Effect of glycyrrhizic acid on phospholipid membranes in media with different pH*

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Glycyrrhizic acid (GA) is the active ingredient in licorice root, which exhibits a wide range of biological activities, including anti-inflammatory and antiviral activities. In particular, the virus-inhibiting effect of GA on SARS-associated coronavirus was demonstrated. In addition, GA was found to be capable of increasing bioaccessibility of other drugs when used together. All these effects can be based on the ability of GA to incorporate into cell membranes and change their physical and functional properties. One of the possible mechanisms of the antiviral action of GA against COVID-19 is also considered to be the prevention of fusion of the virus envelope with the plasma membrane of the host cell. The interaction of GA with model lipid membranes was studied by the NMR method. Different factors influencing the incorporation of the GA molecule into the lipid bilayer (phospholipid structure, pH of the medium) were examined.

Key words: glycyrrhizic acid, lipid membranes, micelles, liposomes, antiviral activity, NMR.

A biological membrane forms a boundary between the intra- and innercellular environments and performs numerous functions necessary for cell viability. The membrane is the site of arrangement of numerous proteins performing the transport and protection functions. Lipids contribute to from 25 to 75% of the cell membrane weight. Phospholipids are among the main components of the most part of membranes. Phosphtidylcholine is most abundant (up to 50% of the overall amount of lipids) in animal tissues. Fatty acids with the linear chain and 16 and 18 carbon atoms are most abundant natural compounds in organisms of animals and plants.[1]

It is known that some properties of membranes, particularly, the density of the surface charge of the membrane and transmembrane potential, depend strongly on the pH of the medium.[2] At low pH the membrane becomes more flexible. In addition, a change in the pH can induce a change in the phase transition temperature by tens degrees.[3] It is also established that for a mixture of lipids (phosphatidic acid and phosphatidylcholine) the appearance of domains in the liquid crystalline phase depends on pH. Experiments showed that at pH 7, when phosphatidic acid is negatively charged, electrostatic effects favors mixing of two components.[4] The partial protonation of phosphatidic acid at pH 4 results in strong changes in miscibility: the molecules undergo clusterization. Phosphate groups are deprotonated at pH ≈ 1—2. In spite of this, the conformation of phospholipids changes with the further decrease in the pH of the medium.[5] In addition, the acidity of the medium plays a significant role for binding a viral species with the membrane of the host cell. The proteins located on the membrane surface of a viral species undergo rearrangement and self-association at a lowered acidity that is maintained in lysosomes, which plays a significant role in the binding of the viral species to the membrane of host cell.[6]

The following phospholipids were used in this work: dioleoylphosphatidylcholine (1,2-di-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphocholine, DOPC) and dimyristoylphosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC). Their structural formulas are presented below. Lipid bilayers formed by these lipids differ primarily by the degree of ordering, and one of the purposes of this work was to compare the behavior of glycyrrhizic acid molecules as a membrane-modifying agent in ordered and disordered membranes. It is known, for example, that saturated lipids and cholesterol form lipid rafts in the cell membrane.[7] Lipid rafts are targets for the penetration of various viral species into the cell.[8]

Glycyrrhizic acid (GA, glycyrrhizin) is triterpenoid glycoside from licorice roots. Glycyrrhizin is mainly known due to its anti-inflammatory and antiviral activities, although there are data on its positive effect when treating diverse diseases. Interest in GA increased especially during the recent year, which is associated with the discovery of its virus-inhibiting effect on SARS-associated coronavirus (pathogene of COVID-19).[9—11] In addition, GA has a number of properties providing its prospects for drug delivery. This concept is based on the fact that GA is an

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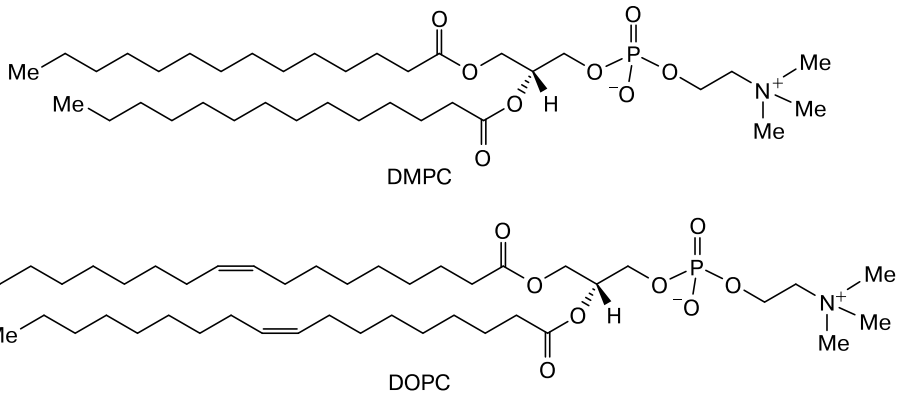


