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CELL BIOPHYSICS

The Combined Effects of Nitrates on Multibilayer Lipid Membranes: Thermodynamic Effects

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Abstract—Model multibilayer membranes based on *L*-α-dimyristoylphosphatidylcholine containing nitrates of silver, sodium, potassium, and copper as $AgNO_3$–$NaNO_3$, $AgNO_3$–KNO_3, and $AgNO_3$–$Cu(NO_3)_2$ pairs were investigated. In each system studied the molar fraction of nitrates relative to the lipid was kept unchanged at 0.35, whereas the molar fraction of silver nitrate (x_{Ag}) was varied from 0.0 to 1.0 within the pair. Thermodynamic parameters of the main phase transition and pre-transition of the model membranes were determined using differential scanning calorimetry. Positive deviations from additivity as a function of x_{Ag} for a number of these parameters were detected, including changes in the main phase transition and the pre-transition temperatures of up to 0.5 and 2.7°C, respectively; the deviation for hysteresis and half-width of the main phase transition reached up to 30%. The physicochemical mechanisms of competitive interactions between cations in membranes composed of *L*-α-dimyristoylphosphatidylcholine are discussed.

Keywords: silver nitrate, multibilayer lipid membranes, differential scanning calorimetry, combined effect

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INTRODUCTION

Membranes of living cells are surrounded by a multitude of different compounds that can change their properties and functionality [1–7]. Ions, which directly influence the protein-carbohydrate part of the membranes as well as their lipid bilayer, represent one of the regular components of the membrane environment [8–13]. In addition to their role in physiological processes, membranotropic properties of ions can manifest at the organism level as either therapeutic or toxic. As an example, the bactericidal action of silver ions, which are traditionally and widely used in pharmacological preparations [14], is largely related with their interactions with cell membranes [15].

In pharmacology it is widely accepted that the initial effect of pharmacological preparations can be enhanced or weakened in the presence of various compounds, including drugs, food, and cellular components; this is very important in pharmacological therapy [16–18]. The mechanisms of combined effects of various compounds can be formally divided into direct (formation of compounds, complexex, etc.) and indirect (contribution to the same physiological process, reaction, change of the environmental properties, etc.) interactions. Direct interactions of pharmacological preparations are observed at the level of a lipid bilayer [19, 20]. The outcomes of indirect combined effects of pharmacological compounds at the lipid bilayer level are of the especial interest as they are currently the least predictable and can be mostly determined experimentally. It is apparent that the results of modeling studies cannot be automatically expanded to describe a living organism, however they are valuable in defining certain aspects of various processes. In fact, the importance of this approach is confirmed by the increasing use of model lipid systems in applied investigations [3–5].

The high reactivity of silver ions significantly limits the variety of compounds for studying their indirect combined effects. Among most suitable candidates are metal cations that don't interact directly with silver cations due to electrostatic repulsion. Taking into account that binding of silver ions with the cell membrane occurs in a multicomponent ionic environment that contains sodium and potassium ions, there was of interested to define the combined effects of silver with each of these types of cations. Another task of practical interest was to determine the combined effects of silver and copper cations, which are considered also possesses antimicrobial and membranotropic properties [13, 21, 22].

MATERIALS AND METHODS

Salts of special purity grade $AgNO_3$, $NaNO_3$, KNO_3, and $Cu(NO_3)_2$ were used as a source of cations. Lipid multibilayer membranes, model membranes and were prepared using *L*-α-dimyristoylphos-

The phase transition parameters of model DMPC membranes containing nitrates

DMPC +	Main transition				Pre-transition			
	T_m, °C	ΔH_m, kJ/kg	$\Delta T_m^{1/2}$, °C	h_m, °C	T_p, °C	ΔH_p, kJ/kg	$\Delta T_m^{1/2}$, °C	h_p, °C
–	24.7	16.4	0.82	1.0	15.6	2.9	1.51	2.7
$AgNO_3$	25.1	15.6	0.71	1.2	17.8	2.0	1.27	7.4
$NaNO_3$	24.3	16.7	0.68	0.9	12.3	1.6	2.25	5.6
KNO_3	24.2	15.5	0.68	0.9	12.6	1.1	1.59	4.7
$Cu(NO_3)_2$	25.9	17.0	0.91	1.4	20.7	0.8	2.12	8.1

phatidylcholine (DMPC) from Sigma-Aldrich (USA) and aqueous solutions of corresponding nitrates. To prepare the model membranes, crystalline DMPC was supplemented with double distilled water or an appropriate salt solution. The samples were then incubated for 6–7 days at room temperature with periodic heating to 50°C under intense stirring. The content of the water was 65 wt % and was maintained constant throughout the process of sample preparation and measurements. This was achieved by weighing the samples using XP26 balances (Mettler Toledo, Switzerland) with an accuracy of 0.01 mg and adding water if required.

