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Lyotropic Mesophase of Hydrated Phospholipids as Model Medium for Studies of Antimicrobial Agents Activity

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The lyotropic L_α*-phase of hydrated dipalmitoylphosphatidylcholine has been proposed as a solvent medium for modeling the effects of membranotropic agents upon cell membranes, including interactions between different agents inside the membrane. For quasibinary systems containing bisquaternary ammonium compounds decamethoxinum or aethonium and 2,5-dihydroxybenzoic acid, calorimetry data allowed us to establish the stoichiometry of the intermolecular complexes incorporated in the membrane. The interaction mechanisms have been clarified using additional data obtained with tetramethylammonium and* l*-menthol, which model the moieties of the decamethoxinum molecule separated by the alkyl chain. Possible implications of the obtained results for pharmacology are discussed.*

Keywords Combined drugs activity; differential scanning calorimetry; drugs effects; lyotropic mesophases; phase transitions; phospholipid model membranes

1. Introduction

Lamellar structures formed in water dispersions of certain phospholipids are an example of mesomorphic systems with a "dual" character of their liquid crystalline (LC) behavior. Since the mesophases in such systems are formed by ordered amphiphilic molecules in the water medium, they are naturally considered as lyotropic. From another point of view, the apparent behavior of hydrated phospholipids can be very similar to thermotropic LC systems. Typical examples are dialkanoylphosphatidylcholines and ethanolamines. Thus, hydrated DPPC (1,2-dipalmitoyl-*sn*-glycero-phosphocholine) shows clear and well-reproducible LC phase sequence L_β'—P_β'—L_α with two mesomorphic phase transitions at ~36°C and ~42°C [1,2].

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The phase transition temperatures are practically the same in a very wide range of water content (~20–80% or even wider), and structures of the LC phases are very similar to thermotropic smectic-A (L_α) or smectic-H (L_β').

Hydrated DPPC was often used as a matrix for introduction of chemical substances of pharmacological relevance [3–5]. It was assumed that the effect of the introduced substance upon LC phase transitions could be related to its pharmacological activity, since the lamellar LC structure of hydrated phospholipids closely resembled the structure of cell membranes. Using for these introduced substances the term "membranotropic agents" (MTA), one can note a close analogy between MTA and commonly known non-mesogenic dopants (NMD) introduced in conventional thermotropic mesophases (nematics, cholesterics, etc.) and affecting their LC transition temperatures. Within this approach, in our previous works [6,7] we used decamethoxinum and aethonium as MTA in hydrated DPPC; effects of MTA introduction upon temperature and enthalpy of P_β'—L_α transition were specified and discussed with relationship to possible mechanisms of antimicrobial activity of these substances.

The idea of our present study was to use MTA of two different types which could presumably interact with each other, and this interaction could probably be reflected in non-additivity of the recorded effects on phase transitions of hydrated DPPC.

The MTA selected for such model studies were two antimicrobial agents based on bisquaternary ammonium salts – decamethoxinum and aethonium, and 2,5-dihydroxybenzoic acid (DHB). The latter compound bearing a trivial name of gentisic acid is a known metabolite of aspirin [8]. These two types of compounds can meet simultaneously in a living organism on its treatment from microbial infections and inflammations.

Similar approaches (the method of quasi-binary systems, when the ratio of two interacting solutes is varied, and the total concentration of the solutes in a presumably indifferent solvent is kept constant) were used for studies of intermolecular interactions in thermotropic LC systems using isotropic [9], cholesteric [10] and smectic [11] solvents.

2. Experimental Methods

The liquid crystalline lamellar lyotropic L_α-phase was formed in water dispersions (50:50) of dipalmitoylphosphatidylcholine (DPPC) obtained from Alexis Biochemicals, Switzerland. As membranotropic agents (MTA), we used bisquaternary ammonium compounds (BQAC)—decamethoxinum and aethonium (synthesized in the Institute for Bio-organic Chemistry of NAS of Ukraine), as well as 2,5-dihydroxybenzoic acid (DHB) obtained from Sigma, Germany. The chemical structures of decamethoxinum, aethonium and DHB are shown in Figure 1.

