounds showed significantly lower value of IC_{50} against D-17 cell line. DBTsbz compound seems to be the most interesting among those which were investigated because it is much more active than carboplatin (about 100 times smaller doses are needed to yield similar effect) and at the same time it was the least toxic against normal cell lines [10].

We plan to continue the research by investigating lipid-BTsbz formulations and in particular—check effectiveness of such formulations as anticancer agents. In vitro and in vivo investigation of cisplatin and carboplatin shows that lipid–compound formulations are more toxic against tumor cell lines than against healthy ones. Data on mouse model suggest that liposomes containing dipalmitoylphosphatidylcholine (DPPC) formulation of tested compound significantly decreased tumor size and increased survival probability compared with cisplatin [11, 12]. In [12] authors conclude that DPPC liposome, the main phase transition of which is nearly close to the body temperature, improves drug retention in blood circulation and is therefore extravasated through permeable microvasculature where it remains confined to the extracellular fluid of the tumor. Temperature-sensitive liposomes are particularly promising option, as tumors can be heated in a controlled and predictable manner with external energy sources. Traditional thermosensitive liposomes are composed of lipids that undergo a gel-to-liquid phase transition at several degrees above physiological temperature [13].

It is well known that drugs can be encapsulated in the water area of liposomes or alternatively can be incorporated directly in the lipid bilayer—depending on the hydrophilicity of a given substance. Therefore, the objective of the research summarized in this paper was to check not only where the butyltin complexes with 2-sulfobenzoic acid locate in the lipid bilayer but also, what's more important, how BTsbz influence the thermotropic phase behavior of DPPC model membranes. For this lipid-BTsbz interaction was studied in the temperature range from 20 to 60 °C, i.e., in the gel and fluid phases of DPPC lipid bilayers. Thermal parameters that were monitored include the temperature at the pretransition (T_p) and the main transition peak (T_m), the half-width ($\Delta T_{1/2}$) and change of the enthalpy (ΔH) of the main transition on DPPC.

The effect of the BTsbz on the lipid bilayer phase transition was examined mainly by the differential scanning calorimetry (DSC)—a method useful for studying the nature of biological systems such as lipids or proteins [14–17], and additionally by fluorescence and infrared spectroscopy. The above-mentioned methods have been employed to systematically monitor the influence of the BTsbz on different membrane regions. The degree of packing order of the hydrophilic phase of the lipid bilayer was determined by the Laurdan, Prodan and MC540 fluorescence probes, whereas the fluorescence anisotropy of the hydrophobic phase—with the DPH probe.

Materials and methods

Chemicals

The compounds $[Sn(C_4H_9)_2\{O_3SC_6H_4COO-2\}(H_2O)]\cdot(C_2H_5OH)$ (DBTsbz), $[Sn(C_4H_9)_3\{O_3SC_6H_4COOH-2\}]$

(TBTsbz) and $[Sn_2(C_4H_9)_6\{\mu-O_3SC_6H_4COO-2\}]$ (DTBTsbz) were prepared by procedures reported earlier [5]. The schematic structures of compounds in solutions are presented in Figs. 1–3.

The 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) lipid and Merocyanine (MC540) were purchased from Sigma Aldrich, Steinheim, Germany. The fluorescent probes: 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), 6-propionyl-2-dimethylaminonaphthalene (Prodan), 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Molecular Probes, Eugene, Oregon, USA.