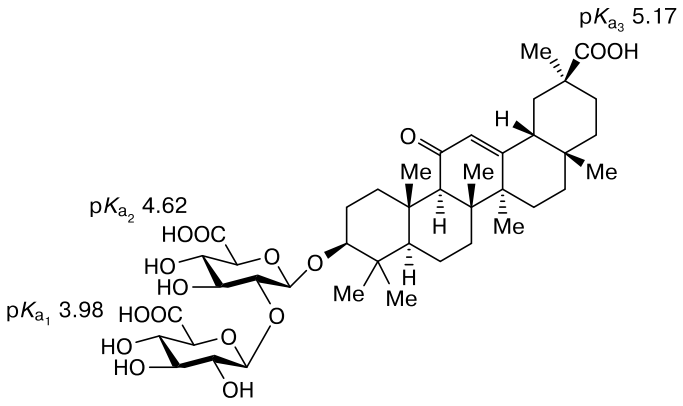
amphiphilic molecule: the hydrophilic moiety is presented by glucuronic acid residues, and the hydrophobic moiety is presented by the glycyrrhetic acid residue.

Owing to this, GA can form micelles in aqueous and aqueous-alcohol solutions and host—guest complexes with various hydrophobic molecules.[12—15] Our recent studies showed that the formation of GA complexes with drugs resulted in a substantial increase in the solubility and bioaccessibility and enhancement of the therapeutic effect and, as a consequence, a decrease in the therapeutic dose of the latter.[12,16,17] We assume that all these effects can be based on the ability of GA to incorporate into cell membranes and to change their physical and functional properties.[18—20] Note that prevention of the fusion of the virus envelope with the plasmatic membrane of the host cell is considered to be one of possible mechanisms of the antiviral effect of GA.[21—23] We have previously[18—20,24—26] shown that GA can affect the phase transition temperature in model lipid membranes, enhance membrane permeability for small molecules, and change the elasticity modulus of living cells and transmembrane potential. A substantial dependence of GA self-association and complex formation with drug molecules on the acidity of the medium was found.[27,28] Continuing these studies, the interaction of GA with model lipid membranes at different pH values of the medium was studied by NMR in this work in order to reveal factors affecting the incorporation of the GA molecule into the lipid bilayer.

Experimental

The studies were carried out on liposomes of two types consisting of saturated or unsaturated phospholipids: dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC, >99%, Avanti Polar Lipids). The preparation of liposomes included their dissolution in chloroform followed by solvent evaporation and hydration of the lipid film in D_2O (99.9% D, Aldrich). The final concentration of the lipid was 13 mmol L^{-1}. The suspension was treated with ultrasound (37 kHz, 1 h) to obtain single-layer liposomes. ^1H NMR spectra were recorded for 0.5-mL samples containing $PrCl_3$ (4 mmol L^{-1}) in order to separate the signal from those of the external and internal semilayers of liposomes.[29] After the addition of $PrCl_3$, GA (0.5 mmol L^{-1}, 98%, Shaanxi Pioneer Biotech Co., Ltd., China) was added to the samples, and DCl and KOD was introduced in concentrations necessary for achievement of a required pH value.

NMR spectra were recorded on a Bruker AVANCE III spectrometer at a frequency of 500 MHz at 30 °C. The standard Carr—Purcell—Meiboom—Gill sequence was used to determine spin-spin relaxation times T_2. The spin-lattice relaxation time (T_1) was measured using the inversion—recovery sequence.



GA

Results and Discussion

The ^{1}H NMR spectra of suspensions of DOPC and DMPC liposomes in the absence and in the presence of GA (0.5 mmol L^{-1}) are shown in Fig. 1. The signal of the $N^+(CH_3)_3$ groups of the lipids splits into two components, which is caused by the addition of the shift reagent $PrCl_3$ to suspensions of liposomes. The splitting is due to the binding of the Pr^{3+} ions to the phosphate group of the lipids forming an external semilayer of the liposome, and thus they do not penetrate into the liposome.[29]

The signal intensity of all functional groups of phospholipid decreases upon the addition of GA to suspensions of liposomes. In the case of the DMPC-based liposomes, the splitting of the signals from the $N^+(CH_3)_3$ groups of the lipids disappears. A decrease in the signal intensity can be due to the enlargement of liposomes. The disappearance of splitting of signals from the external and internal $N^+(CH_3)_3$ groups of the lipid is caused by the penetration of Pr^{3+} ions into the liposomes. Note that this effect is not observed for the DOPC-based liposomes.