The model DMPC membranes were investigated containing the following pairs of nitrates: $AgNO_3$–$NaNO_3$, $AgNO_3$–KNO_3, and $AgNO_3$–$Cu(NO_3)_2$. The molar fraction of nitrates relative to *L*-α-dimyristoylphosphatidylcholine was 0.35, whereas the molar fraction of $AgNO_3$ (x_{Ag}) within each pair was varied from 0.0 to 1.0.

Differential scanning calorimetry (DSC) studies were carried out using a DSC-1 calorimeter (Mettler, Switzerland). The samples (15–25 mg) were placed into aluminium crucibles and sealed. For each sample two successive cycles of heating and cooling were performed at a temperature scanning rate of 2 K/min which is commonly used for such systems [24]. Based on the DSC thermograms the following several parameters of phase transitions $L_{\beta'} \leftrightarrow P_{\beta'}$ (pre-transition, p index) and $P_{\beta'} \leftrightarrow L_\alpha$ (main transition, m index) were determined: temperature (T_m, T_p), enthalpy (ΔH_m, ΔH_p), hysteresis (h_m, h_p), and half-width ($\Delta T_m^{1/2}$, $\Delta T_p^{1/2}$). The hysteresis of phase transitions was determined as the difference in transition temperatures of heating and cooling scanning processes: $h_m = T_m^{heat} - T_m^{cool}$ and $h_p = T_p^{heat} - T_p^{cool}$. The experimental errors were as follows: $\delta T = 0.1$°C, $\Delta H = 1.2$ kJ/kg, $\delta \Delta T^{1/2} = 0.05$°C, and $\delta h = 0.1$°C.

RESULTS AND DISCUSSION

Initially various parameters of the model DMPC membranes in the presence of individual nitrates were determined (table). As can be seen from the table, the phase transition temperatures were reduced in the presence of $NaNO_3$ and KNO_3 and increased in the presence of $AgNO_3$ and $Cu(NO_3)_2$ with the absolute value effects of KNO_3 and $Cu(NO_3)_2$ being higher than those of $NaNO_3$ and $AgNO_3$, respectively. The pre-transition was found to be more sensitive to the action of nitrates than the main transition. The hysteresis of both transitions in the presence of $AgNO_3$ and $Cu(NO_3)_2$ was significantly higher than in the presence of $NaNO_3$ and KNO_3. In all the systems certain correlations were observed between the parameters reflecting the degree of molecular order, namely, hysteresis and half-width. The transition enthalpies showed essentially no change within the accuracy of measurements. The changes in thermodynamic parameters of the model DMPC membranes containing nitrates are in good agreement with the results of studies that employed membranes based on *L*-α-dipalmitoylphosphatidylcholine containing nitrates [13] and DMPC membranes in the presence of chlorides [25].

The DSC profiles of DMPC membranes upon introduction of equimolar amounts of $AgNO_3$ and KNO_3 closely resemble those of DMPC membranes that contain $AgNO_3$ alone (Fig. 1, curves *2* and *3*). Similar dependencies were observed for other pairs of nitrates. These results indicate that the competitive binding of the investigated nitrates with the lipid membranes might take place.