Differential scanning calorimetry

DSC studies were performed as described previously [17]. Samples for DSC were prepared from multilamellar liposomes (MLVs) of DPPC. The final concentration of lipids was 25 mg cm^{-3}. Pure lecithin MLVs (control sample) and lecithin with BTsbz were placed in Mettler Toledo standard aluminum crucibles of 40 μl capacity. Tightly closed vessels were incubated for 2 days at 4 °C. Measurements were performed with Mettler Toledo Thermal Analysis System D.S.C. 821^e, operated at a heating rate of 2 °C min^{-1} from 20 to 60 °C. Thermal cycles were repeated three times. The experimental error in temperature and thermal response was ±0.2 °C and ±5 %, respectively. Data were analyzed using original software provided by Mettler Toledo.
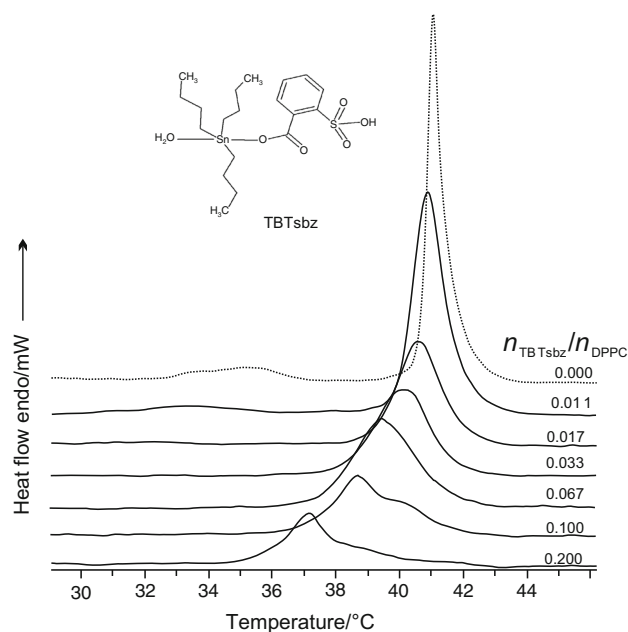

Fig. 1 DSC transition curves of MLVs formed from DPPC (*dotted line*) and DPPC modified with the TBTsbz (schematic structure of TBTsbz in solutions shown in the *inset*)

Fig. 2 DSC transition curves of MLVs formed from DPPC (*dotted line*) and DPPC modified with the DBTsbz (schematic structure of DBTsbz in solutions shown in the *inset*)
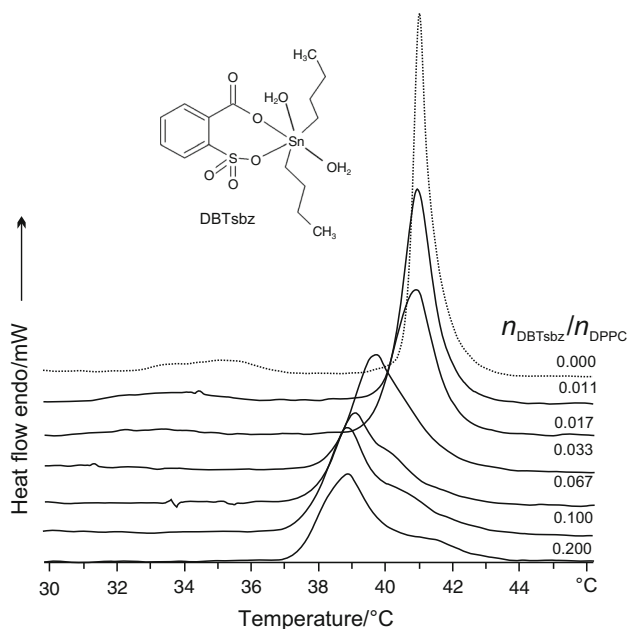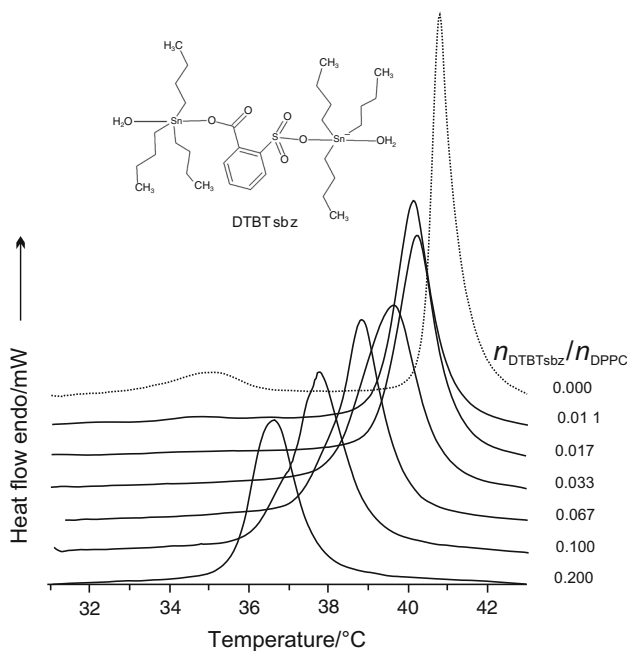