The sample preparation included mixing of MTA with DPPC in the proper ratios, adding bi-distillated water (DPPC:water 50:50 by mass) and then preparing the multilamellar model membranes according to a standard procedure described in [3,6]. In some experiments, we used a quaternary ammonium compound (QAC) tetramethylammonium chloride (TMA:Cl) and *l*-menthol as MTA molecules modeling some parts (specific moieties) of the decamethoxinum molecule; their concentrations were chosen to ensure approximately the same volume fraction as 5 wt.% of decamethoxinum.

DSC thermograms were measured using a Mettler TA 3000 thermoanalytical system (Switzerland). The samples investigated (15 to 25 mg) were placed into an

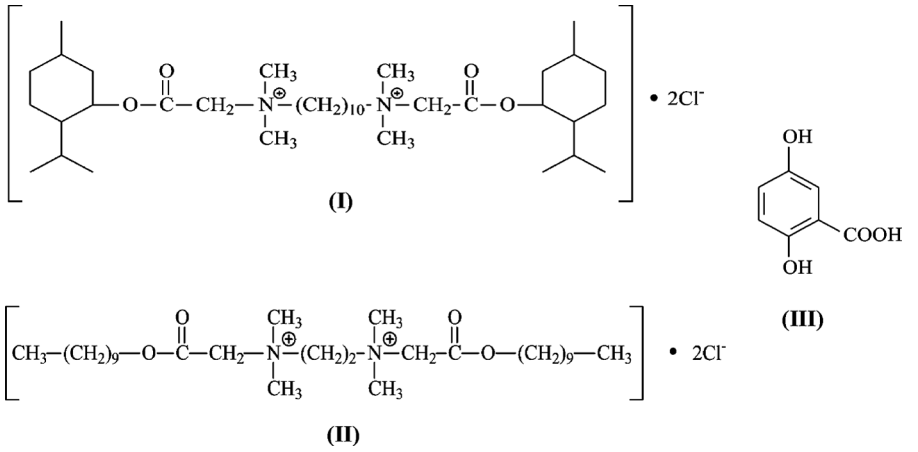

Figure 1. Molecular structure of bisquaternary ammonium agents decamethoxinum and aethonium (I, II) and 2,5-dihydroxybenzoic acid (III).

aluminum pan and subjected to several series of heating and cooling at the scanning rate 2 K/min. The values of phase transition temperatures were obtained using the calculation algorithm of the instrument software.

The MTA activity parameter was determined from the ratios:

$$a_{wt} = \Delta T_m / c_{wt}; \quad a_{mol} = \Delta T_m / c_{mol}, \tag{1}$$

were a_{wt} and a_{mol} are, respectively, weight and molar activity parameters, ΔT_m is the shift of the main transition (P_β'—L_α) temperature in the DPPC model membrane due to MTA introduction, c_{wt} and c_{mol} are the weight and molar concentrations of the corresponding MTA in DPPC.

The peak asymmetry parameter a_r was obtained from the ratio:

$$a_r = \frac{l_r}{l_r + l_l}, \tag{2}$$

where l_r and l_l are the right and the left parts of the half-width of the DSC peak related to the center of the peak, correspondingly.

3. Results and Discussion

3.1. *Method of Quasi-Binary Systems Based on DSC Data*

In order to answer whether the complexes of the cations of BQAC/QAC with DHB, (recorded and theoretically considered in our previous works [12,13]), are also formed in a model phospholipid medium, we used the method of quasi-binary systems [14]. This method is based on the concept of presumed additivity of thermodynamical parameters (e.g., temperatures) of a mesomorphic phase transition over the weight (volume) ratio of the dopant components, i.e., the ratio of MTAs introduced into the phospholipid solvent (matrix) in the case of absence of specific intermolecular interactions and complex formation. That is, a diagram in coordinates

'parameter vs. dopant fraction' should appear as a straight line. Any specific interactions or formation of intermolecular complexes in the system should lead to deviations from linearity on the quasi-binary diagram. The component ratio corresponding to the maximum deviation indicates the most probable stoichiometry of the complex.

