

The Influence of Lipid Matrix Composition on the Microenvironment of Levofloxacin in Liposomal Forms

I. M. Le-Deygen^{a, 1}, A. S. Safronova^a, I. M. Kolmogorov^a, A. A. Skuredina^a, and E. V. Kudryashova^a

^a Chemistry Department, Moscow State University, Moscow, 199991 Russia

Received November 15, 2021; revised December 17, 2021; accepted December 21, 2021

Abstract—We have studied the interaction of the antibacterial drug levofloxacin with lipid bilayers of various compositions: 100% DPPC and with the addition of 20% cardiolipin. For DPPC liposomes, levofloxacin was found to penetrate into the subpolar region at the lipid–water interface. The role of the anionic lipid in the interaction of an active molecule with a bilayer has been established: levofloxacin enters the microenvironment of the phosphate group, displacing water, and does not penetrate into the hydrophobic part of the bilayer. For the first time, the study of the microenvironment of levofloxacin in the liposome by IR and CD spectroscopy was carried out. Such an approach based on a combination of several spectral methods opens up new prospects for the creation of new medicinal properties and the possibility of predicting the nature of the interaction of active molecules with biomembranes in order to predict their efficacy and potential side effects.

Keywords: liposomes, fluoroquinolones, levofloxacin, FTIR spectroscopy, CD spectroscopy

DOI: 10.1134/S1068162022040148

INTRODUCTION

The treatment of severe infectious diseases, such as tuberculosis and pneumonia, including secondary bacterial pneumonia in community-acquired COVID-19-related cases is an urgent task both in the Russian Federation and worldwide. The fluoroquinolones are potent and broad-spectrum antimicrobials, and hence, they appear to be attractive agents to treat lung infections. Thus, the fluoroquinolone levofloxacin (LF) has been included in the treatment of tuberculosis and secondary bacterial pneumonia. For example, levofloxacin has been included in the antibiotic therapy for SARS-CoV-2-induced lung injury in patients with COVID-19.

The inhaled levofloxacin holds promise for improving therapeutic efficacy and reducing adverse effects [1]. Clinical trials of the Aeroquin solution formulation of levofloxacin for aerosol delivery (MP-376, ClinicalTrials.gov Identifier: NCT01180634) to treat infections, including those caused by *Pseudomonas aeruginosa*, in lung fibrosis [2] still continue (at phase III). There is a need to create a delivery system of levofloxacin for aerosol administration, and the task is to increase the drug's affinity to lung tissue [1]. Liposomes are an attractive option. Liposomes can trap

hydrophobic, hydrophilic, and amphiphilic compounds. In order to create delivery systems based on liposomal levofloxacin, close understanding of molecular mechanisms involved in the interaction of a lipid bilayer with an active molecule is necessary. It is known that the membrane lipid composition has a significant effect on such interaction [3]. We showed previously this effect for moxifloxacin, a fourth generation fluoroquinolone [4].

The charged lipid groups of membrane, primarily phosphate and carbonyl groups, have the ability to establish electrostatic bonds with fluoroquinolones. It is shown that electrostatic interactions make a significant contribution to the binding of active molecules with a lipid bilayer [5]. Electrostatic interaction may also increase a drug loading content and improve retention of LF in the inside of the liposome membrane.

A solid understanding of the molecular interaction of LF with liposomes of different composition will allow one to optimize liposomal delivery systems in order to control the release and adjust doses of encapsulated antibacterial LF. The purpose of this work is to investigate the interaction of levofloxacin with the liposome membrane depending on its composition.

EXPERIMENTAL

Reagents. Levofloxacin, 1,4-dioxane (Sigma-Aldrich, United States); HCl, solid citric acid, diso-

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; CL, cardiolipin, CD spectroscopy, circular dichroism spectroscopy; LF, levofloxacin; LFL, liposomal form of levofloxacin.