Fig. 3 DSC transition curves of MLVs formed from DPPC (*dotted line*) and DPPC modified with the DTBTsbz (schematic structure of DTBTsbz in solutions shown in the *inset*)

Fluorescence spectroscopy

Samples for steady-state fluorimetry consisted of DPPC MLVs with BTsbz. MLVs were prepared identically as for DSC measurements. The DPPC, compounds and

fluorescence probes (Laurdan, Prodan, MC540 and DPH) were dissolved in chloroform and in ethanol, respectively. Chloroform and ethanol were very carefully evaporated to dryness under nitrogen and thin film was formed on the flask wall, after which distilled water was added. The lipid film was dispersed by agitating the flask on a vortex mixer to give a milky suspension of liposomes at a temperature above the main phase transition of DPPC. Samples were incubated for 30 min in darkness at room temperature. Final lipids' concentration in the samples was 450 μM, molar ratio of BTsbz and lipids was 0.2, and the concentration of fluorescence probes was 10 μM. Measurements were made at different temperatures—above and below the main phase transition of DPPC. Thermal cycles (20–60 °C) were repeated three times.

Measurements were conducted with a CARY Eclipse of VARIAN fluorimeter equipped with a DBS Peltier temperature controller (temp. accuracy ±0.1 °C). The excitation and emission wavelengths for DPH probe were $\lambda_{ex} = 360$ nm and $\lambda_{em} = 425$ nm. The excitation wavelength for Laurdan and Prodan was 360 nm, and the emitted fluorescence was recorded at two wavelengths: 440 and 490 nm. The excitation and emission wavelengths for MC540 probes were $\lambda_{ex} = 540$ nm and $\lambda_{em} = 585$ nm, respectively.

Fluorescence anisotropy (A) for DPH probes was calculated using the formula $A = (I_{II} - GI_\perp)/(I_{II} + GI_\perp)$, where I_{II} and $I_\perp$ are fluorescence intensities observed in the direction parallel and perpendicular to the polarization direction of the exciting wave. G is an apparatus constant dependent on the emission wavelength [20]. Changes in the polar group packing arrangement of the hydrophilic part of the membrane were investigated using Laurdan and Prodan probes, on the basis of generalized polarization (GP), and were calculated with the formula $GP = (I_g - I_l)/(I_g + I_l)$, where I_g and I_l are the fluorescence intensities at the gel and the fluid phase, respectively [18, 19].

Attenuated total reflectance Fourier transform infrared spectroscopy

The method was applied as described earlier [14, 20] with a few modifications. Mixtures of DPPC dissolved in chloroform (1 mg) and butyltin complexes dissolved in ethanol were placed on ZnSe crystal and were dried under nitrogen for a few minutes and under vacuum for 24 h. The dried films of DPPC or BTsbz/DPPC were hydrated in distilled water above the main phase transition of DPPC for 4 h. Molar ratio of BTsbz/DPPC was 0.2. Measurements were performed using Thermo Nicolet 6700 MCT spectrometer (Thermo Fisher Scientific, Waltham, MA) with ZnSe crystal at a heating cycle from 20 to 50 °C. Each single spectrum was obtained from 128 records at 2 cm^{-1}

resolution in the range of 700–4000 cm^{-1}. Preliminary elaboration of a spectrum was done using the EZ OMNIC v 8.0 program, also by Thermo Nicolet. After filtering the noise out of the extract spectrum, the spectrum of the water solution was removed, and the baseline corrected.