The interaction of GA with the model lipid bilayer was studied by analysis of the GA effect on the mobility of individual fragments of lipid molecules by the NMR relaxation method. The decay kinetics of the echo signal for

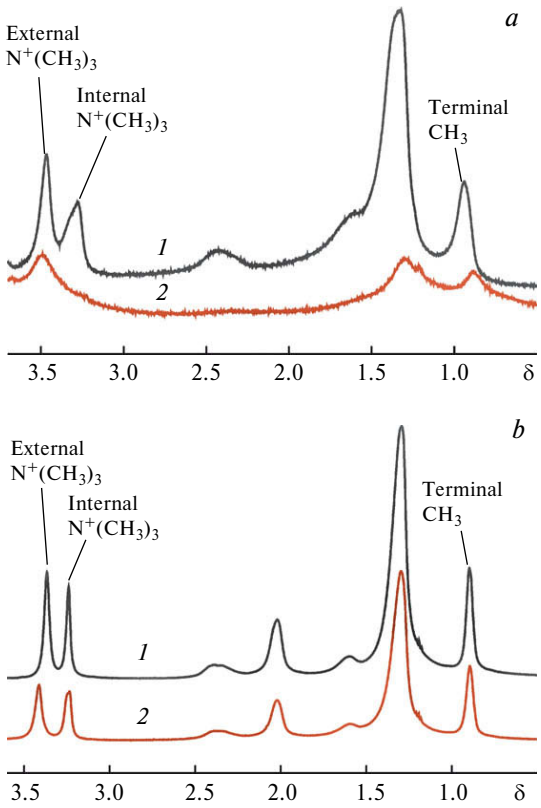


Fig. 1. ^{1}H NMR spectra of suspensions of DMPC (*a*) and DOPC (*b*) liposomes in the absence (*1*) and in the presence of GA (0.5 mmol L^{-1}, *2*); pH 7.

liposomes is biexponential. According to the earlier published data,[30] the spin-spin relaxation time of lipids in liposomes is determined by the rotation of the whole vesicle and lateral diffusion of lipids. The observed dependences of the "long" T_2 component on the pH of the medium in the absence and in the presence of GA (0.5 mmol L^{-1}) are shown in Fig. 2. No influence of GA on the "short" component of T_2 was revealed. This can be caused by the fact that the "short" component is determined by vesicle rotation that is not affected by GA.[19,31]

In the case of DOPC phospholipid, the addition of GA affects the relaxation times of the external $N^+(CH_3)_3$ groups of phospholipid only (see Fig. 2, *a*). A decrease in the relaxation time is observed for all functional groups of DMPC phospholipid. The dependences of the spin-spin relaxation time (T_2) on the pH of the medium for the terminal CH_3 groups of DOPC and DMPC phospholipids are shown in Figs 2, *c*, *d*.

It was shown previously[18,19] by molecular dynamics that GA can form associates inside the lipid bilayer, and the affinity to association increases with the increase in lipid ordering in the bilayer. Probably, the effect on the spin-spin relaxation time is determined by the presence of GA associates inside the membrane, and the GA associates impede lateral diffusion of lipids. No significant effect of GA on the proton mobility in the hydrophobic moiety of the lipid is observed in a less ordered bilayer of DOPC lipid containing double bonds in each acyl chain. The more ordered bilayer of DMPC containing no double bonds exhibits a substantial effect of GA on the proton mobility in the hydrophobic moiety of the lipid, in particular, of the terminal CH_3 groups, and the T_2 time increases with pH, which can indicate that the amount of associates inside the bilayer decreases with the gradual deprotonation of GA. However, an appreciable decrease in the relaxation time in the presence of GA even at pH 7 suggests that GA does not completely shift from the hydrophobic moiety of the bilayer. Probably, the formation of associates prevents GA escape to the aqueous environment and deprotonation.

The dependence of the spin-lattice relaxation time of protons (T_1) of DOPC and DMPC on the pH in the absence and in the presence of GA (0.5 mmol L^{-1}) was measured. The times T_1 for the terminal CH_3 groups of the lipids are given in Fig. 3.