¹ Corresponding autor: phone: +7 (999) 800-89-55; e-mail: i.m.deygen@gmail.com.

dium hydrogen phosphate dodecahydrate, borax $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (Reakhim, Russia); tablets for preparation of sodium phosphate buffer solution of pH 7.4 (PanEco, Russia); cardiolipin (CL) disodium salt, solution in chloroform (25 mg/mL); dipalmitoylphosphatidylcholine (DPPC), solution in chloroform (Avanti Polar Lipids, United States). Buffer solutions included 0.1-M hydrochloric acid buffer (pH 4.0); 0.02-M sodium phosphate buffer (pH 7.4); 0.01-M borate buffer (pH 9.2).

Liposome preparation. A solution of lipids in chloroform was taken in the ratio (DPPC : CL 80 : 20%). The organic solvent was carefully removed using a vacuum rotary evaporator (Ika, Germany) at a temperature not exceeding 55°C. The resulting thin film of lipids was dispersed in 0.02-M sodium phosphate buffer, pH 7.4. The suspension was exposed to an ultrasonic bath (37 Hz) for 5 min. Next, the suspension was sonicated (22 kHz) for 600 s (2×300 s) in a continuous mode with constant cooling using a 4710 disperser (Cole-Parmer Instrument, United States).

Preparation of liposomal levofloxacin. To obtain levofloxacin-loaded liposomes (LFL) by passive loading, the previously described method was used [4]. The lipid film was dispersed with 0.01-M sodium phosphate buffer solution, pH 7.4, containing levofloxacin (4 mg/mL). The resulting suspension was exposed to an ultrasonic bath (37 Hz) for 5 min; then it was treated with ultrasound (22 kHz) for 600 s (2×300 s) in continuous mode with constant cooling using a 4710 disperser (Cole-Parmer Instrument, United States). Free levofloxacin was separated by dialysis against sodium phosphate buffer saline (Serva MW cut-off 3500) for 2 h, followed by determination of the degree of LF incorporation into the vesicles.

UV spectroscopy. UV spectra of LF were recorded using a UV-visible UltraSpec 2100 pro spectrometer (Amersham Biosciences, United States) in the range from 200 to 400 nm in a 1-mL quartz cuvette (Hellma Analytics, Germany). The main characteristic peak of LF was observed at 287 nm.

IR spectroscopy. The spectra were recorded using a Tensor 27 IR Fourier spectrometer (Bruker, Germany) equipped with an MCT detector cooled with liquid nitrogen and a thermostat (Huber, United States). The measurements were carried out in a Bio-ATR II thermostated cell (Bruker, Germany) using a single reflection ZnSe element at 22°C and continuous purging of the system with dry air using a compressor (JUN-AIR, Germany). An aliquot (50 μL) of the corresponding solution was applied to the internal reflection element. The spectrum was recorded three times in the range from 4000 to 950 cm^{-1} with a resolution of 1 cm^{-1} ; 70-fold, scanning and averaging were performed. The background was registered in the same way and was automatically subtracted by the program. The spectra were analyzed using the Opus 7.0 software (Bruker, Germany). When recording the IR

spectra of liposomes loaded with LF, a solution of LF in equal concentration was used as a background solution. When recording the IR spectra of LF in liposomes, spectra of the corresponding unloaded liposomes were subtracted as a background solution.

CD spectroscopy. CD spectra were recorded using a J-815 circular dichroism spectrometer (Jasco, Japan) equipped with a thermostated cell holder in the wavelength range of 310–220 nm. In a typical experiment, 0.25–0.5 mg/mL of sample with the fluoroquinolone in an appropriate solvent was placed at the desired temperature in a quartz cuvette with a 1-cm path length (Hellma Analytics, Germany).

Dynamic light scattering. The sizes and ζ -potentials of particles were measured using the Zetasizer Nano S particle size analyzer (Malvern, England; light source 4-mW HeNe laser, 633 nm) in a temperature controlled cell at 22°C using the Malvern Zetasizer software.