Results and discussion

The influence of butyltin complexes with 2-sulfobenzoic acid with thermotropic parameters of lipid membranes was investigated by the means of differential scanning calorimetry and additionally by spectroscopy methods. The molecular model membranes are multilamellar liposomes formed from DPPC. Phospholipids show thermotropic and lyotropic phase behavior. When dispersed in excess water, these lipids form hydrated bimolecular lamellar phases, in which the lipid molecules either are packed in a quasi-crystalline structure, the so-called gel phase, or remain in lamellar arrangement but show higher two-dimensional fluidity, i.e., the liquid-crystalline phase (L$_\alpha$). Transition between these phases, called the main phase transition, can be induced by a temperature change, changes in hydration or pH changes [21]. DPPC molecules show more complicated phase behavior as at least two different gel phases are observed: so-called intermediate P$_{\beta'}$ phase, in which the surface of the bilayer is distorted by a periodic ripple, and L$_{\beta'}$ phase in which the molecules are tilted at an angle of about 30° relative to bilayer normal. With the help of DSC, the heat signal associated with the transition from L$_{\beta'}$ to P$_{\beta'}$ and from P$_{\beta'}$ to L$_\alpha$ was examined. The influence of TBTsbz, DBTsbz and DTBTsbz on the thermotropic phase behavior of DPPC liposomes is compared in Figs. 1–3, respectively. For pure DPPC, a broad pretransition can be observed at $T_p = 35$ °C which corresponds to the transition from the lamellar gel phase to the ripple gel phase and then—a sharp and symmetric main phase transition peak at $T_m = 41.2$ °C that corresponds to the transition from the

ripple gel phase to the lamellar liquid phase. The measured transition temperatures and enthalpies for pure DPPC are summarized in Table 1; these are in good agreement with literature values [21].

As for the impact of individual compounds on cooperativity of the main phase transition then for both TBTsbz and DBTsbz for a molar ratio BTsbz/DPPC = 0.067, the peak of the main phase transition became asymmetrical. At higher concentrations of these complexes, the main phase transition peak increasingly widened and two components could be identified: the first (major) shifted toward lower temperature and second (minor) corresponding to the T_m of pure DPPC. These results may indicate formation of different domains consisting of different BTsbz/DPPC compositions and consequentlywith different T_m values. No such effect was observed for DTBTsbz; however, this compound caused the T_m to be decreased the most (Table 1). For increasing concentration, all investigated compounds abolished the pretransition and decreased T_m of the main phase transition.

Supplementary methods of determining phase transition temperatures of phospholipids that constitute liposomes are spectroscopic methods, such as fluorescence spectroscopy and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). These methods are applied in a parallel with DSC to monitor the thermal properties of biomolecules [22–26].

The infrared spectroscopy was used to check the influence of DBTsbz and DTBTsbz on phase transitions of DPPC. The application of FTIR to the study of the phase behavior of DPPC membranes allows monitoring of various functional groups in order to obtain information about lipid–compound interactions at the molecular level. The infrared spectra of phospholipids can be divided into spectral regions that originate from the molecular vibration of the hydrocarbon tail, the interface region and the head group. In the previous paper, we employed FTIR and NMR methods to analyze molecular interactions between

Table 1 Temperature values of: (T_m) main phase transition, ($\Delta T_{1/2}$) peak half-width and enthalpy change (ΔH) of the main phase transition for DPPC liposomes, in the presence of selected concentrations of butyltin complexes

	TBTsbz			DTBTsbz			DBTsbz		
	T_m/°C	$\Delta T_{1/2}$/°C	ΔH/kJ mol^{-1}	T_m/°C	$\Delta T_{1/2}$/°C	ΔH/kJ mol^{-1}	T_m/°C	$\Delta T_{1/2}$/°C	ΔH/kJ mol^{-1}
0.00	41.1	0.6	36.1	41.1	0.6	36.1	41.4	0.6	36.1
0.011	41.0	1.2	35.5	40.2	1.0	34.8	41.0	1.1	32.0
0.017	40.8	1.3	34.2	40.3	1.1	34.3	40.9	1.4	28.0
0.033	40.7	2.0	28.9	39.7	1.6	29.6	39.8	1.5	28.0
0.067	39.5	2.2	21.2	38.4	1.2	30.1	39.6	2.0	30.1
0.100	38.8	2.5	20.3	37.7	1.4	25.8	38.9	1.8	24.0
0.200	37.7	2.5	17.5	36.6	1.3	21.9	38.3	1.9	20.1