According to the published data,[30,32] the spin-lattice relaxation time of lipids in liposomes is determined by high-frequency vibrations of the acyl chain. The effect of GA on the spin-lattice relaxation times of the terminal CH_3 groups is observed for both lipids. This possible implies that GA is incorporated inside the lipid bilayer. The spin-lattice relaxation time increases in the presence of GA with an increase in the pH. It is most likely that on gradual deprotonation GA moves closer to the bilayer surface and exerts a lower effect on the motion of the terminal CH_3 groups.

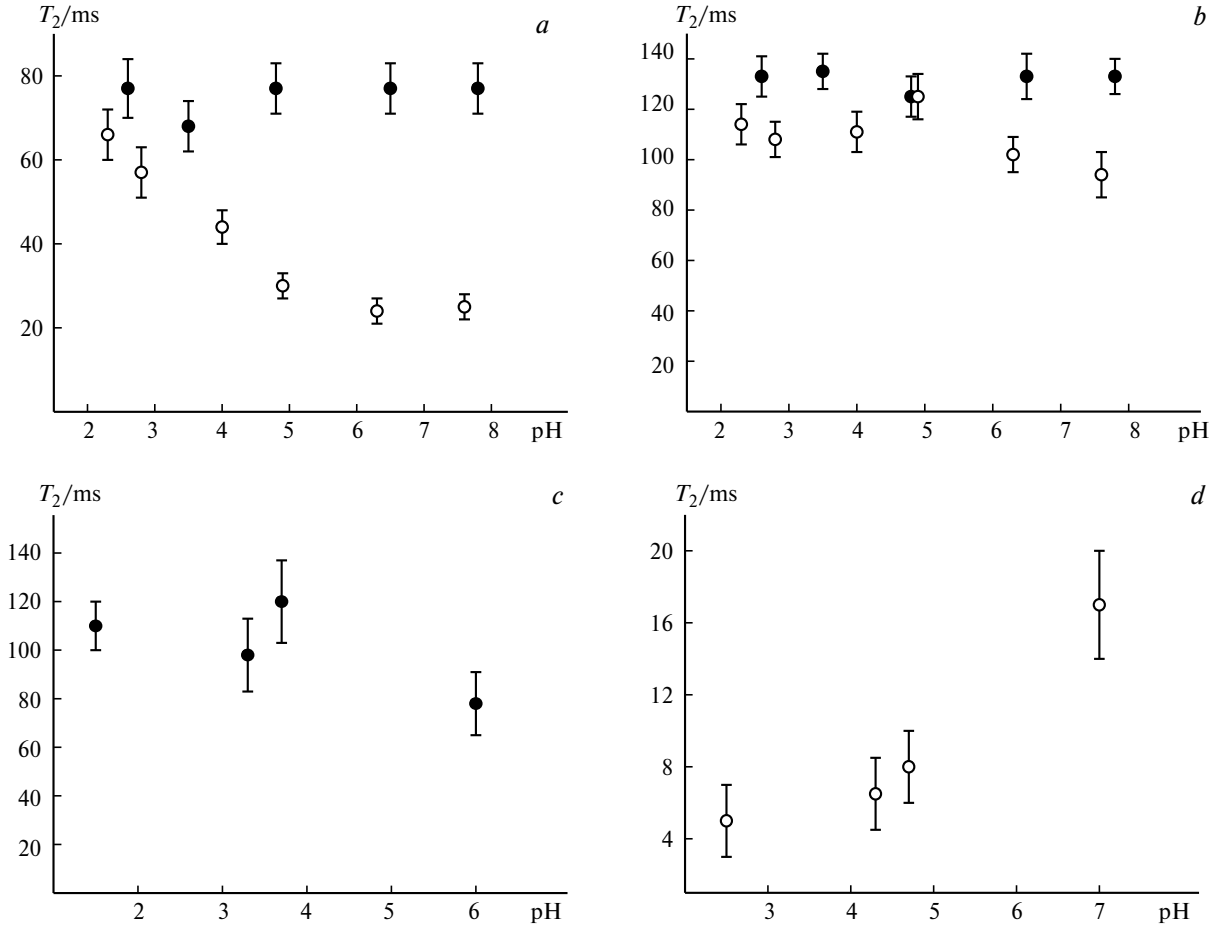


Fig. 2. Dependences of the spin-spin relaxation time (T_2) on the pH of the medium for the $N^+(CH_3)_3$ groups (*a*) and terminal CH_3 groups of phospholipid DOPC (*b*) and terminal CH_3 groups of phospholipid DMPC in the absence (*c*) and in the presence of GA (0.5 mmol L^{-1}, *d*); dark points correspond to pure phospholipid, and light points indicate phospholipid with the addition of GA (0.5 mmol L^{-1}).