RESULTS AND DISCUSSION

To establish the nature of the interaction of LF with liposomes and determine the factors that influence such interaction, the following tasks were suggested: (1) to determine how the nature of LF interaction with liposomes changes depending on the lipid composition, to identify the main binding sites, to determine the loading efficiency; (2) to study the state and microenvironment of LF functional groups using circular dichroism spectroscopy (CD spectroscopy).

LF exists in several forms depending on pH (Fig. 1). We revealed previously [4] that moxifloxacin (an antibacterial drug of the same class as LF) interacts with the lipid matrix mainly via the protonated nitrogen of the heterocycle moiety. However, in LF, protonated nitrogen (HX^{+-} , H_2X^+ forms [6, 7]) is shielded by a methyl group, with the result that the nature of LF interaction with the lipid bilayer may be different. Ionogenic groups are potential binding sites with the lipid bilayer, while deep movement of small organic molecules with a large number of polar groups into a bilayer is unlikely, based on data of a computer simulation study conducted for the model of a dipalmitoylphosphatidylcholine (DPPC) bilayer and doxorubicin [8].

The 100% DPPC liposomes with phospholipid headgroups shielded from interaction by positively charged choline groups and liposomes DPPC : cardiolipin (CL) 80 : 20% (m/m) with strong anionic properties represent convenient and well-studied model systems for elucidating the role of electrostatic interactions when loading small organic molecules into liposomes. Presumably, an additional negative charge on the vesicles because of CL addition may change the nature of liposome interaction with LF due to formation of additional electrostatic interactions with the fluoroquinolone heterocycle.

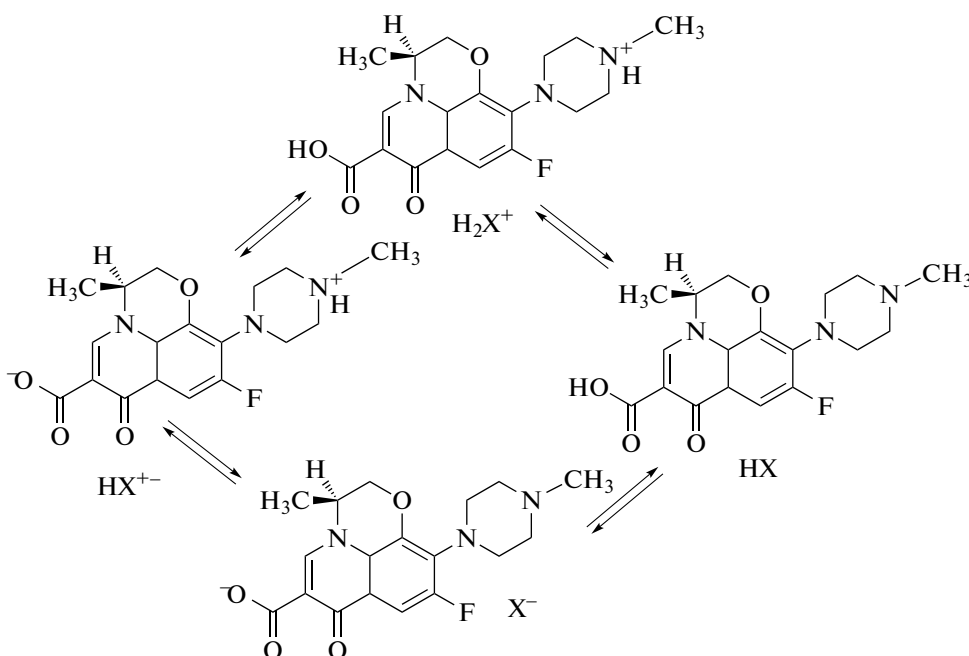


Fig. 1. Ionogenic forms of levofloxacin in a solution. The pK_a values: $pK_{a1} = 7.31$; $pK_{a2} = 8.22$; $pK_{a3} = 7.14$; $pK_{a4} = 6.29$ [6, 7].