investigated compounds and DPPC. FTIR measurements were conducted at room temperatures for dry lipid films as well as for lipids hydrated with deuterated water [10]. In this paper, we analyze the influence of BTsbz on lipid phase transitions focusing in particular on properties of hydrophobic part of the DPPC-formed lipid bilayer because
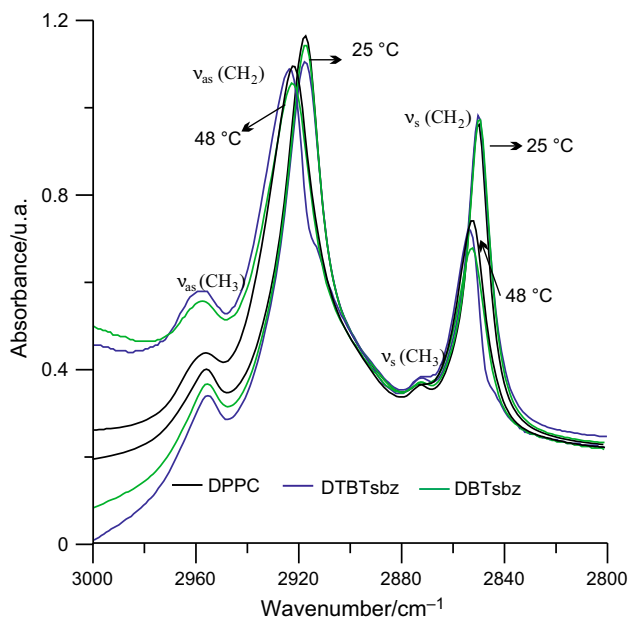
the physical stability of liposomes has been found to be a function of lipid acyl chain length [27]. The most intense vibrations of lipid systems are caused by carbon–hydrogen stretching vibrations and known as asymmetric and symmetric CH_2 stretching modes in the spectral region of 3000–2800 cm^{-1}. The frequencies of this group of alkyl chains depend on mobility (fluidity) of the chains and increase, e.g., with increasing temperature or during transition from the gel to the liquid-crystalline state. The increase in the wave number of these bands testifies to an increased liquidity of the hydrophobic part of the membrane. The CH_2 asymmetric stretching located at about 2920 cm^{-1} and symmetric located about at 2850 cm^{-1} are strong lipid bands which are sensitive to conformational changes. They respond to any difference occurred in the trans/gauche ratio in acyl chain. Conformational changes can also be detected by examining asymmetric CH_3 at 2965 cm^{-1} and symmetric CH_3 at 2872 cm^{-1} bands which are stretching modes of the terminal methyl group (Fig. 4). No changes in the methylene group frequencies were observed for the investigated BTsbz/DPPC systems.

Dependence of the asymmetric and symmetric CH_2 stretching vibration in pure DPPC and BTsbz/DPPC systems as a function of temperature is shown in Fig. 5a, b, respectively. It is apparent that at a room temperature (25 °C) investigated complexes do not significantly change the ordering of hydrocarbon chains, which is in agreement with earlier conclusions [10]. Significant changes start to appear with increasing temperature: Both DTBTsbz and DBTsbz compounds change the frequency of vibrations of
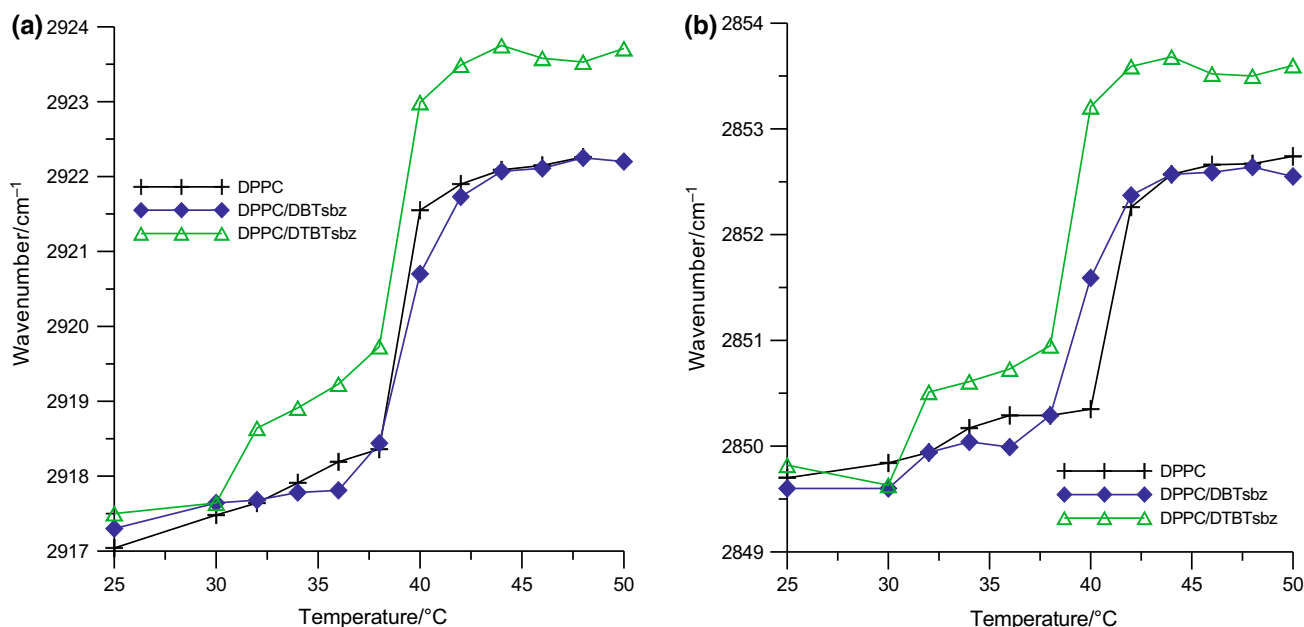