To study this effect further, the method of quasi-binary diagrams was employed [26]. In accordance to this method a phospholipid medium is considered as a matrix, in which interactions between two compounds introduced can occur [23]. In the absence of specific interactions between the components, various param-

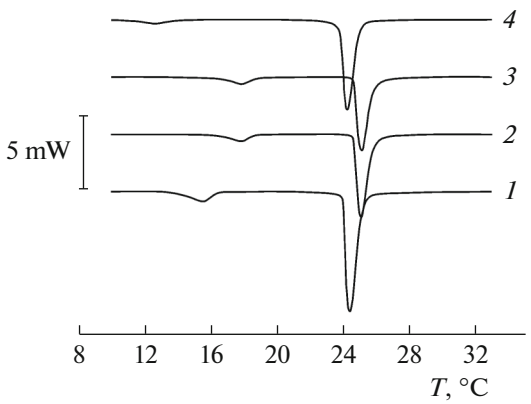

Fig. 1. The DSC thermograms of model DMPC membranes with (*1*) no additives or containing (*2*) $AgNO_3$, (*3*) $AgNO_3$ and KNO_3 at a molar ratio of 1 : 1, and (*4*) $AgNO_3$.

eters of the system should change according o linear low. Consequently, deviations of the parameters from concentration additivity indicate the existence of their specific interactions between the components.

The phase transition temperature, T_m, is especial sensitive and at the same time highly reproducible thermodynamic parameter. For each of the nitrates investigated, the T_m parameter exhibits additive dependence by the molar fraction of up to 0.35 [25, 27]. The relationships between the additivity dependence of T_m and the absence of specific interactions between the components introduced into a membrane have been described in [28].

The shift of the main transition temperature of the DMPC membrane in the presence of various nitrates (ΔT_m) is shown in Fig. 2a as a function of the molar fraction of $AgNO_3$ relative to the total content of nitrates in the membrane (x_{Ag}). The straight line connecting data points correspond to single-nitrate systems containing $AgNO_3$ alone ($x_{Ag} = 1$) and the second nitrate only ($x_{Ag} = 0$) represents the additive dependence of T_m. Deviations from linearity (δ_m) were detected for all systems and were statistically significant (($\delta_m = 0.2–0.5$°C) and positive (Fig. 2b). The maximum deviation from additivity for the $AgNO_3$–$Cu(NO_3)_2$ system was observed at $x_{Ag} \sim 0.3$, which corresponds to a molar ratio of nitrates of 2 : 1 (shown as dotted line in Fig. 2b). The deviation maxima of the $AgNO_3$–$NaNO_3$ and $AgNO_3$–KNO_3 systems were observed at approximately the equimolar content of nitrates. It is important to mention that similar ratio of $AgNO_3$ and KNO_3 is used in caustic pencils [19].

Positive deviations of the pre-transition temperature from linearity (δ_p) were also detected in all systems and these deviation values ($\delta_p = 0.3–2.7$°C) were significantly higher than those in the case of δ_m (Fig. 3). It should be noted that the δ_p maxima correspond to lower x_{Ag} values compared to the δ_m maxima. This may result from the different distribution of ions in the membrane in the $L_{\beta'}$- and L_α-phases.

Deviations from additivity were observed for several other thermodynamic parameters of the systems. The hysteresis and half-width values of the main phase transition for the $AgNO_3$–$Cu(NO_3)_2$-containing sys-