Preparation of liposomal forms of levofloxacin and determination of the encapsulation efficiency.

It is known from the literature that the lipid composition has a significant effect on the physicochemical properties of liposomal forms of small medicinal molecules [9], including antibacterial drugs. It was shown for levofloxacin that addition of cholesterol to egg yolk phosphatidylcholine liposomes significantly reduces the LF encapsulation efficiency in passive loading [10] from 20 to 9%, which indicates a negative effect of increased membrane stiffness on loading efficiency.

The paper considers 100% DPPC liposomes with a characteristic ζ -potential of -10 mV and liposomes containing 20% CL with an electrokinetic potential of -20 mV. The inclusion of levofloxacin hardly affected the ζ -potential of DPPC liposomes and insignificantly (comparable to the error value) reduced the ζ -potential of anionic liposomes (Table 1). The inclusion of LF in liposomes did not significantly change the size of vesicles, which was observed earlier for the liposomal moxifloxacin [4].

The amount of levofloxacin encapsulated into liposomes was determined by mass balance and recording UV spectra of the washings after dialysis. The absorption band with the maximum intensity at $\lambda = 285\text{--}287$ nm (depending on pH) is characteristic of LF and corresponds to absorption by the aromatic backbone of fluoroquinolone. This peak is convenient to analyze the drug content in solution.

The high LF encapsulation efficiency was achieved, i.e., $\sim 50\%$, which corresponded to $\sim 4\%$ LF : lipids mass ratio. It is known that the maximum possible 10% ratio for small drug molecules corresponds to the formation of drug nanocrystals in an aqueous core of the lipid bilayer [11]. Previously, a mass ratio of 5–6% depending on the lipid membrane composition was achieved for moxifloxacin [4]. In experiments with LF, addition of anionic lipid to a bilayer did not significantly increase the loading efficiency, which indicates an insignificant contribution of electrostatic interactions. However, further study of liposomal LF using spectral analysis is needed in order to clarify the nature of the interaction.

Table 1. The main characteristics of liposomal forms of levofloxacin (LFL) compared to control unloaded liposomes

Specimen	Encapsulation efficiency, %	ζ -Potential, mV	Dh , nm (Z-Ave)
100% DPPC liposomes	—	-10.1 ± 0.5	103 ± 2
DPPC 100% LFL	55 ± 2	-10.3 ± 0.2	100 ± 2
DPPC: CL 80 : 20 liposomes	—	-20.1 ± 1.0	100 ± 2
DPPC : CL 80 : 20 LFL	51 ± 7	-21.5 ± 1.0	103 ± 3

0.01-M sodium phosphate buffer solution (pH 7.4), 22°C. Dh is the hydrodynamic radius.