Fig. 4 Infrared spectra of DPPC bilayer and DPPC with butyltin compounds in the region between 3000 and 2800 cm^{-1} at the gel (25 °C) and liquid-crystalline (48 °C) phases

Fig. 5 Temperature dependence of the frequency of the CH_2 asymmetric (**a**) and symmetric (**b**) stretching mode in the presence and absence of butyltin compounds for DPPC bilayers

methylene groups of acyl chains of DPPC, however, in a different way. The most pronounced changes of frequencies of CH_2 were observed in the presence of the DTBTsbz. DTBTsbz causes an increase of the wave number for the gel and liquid phase and also lowers the T_m (Fig. 5a, b). DBTsbz also causes lowering T_m, except that in comparison with DTBTsbz, the change is less pronounced and different in character because DBTsbz slightly abolished the sharp phase transition from the gel phase to the liquid crystal phase (Fig. 5b). This is supported by the broadening of the main transition peak observed in DSC measurements for this compound.

In summary, FTIR and DSC results revealed that BTsbz eliminate the pretransition and shift the main phase transition to lower temperatures as compounds concentrations are increased. By monitoring the CH_2 stretching vibrations in infrared spectra, it was revealed that DTBTsbz increases acyl chain flexibility, i.e., increases disorder of DPPC bilayer in both the gel and liquid-crystalline phase.

In addition to IR methods, the impact of BTsbz on phase transition and fluidity of multilamellar liposomes (MLVs) formed with DPPC was examined with the help of the steady-state fluorimetry. Fluorescence intensity was measured using four probes: Laurdan, Prodan, MC540 and DPH. These fluorescent probes were used because each of them incorporates in a different region of lipid bilayers [20].