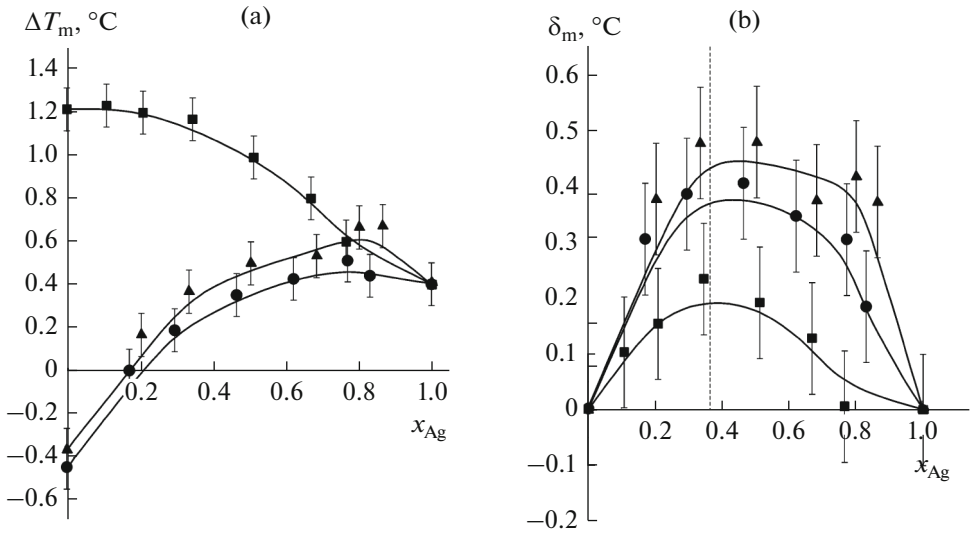

Fig. 2. The main phase-transition temperature dependence on the molar fraction of silver nitrate relative to the total nitrate content (x_{Ag}) for DMPC membranes containing $AgNO_3$–$Cu(NO_3)_2$ (squares), $AgNO_3$–$NaNO_3$ (triangles), and $AgNO_3$–KNO_3 (circles): a, shift relative to the DMPC membrane, ΔT_m; b, deviation from additivity of the ΔT_m (x_{Ag}) dependence, δ_m. Dashed line represents $\delta_m(x_{Ag})$ for the $AgNO_3$–$Cu(NO_3)_2$ pair.

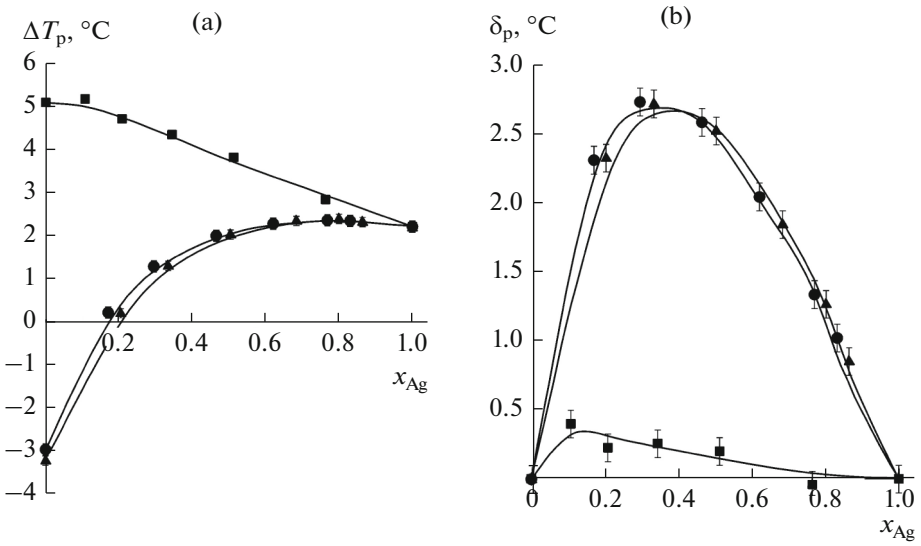

Fig. 3. The pretransition temperature as a function of the molar fraction of silver nitrate relative to the total nitrate content (x_{Ag}) for DMPC membranes containing $AgNO_3$–$Cu(NO_3)_2$ (squares), $AgNO_3$–$NaNO_3$ (triangles), and $AgNO_3$–KNO_3 (circles): a, the shift relative to the DMPC membrane (ΔT_p); b, the deviation from additivity of the (ΔT_p (x_{Ag}) dependence, δ_p.

tem are shown in Fig. 4. The deviations of both values from additivity were of up to 30%, while the $h_m(x_{Ag})$ and $\Delta T_m^{1/2}$ maxima correspond to a molar ratio of 2 : 1 as was observed for ΔT_m (see Fig. 2). Positive deviation values indicate disordering of lipids in the membrane, which may provide an additional positive contribution to their bactericidal effect. It should be noted that similar deviations of these parameters were observed for $AgNO_3$–$NaNO_3$ and $AgNO_3$–KNO_3; however the effects were less pronounced.