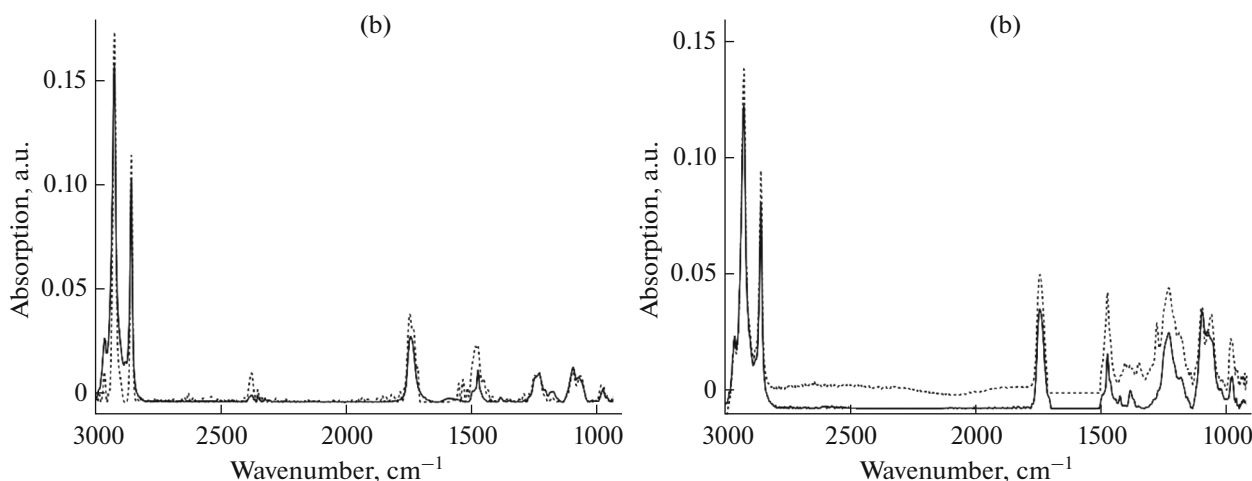


Fig. 2. ATR-FTIR spectra of liposomes: (a) 100% DPPC (full line) and DPPC 100% loaded with levofloxacin (dotted line); (b) DPPC : CL 80 : 20% (full line) and DPPC : CL loaded with levofloxacin (dotted line). Conditions: 0.02-M sodium phosphate buffer solution (pH 7.4), 22°C; lipid concentration is 5 mg/mL.

Determination of binding sites of liposomes of various compositions with levofloxacin by IR spectroscopy.

To study the molecular LF interaction with the liposome membrane and to identify the main binding sites and differences in the structure of loaded liposomes of different compositions, Fourier transform infrared spectroscopy was used.

This method is a highly informative spectral technique suitable for studying complex colloidal systems, including liposomes [12, 13]. This method allows a detailed study of the LF interaction with various functional groups of lipids. The IR spectra of liposomes contain a number of main absorption bands, which are informative in the analysis of the interaction of liposomes with various molecules. Figure 2 shows the IR spectrum of 100% DPPC liposomes. Symmetric and asymmetric stretching vibrations of the CH₂ group correspond to bands in the region of $2850 \pm 1 \text{ cm}^{-1}$ and $2919 \pm 1 \text{ cm}^{-1}$. These absorption bands are sensitive to changes in liposome acyl chain packing [14]. The absorption band of the carbonyl group is located in the region of $1715\text{--}1750 \text{ cm}^{-1}$ and is sensitive to changes in the microenvironment on the lipid–water surface. The phosphate group of phospholipids is characterized by two stretching vibration bands: νPO_2^- at 1088 cm^{-1} and νPO_2^- as $1250\text{--}1230 \text{ cm}^{-1}$. The analytically significant νPO_2^- as band is of greatest interest as it is sensitive to the interaction of cationic ligands with the polar head of liposomes. A change in the position of absorption bands and their shape indicates a change in the microenvironment of the corresponding functional groups. Hence, the analysis of IR spectra makes it possible to identify the main binding sites of ligands including small organic molecules [15].

Next, we will consider the differences in IR spectra of DPPC liposomes and levofloxacin-loaded DPPC liposomes (LFL) (Fig. 2, Table 2). The absorption bands of CH₂ groups do not undergo significant changes when LF is included in liposomes, which indicates that the hydrophobic part of the bilayer is not involved in the interaction with LF. It is predictable, since molecules with a large hydrophobic fragment, for example, hydrophobic doxorubicin, commonly have a significant effect on the packing density of chains [16]. For the phosphate group band, there is an insignificant change due to an increase in the intensity of the 1240-cm^{-1} peak shoulder. These changes in the spectrum are associated with a decrease in the hydration degree of the phosphate group and may indicate two processes. Electrostatic binding of phosphate groups to the cationic ligand is possible, and the 1223-cm^{-1} main peak is usually shifted [17]. In addition, 1240-cm^{-1} shoulder may also be related to displacement of water from the microenvironment of a phosphate group. Since in both LF and DPPC, the charge is shielded from interaction, and the 1260-cm^{-1} shoulder is not observed in the spectrum, we suggest that the changes in the absorption region of phosphate groups are associated with the deep movement of LF into the bilayer, including to the lipid–water interface. This is also evidenced by changes in the absorption region of the carbonyl group: this band undergoes a characteristic splitting described previously [17]. The 1740-cm^{-1} band corresponding to low-hydrated carbonyl groups is characteristic for the electrostatic interaction at the lipid–water interface. Apparently, LF partially displaces water from the microenvironment of carbonyl and phosphate groups. A similar nature of the interaction was observed for moxifloxacin [4]: the DPPC membrane is not capable of significant electrostatic interaction with fluoroquinolones.