Laurdan is a membrane fluorescent probe that has the unique advantage of being sensitive to the phospholipid phase state. It is located in the hydrophilic–hydrophobic interface of the bilayer with the lauric acid tail anchored in the phospholipid acyl chain region [28]. Calculated values of general polarization (GP) of the Laurdan probe for MLVs formed from DPPC are presented in Fig. 6a. All investigated compounds slightly lower the GP coefficient values in the gel phase; however, DBTsbz induces bigger change in the liquid-crystalline phase. Lower values of GP for MLVs in the absence/presence of BTsbz, in comparison with pure DPPC, may indicate slightly increasing disorder of the polar heads of lipid bilayers, which would testify that these compounds locate in the hydrophilic part of the bilayer. Most probably, such location of compounds causes slight lowering of T_m which can be observed in Fig. 6a. Experiments performed with Prodan probe lead to similar conclusions. Furthermore, as a result of its location in the bilayer, Prodan should be also sensitive to the pretransition occurring in the lipid polar head groups region [28]. In Fig. 6b values of GP coefficients calculated on the basis of fluorescence intensity measured at different temperatures for the Prodan probe are presented. For DBTsbz, large changes in GP values are observed in both gel and liquid phases of the DPPC bilayer. The other two compounds: TBTsbz and DTBTsbz cause slightly smaller changes of GP in the gel phase. These results suggest that DBTsbz strongly influences the gel state of the DPPC bilayer. All compounds reduce the pretransition and slightly decrease fluidity of hydrophilic part of bilayers—shift toward lower temperatures of the main transition phase. Similar
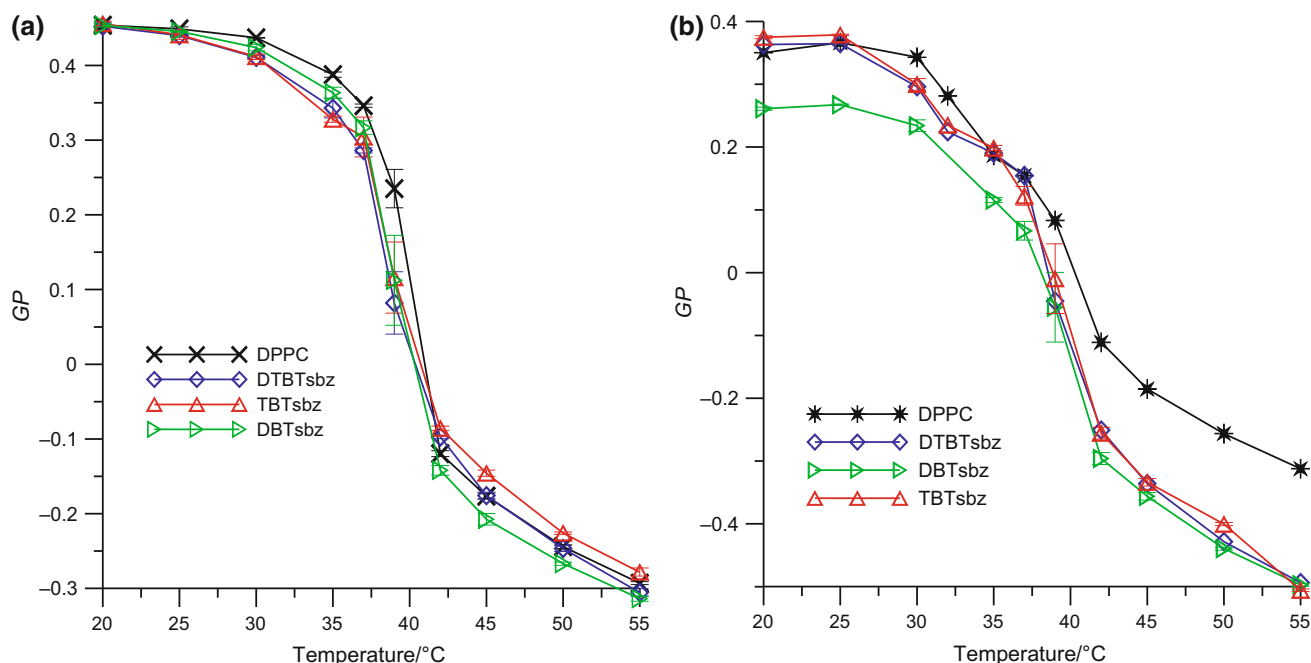