The quasi-binary diagrams obtained (Fig. 2) bear witness to formation of an intermolecular complex in the system "BQAC + DHB" in the phospholipid model membrane medium. The most probable stoichiometry appears to be BQAC:DHB 1:2. Taking into account the structure of the molecules involved (see Fig. 1) and their dissociation in the water medium, such ratio corresponds to incorporation into the membrane of a fully neutral complex with two ammonium centers bound to the deprotonated form of DHB–$(DHB\text{-}H)^-$. A similar quasi-binary diagram was obtained for the system "TMA + DHB", suggesting the most probable stoichiometry QAC:DHB 2:1.

It was of substantial interest to compare our DSC results for "BQAC/QAC + DHB" complexes in the phospholipid medium with other results for such systems obtained in our previous studies (Table 1). As to QAC TMA, the complexes forming in the phospholipids medium appear to be similar to those observed in a low density gas phase by means of mass spectrometry [12] rather than to structures predicted by quantum-chemical calculations for the liquid (water) phase [13]. On the basis of that, one can suppose that in the phospholipid membrane medium the complex formed is located not in the interlayer "bulk" water, but inside the bilayers. In the case of BQAC + DHB, this supposition is even more justified, because in the vacuum medium complexes BQAC:DHB 1:2 and 1:3, similar to those observed into DPPC multilayers, have been observed just in the presence of DPPC. Thus, it can be

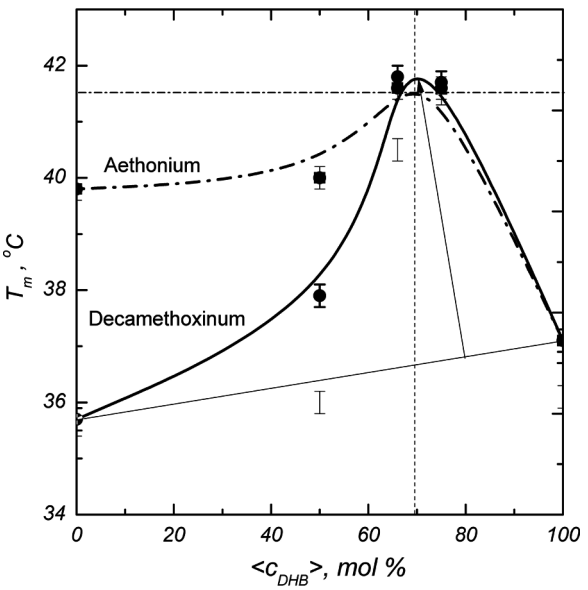

Figure 2. Quasi-binary phase diagram for the systems BQAC + DHB in the DPPC model membrane medium. The vertical dotted line marks the stoichiometry of the most probable complex formation.

Table 1. BQAC/QAC+DHB optimum stoichiometry determined by different methods in various media

Medium \ Method	Mass-spectrometry	DSC	Theoretical calculations (DFT, MP2, ab initio)
Vacuum approach (low density gas phase)	$BQAC^{2+}$:$[DHB\text{-}H]^{-}$ $[BQAC\text{-}H]^{+}$: $2[DHB\text{-}H]^{-}$* $BQAC^{2+}$:$3[DHB\text{-}H]^{-}$* $2TMA^{+}$:$[DHB\text{-}H]^{-}$		TMA^{+}:DHB^{0} TMA^{+}:$[DHB\text{-}H]^{-}$ $2TMA^{+}$:$[DHB\text{-}H]^{-}$
Water environment (isotropic liquid phase)			TMA^{+}:DHB^{0} TMA^{+}:$[DHB\text{-}H]^{-}$
Phospholipid model membrane (liquid crystal phase)		$2TMA^{+}$:DHB $BQAC^{2+}$:2DHB	

*In the ternary system $\langle\langle BQAC + DHB + DPPC\rangle\rangle$.

summarized that complex formation between BQAC/QAC and DHB is sensitive to the surrounding medium. It is of great importance from the pharmacological point of view, since interactions between various pharmacological agents occur in the complex medium of human body bearing some features of liquid crystal ordering (e.g., in cell membranes), while most *in vitro* studies are carried out in standard isotropic solvents.