Table 2. Position of main absorption bands in ATR-FTIR spectra of levofloxacin-loaded DPPC 100% liposomes compared to unloaded liposomes

Specimen	νCH_2 as, cm^{-1}	νCH_2 s, cm^{-1}	νCO , cm^{-1}	νPO_2^- as, cm^{-1}
DPPC 100% liposomes	2918	2850	1737	1223
Interpretation	Gel-like state			
DPPC 100% LFL	2918	2850	1740, 1725	1221, 1240
Interpretation	Inclusion of LF does not influence the hydrophobic part of a bilayer		Interaction with LF at the lipid-water interface	Displacement of water from the microenvironment of phosphate groups

0.01-M sodium phosphate buffer solution, pH 7.4, 22°C. Error in determination of the maximum of an absorption band is 1 cm^{-1} .

Table 3. Position of main absorption bands in IR spectrum of levofloxacin-loaded DPPC : CL 80 : 20% liposomes compared to unloaded liposomes

Specimen	νCH_2 as, cm^{-1}	νCH_2 s, cm^{-1}	νCO , cm^{-1}	νPO_2^- as, cm^{-1}
DPPC: CL 80 : 20 liposomes	2919	2851	1739, 1723 (shoulder)	1225
Interpretation	Gel-like state			
DPPC: CL 80 : 20 LFL	2918	2851	1740	1220, 1225, 1265
Interpretation	Inclusion of LF does not influence the hydrophobic part of a bilayer		Interaction with LF at the lipid-water interface	Displacement of water from the microenvironment of phosphate groups

0.01-M sodium phosphate buffer solution (pH 7.4), 22°C. Error in determination of the maximum of an absorption band is 1 cm^{-1} .

Anionic liposomes with the composition of DPPC : CL 80 : 20% yielded a different spectral pattern (Table 3). A comparison of the position of the main absorption bands in the spectra of unloaded liposomes of DPPC and DPPC : CL 80 : 20% revealed significant differences in the regions characteristic of the carbonyl and phosphate groups. These differences are predictable and correspond to inclusion of anionic CL lipid in the composition of liposomes.

The absorption band of the carbonyl group in the spectrum of DPPC : CL 80 : 20% liposomes is split into two components corresponding to the high (1723 cm^{-1}) and low hydrated (1739 cm^{-1}) states, and in the DPPC liposome spectrum the absorption band represents a single peak. The inclusion of LF in the bilayer changes the band appearance in both cases. In the DPPC membrane, the band was split due to the emergence of a component related to the low-hydrated carbonyl groups. For mixed anionic liposomes, the inclusion of LF in the bilayer leads to a uniform band shift to 1740 cm^{-1} . The component of the highly hydrated state, apparently corresponding to the unbound carbonyl groups of CL, disappears. Probably, these spectral changes are associated with the interaction of these functional groups with LF and displacement of water molecules from their microenvironment. Thus, for both 100% DPPC and DPPC : CL

80 : 20% liposomes, carbonyl groups of lipids are an important binding site of LF.

The changes in the absorption region of phosphate groups are notable. Here, the changes in the bilayer are even more marked in comparison with 100% DPPC liposomes: the proportions of components corresponding to low-hydrated phosphate groups bound with LF significantly increase. Obviously, the inclusion of CL in the bilayer causes a more intense interaction of LF with vesicles on the surface.