Thus, the pH dependence of the interaction of GA with the lipid bilayer was studied for the model of single-layer liposomes of two types consisting of DOPC or DMPC lipids. The presence of GA was found to exert different effects on the spin-spin relaxation time of the proton of the lipids of the liposomes. We assume that the differences

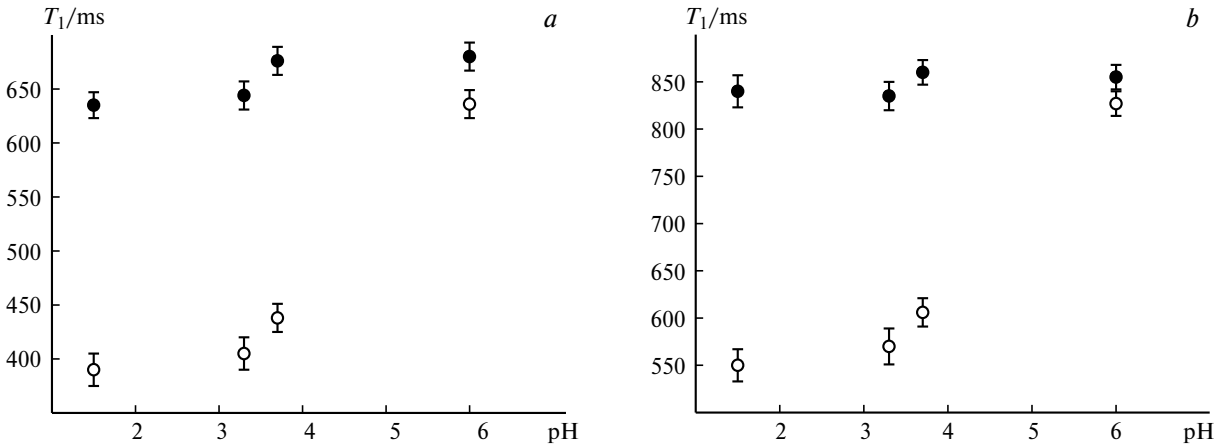


Fig. 3. Dependences of the spin-lattice relaxation time (T_1) of the protons of the terminal CH_3 groups of DMPC (*a*) and DOPC (*b*) on pH in the absence (dark points) and in the presence of GA (0.5 mmol L^{-1}, light points).

are related to the ability of GA to form self-associates in a more ordered lipid bilayer and a probable influence of these self-associates on the spin-spin relaxation time of the lipid protons. The character of the GA effect on the spin-lattice relaxation time of the protons of both lipids is similar. Possibly, this is due to the fact that GA penetrates inside the bilayer of both lipids thus retarding motions of the acyl chains of phospholipids. The pH dependences of the spin-spin and spin-lattice relaxation times of the lipid protons in the presence of GA were also observed. This can be due to the fact that with pH increasing GA is deprotonated and the charged molecule shifts closer to the lipid bilayer surface resulting in a decrease in the GA influence on motions of the acyl chains. In addition, an overall decrease in the signal intensity of the lipids in the NMR spectrum is observed in the presence of GA, which can be explained by the enlargement of lipid vesicles.

According to the literature data on studying the antiviral activity of GA against virus of hepatitis C in experiments on cell cultures, the treatment of the cells with GA does not decrease either the penetration of virus species into the cell or replication of virus species.[33] However, virus species are accumulated on the lipid droplet surface in the endoplasmic reticulum. This allowed one to conclude that the activity of GA against virus of hepatitis C is probably related to the inhibition of the escape of a virus species from the cell.[33] Our results of studying the fusion of lipid vesicles in the presence of GA can be an argument in favor of this assumption. The difference in effects of GA in lipid bilayers with different degree of ordering is also significant. It is known that saturated lipids and cholesterol form lipid rafts in the cell membrane. These regions are targets for the penetration of many viruses into the cell.[8] The penetration of a virus species into the cell depends on the pH of the medium, and data on the pH-dependent effect of GA on the mobility of ordered lipids form a basis for the further study of the mechanism of antiviral activity.

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This paper does not contain descriptions of studies on animals or humans.

The authors declare no competing interests.

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