Fig. 6 Values of general polarization of Laurdan (a) and of Prodan (b) at different temperatures for DPPC liposomes with the tested BTsbz (molar ratio BTsbz/DPPC = 0.2)
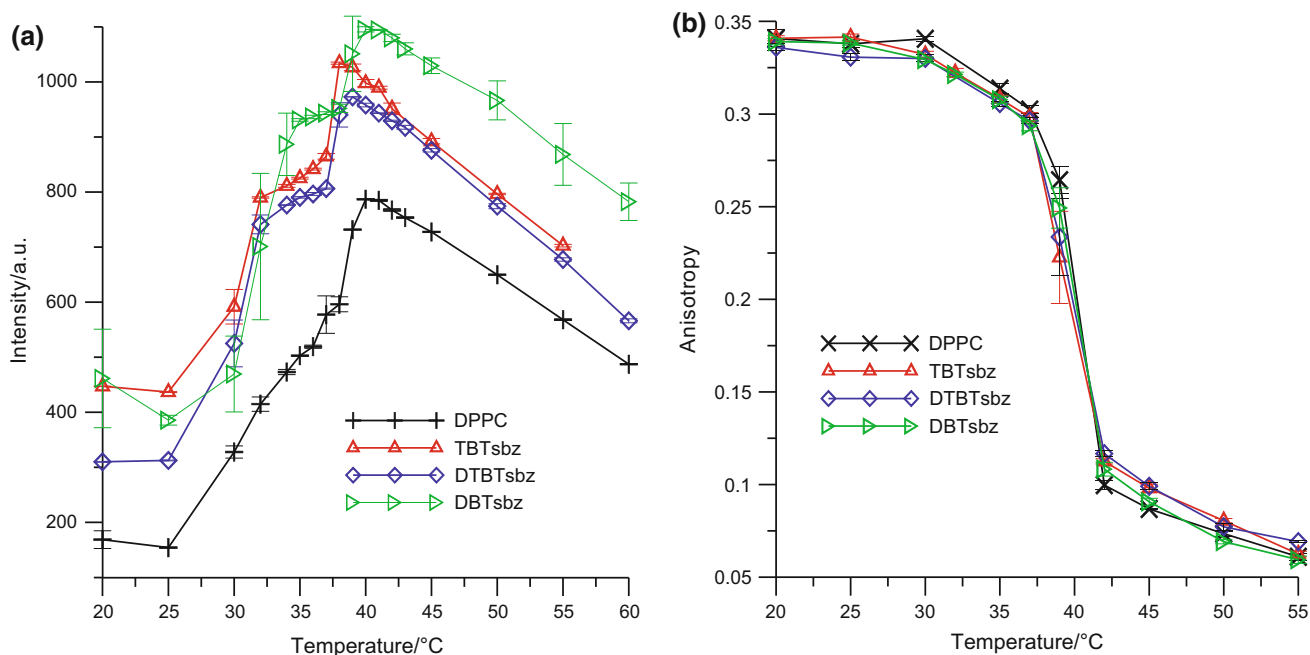
Fig. 7 Fluorescence intensity of MC540 (**a**) and fluorescence anisotropy of DPH (**b**) in MLVs prepared from DPPC as a function of temperature (molar ratio BTsbz/DPPC = 0.2)

conclusions can be drawn from studies with MC540 probe. The negative charge of the MC540 probe determines its location at or near the membrane interface slightly above the domain of the glycerol backbone of phospholipid liposomes [29]. The research results show that fluorescence of MC540 increases in the presence of loosely packed membrane when compared to that in the presence of lipid in the gel phase [29, 30]. In the presence of BTsbz, the fluorescence intensity of MC540 was increasing (Fig. 7a) which suggests decreased organization of lipids, which in turn would indicate increased membrane surface area accessible for the binding of the dye due to the loss of lipid packing [31]. All compounds also cause a slight decrease in the T_m, which can be seen in Fig. 7a as a shift of the maximum intensity toward lower temperatures. Hence, the conclusion is that BTsbz interact mainly with the interfacial and hydrophilic region of the lipid bilayer, which confirms conclusions from our earlier research [10].

Finally, the effect of the BTsbz on the fluidity and the main phase transition of the MLVs formed from DPPC was studied on the basis of anisotropy measured with the DPH probe. The fluorescence steady-state anisotropy is primarily related to the restriction of the rotational motion of the dye to the hydrocarbon chain packing order. Therefore, decrease of the anisotropy parameter can be explained by the structural perturbation of the bilayer hydrophobic region due to incorporation of studied compounds. The dependence of the DPH probe on temperature is presented in Fig. 7b. BTsbz practically do not change fluorescence

anisotropy—we observed only slight decrease in the T_m. These results suggest that BTsbz significantly influence the fluidity in the hydrophobic region of the DPPC bilayers.

Conclusions

The aim of this work was to analyze the effect of newly synthesized butyltin complexes with 2-sulfobenzoic acid on thermotropic phase behavior of DPPC. In this study were used calorimetric and spectroscopic methods to monitor changes in the gel-to-liquid-crystalline transition of DPPC, as this phase change is very sensitive to molecules that interact with the membrane. Obtained results show that all investigated compounds slightly decrease the enthalpy and temperature of the main phase transition of DPPC. TBTsbz and DBTsbz reduce the transition's cooperativity of the peak. With increasing concentration of compounds, the main phase transition gets significantly broader and asymmetrical and at the same time—the pretransition disappears. These results may indicate the BTsbz preferentially intercalate onto head group region and to some extent—into polar–apolar region of the lipid bilayer.

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