3.2. *Membranotropic Activity and Its Changes Under Complex Formation*

In order to correctly compare the results obtained for systems with different MTA concentration, it is reasonable to introduce appropriate parameters of membranotropic activity. Their physical meaning can be determined as the values of effects caused by MTA with respect to their weight (volume) or molar fraction. Table 2 presents both primary data on the P_β'—L_α phase transition temperature shift ($\Delta\mathbf{T_m}$) and the corresponding membranotropic activity parameters obtained by equation (1) (see part **2**). According to the table data, it is possible to determine some regularities as to the MTA action that became especially clear from Figure 3. Thus, for all MTA investigated their action undergoes significant changes in the presence of DHB in certain ratios. For system TMA + DHB, the 1:1 ratio leads to a certain increase of a_{wt}, but 2:1 ratio results in its decreasing, whereas the individual DHB has strong activity of the opposite sign. For the BQAC + DHB system, the ratio corresponding to the most probable stoichiometry becomes an inversion point where the sign of activity changes. Also, there is a remarkable difference between a_{wt} values for BQAC decamethoxinum and aethonium in spite of similarity of their bisquaternary nature. This can be explained taking into account the presence of two menthyl groups in the decamethoxinum molecule and the fact that *l*-menthol as individual substance has very large a_{wt} value.

Table 2. Parameters of MTA effect on calorimetric parameters of model DPPC membrane phase transition: shifts of $P_\beta' - L_\alpha$ phase transition temperatures and corresponding activity parameters calculated by Equation (1)

		MTA activity	
MTA	ΔT_m,°C	a_{wt}, K/wt. %	a_{mol}, K/mol
TMA	1.0	0.38	6.7
DHB	−4.4	−1.54	−36.2
TMA:DHB 1:1	0.5	0.16	6.4
TMA:DHB 2:1	1.4	0.29	25.8
Decamethoxinum	−5.8	−0.95	−95.5
Decamethoxinum:DHB 1:1	−3.6	−1.04	−93.0
Decamethoxinum:DHB 1:2	0.3	0.08	10.2
Decamethoxinum:DHB 1:4	0.2	0.06	10.1
Aethonium	−1.7	−0.32	−27.0
Aethonium:DHB 1:1	−1.5	−0.28	−29.3
Aethonium:DHB 1:2	0.1	0.04	5.0
Aethonium:DHB 1:4	0.1	0.03	3.3
Menthol	−8.5	−3.36	−79.5

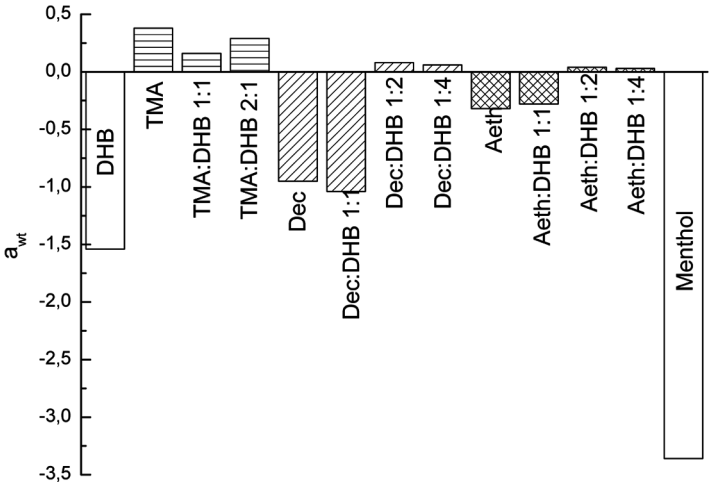

Figure 3. Parameters of MTA activity in DPPC model membrane. Abbreviations: Dec – decamethoxinum, Aeth – aethonium.

Returning to Table 2, certain additional notes should be made. Strictly speaking, T_m as a mesomorphic transition temperature is a volume-additive property, so expression of the MTA activity in weight fraction units is quite correct. Nevertheless, to avoid uncertainties we present both "volume" ("mass") and "molar" activity parameters. It can be useful in the cases when molecules interact not as whole particles (as in mean-field models of mesophases) but by certain active centers (e.g., forming stoichiometric intermolecular complexes). An obvious example can be decamethoxinum and menthol. At equal weight concentrations, the decamethoxinum activity is much less as compared with *l*-menthol. But when significant difference in their molecular weight is taken into account, menthol appears a less active MTA than decamethoxinum. In this way, it becomes clear that it is the presence of menthol moiety that is responsible for higher MTA activity of decamethoxinum as compared to aethonium. In general, most of the features noted for a_{wt} are also characteristic for a_{mol}.