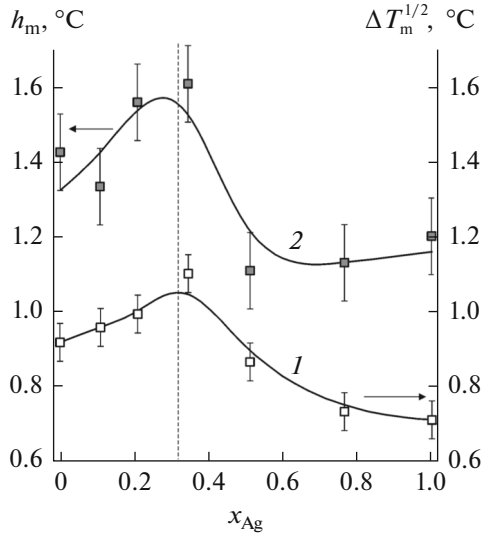

Fig. 4. The half-width (curve *1*) and hysteresis (curve *2*) of the DMPC membrane main phase transition as functions of the molar fraction of silver nitrate relative to the total nitrate content (x_{Ag}) in the $AgNO_3$–$Cu(NO_3)_2$ pair. The maximum deviation from linearity is shown by the dashed line.

Thus, deviations of several thermodynamic parameters from additivity were observed for all the nitrate pairs that were studied and the deviation values increased with elevating in the differences in the individual membranotropic action of the nitrates. It should be noted that at concentrations used in our experiments the nitrates were fully dissolved in water so that the cations and anions were relatively independent of each other. Therefore, the joint membranotropic action of these salts can be represented as a superposition of membranotropic effects of cations and anions as was previously demonstrated for earth metal nitrates and chlorides [25].

We will now attempt to draw a picture of the ion distribution in the near-surface area of the membrane. It is known that cations are preferentially adsorbed on the membrane surface of phosphatidylcholines due to increased accessability of negatively charged phosphate groups [20, 21]. The anions contact with positively charged centers that are shielded by methyl groups ($N(CH_3)_4^+$) and thus are located slightly further away from the middle of the bilayer [22]. The Na^+, Ag^+, and Cu^{2+} cations are considered to be kosmotropic, whereas K^+ is a chaotropic ion [23]. The introduction of any cations of the first group into a membrane leads to an increase in T_m and T_p, the opposite effect being observed upon the introduction of K^+ [25]. It should also be noted that the addition of NO_3^-

induces a reduction in T_m and T_p [13]. Given that the NO_3^- anion is common between all pairs of nitrates examined and direct contact of cations can be excluded due to electrostatic repulsion, the deviations of the membrane parameters are highly likely to result from with the adsorption of cations on its surface.

The deviations of various parameters towards a specific cation indicate its advantage in a competition for binding to the lipid membrane. Then, in the Ag^+–Cu^{2+} pair, the preferential binding to the membrane is observed for Cu^{2+} cations, that could be caused by their higher charge. It should be noted that the double number of moles of the nitrate ion in $Cu(NO_3)_2$, which provides an additional negative contribution to the ΔT_m parameter, should not be considered as an independent factor, as it contributes to the membranotropic effect of copper nitrate (Fig. 2, $x_{Ag} = 0$).

Competitive interactions in the Ag^+–Na^+ and Ag^+–K^+ pairs can be explained by the empirical rule of Peskov–Fayants. According to this rule, an advantage in competitive adsorption gains an ion, which can form a poorly soluble compound with another ion that is a part of the sorbent [34]. An ion that binds cations on the membrane surface is represented by a phosphoric acid residue, while the solubility of silver phosphate is substantially lower than that of potassium and sodium phosphates [35]. Thus, the Ag^+ cations have an advantage in competitive binding to the lipid membrane in Ag^+–Na^+ and Ag^+–K^+ pairs.

Thus, the method that was employed in this work appears to be informative for studying a quasi-binary system, even in the absence of direct interactions between the components.

CONCLUSIONS

The thermodynamic phase transition parameters of DMPC model membranes containing pairs of $AgNO_3$–$NaNO_3$, $AgNO_3$–KNO_3, and $AgNO_3$–$Cu(NO_3)_2$ were determined at the equal total content the nitrates. Deviations from additivity of temperatures of the main phase transition and the pre-transition, as well as their half-width and the hysteresis were detected. The experimental data indicate the existence of competitive adsorption of cations at the lipid-water interface. In the Ag^+–Cu^{2+} pair, the copper cations bind to DMPC membrane preferentially, while in Ag^+–Na^+ and Ag^+–K^+ pairs, the Ag^+ cations have an advantage in binding to the membrane. The $AgNO_3$–$Cu(NO_3)_2$ pair at a molar ratio of components of 2 : 1 is especially interesting and its addition induces disordering of the lipid membrane, which may provide an additional contribution to the bactericidal effect of these nitrates.

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