3.3. *Peak Asymmetry Parameter as a Source of Data on MTA Complex Formation*

It is known that introduction of a dopant into a liquid crystal matrix can result in deformation of mesomorphic transition peaks, e.g., nematic—isotropic, with peak shape becoming asymmetric. It can be explained in terms of affinity of the dopant to low- or high temperature phase of the matrix. In the case of low-temperature phase affinity, one can observe extension of the left shoulder of the melting peak; correspondingly, the right shoulder extension is caused by high-temperature phase affinity of the dopant. In order to quantitatively compare the asymmetry of different peaks, we used a simple but rather objective form of expression (see equation (2) in Chapter **2**). In such form of expression, for fully symmetric peaks $a_r = 0.5$, for low-temperature phase affinity this value decreases and for low-temperature phase affinity it increases.

The asymmetry parameter values were obtained for all the systems investigated, revealing a number of interesting features (some of them are shown in Fig. 4). First

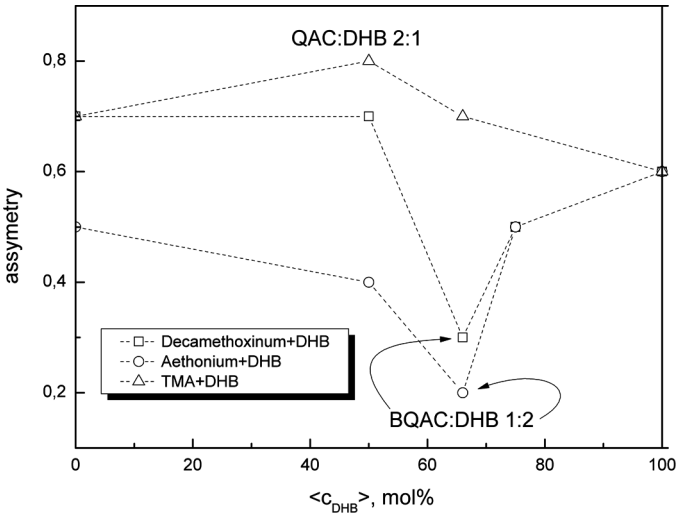

Figure 4. Peak asymmetry parameter changes according to the composition of the MTA introduced.

of all, it should be noted that in all cases a_r values strictly correspond to the sign of MTA activity parameter (Table 2): all MTAs that increase P_β'—L_α phase transition temperature have $a_r > 0.5$, while those decreasing it have $a_r < 0.5$. Then, for all the systems studied, a significant deviation is observed at the DHB concentration corresponding to the most probable stoichiometry of the intermolecular complex. The direction of deviation in each case shows greater peak asymmetry at the point of complex formation, which can be attributed to changes in the effective form of MTA.

4. Conclusions and Perspectives

In this study, it was established that combined introduction of BQAC/QAC and DHB into the liquid crystalline lamellar lyotropic L_α-phase of DPPC (as a model of phospholipid membranes) results in remarkable shifts of the membrane melting temperature, which can be both positive and negative. This can imply possible weakening of the BQAC antimicrobial activity due to formation of intermolecular complexes between BQAC and DHB. Using the physico-chemical approach of quasi-binary systems, we established the stoichiometry of complexes formed in the phospholipid medium—QAC:DHB 2:1 and BQAC:DHB 1:2, which is in agreement with our previous results obtained by other methods (mass-spectrometry, MP2 calculations etc.). The complex activity appears to be much lower, and it has the opposite sign as compared to individual MTA.

Varying the structure of MTA (i.e. using TMA and *l*-menthol as molecules modeling specific moieties of the BQAC examined), separation of the contributions to MTA activity from different fragments of MTA molecule became possible.

The asymmetry parameter a_r proposed for quantitative comparison of calorimetric peak shapes was shown to exhibit remarkable sensitivity to complex formation between MTA examined, as well as to their affinity to high- or low-temperature phase.

Thus, the method of quasibinary systems based on DSC data for phase transitions in phospholipid model membranes (used as solvent medium for pharmacologically relevant substances) could be promising for studies of combined action of various membranotropic agents, which is especially important in the case of drugs.

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