The data indicate that ways of LF binding with liposomes differ for different compositions. LF apparently does not affect significantly the mobility of the hydrophobic chains in a bilayer, and the interaction occurs at the lipid-water interface. The presence of anionic CL lipid probably enhances this interaction due to the formation of electrostatic bonds. However, a comparison of these results with previous data for moxifloxacin [4] shows that the interaction of LF with a bilayer is slightly weaker, which may be explained by two factors. First, moxifloxacin is the most lipophilic fluoroquinolone, and secondly, nitrogen in its heterocycle is not methylated and is open to interaction with lipids. In order to consider in more detail the interaction of LF with a bilayer, we will consider how the microenvironment of the fluoroquinolone itself changes when incorporated into a bilayer using Fourier infrared spectroscopy and CD spectroscopy.

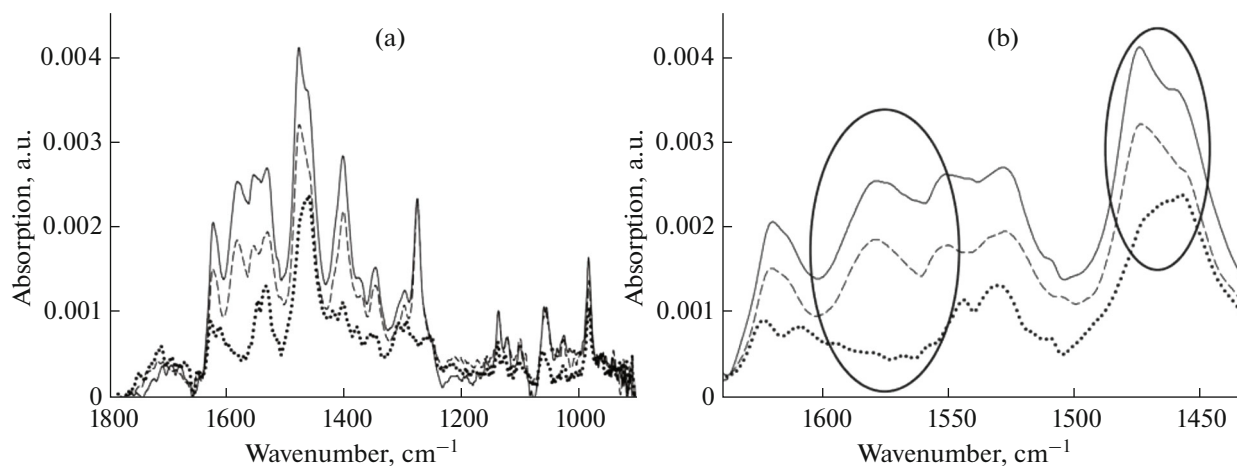


Fig. 3. ATR-FTIR spectra of levofloxacin (1 mg/mL) at pH 7.0 (full line), pH 4.0 (dash line) and pH 2.0 (dotted line) in the regions of 1800–950 cm^{-1} (a) and 1650–1450 cm^{-1} (b).

Investigation of levofloxacin state in liposomes using ATR-FTIR spectroscopy. Comparison of free LF spectra with LFL spectra will allow correlating the spectral data for liposomal forms with the ionic state of fluoroquinolone.

The IR spectrum of LF is most informative in the range of 1800–950 cm^{-1} containing absorption bands at 1456 and 979 cm^{-1} corresponding to vibrations of the aromatic backbone and piperazine fragment [18]. These bands are analytically significant and can be used to determine the content of LF, as well as its right optical stereoisomer ofloxacin in the system.

Next, we will consider the effect of pH on LF spectra, which will allow correlating the IR spectra of LF in matrices of delivery systems with the ionogenic state that will prevail in the system. Two ionic groups are present in the LF molecule: a carboxyl group conjugated to the aromatic structure of quinolone and a piperazine group.

The change in pH significantly affects the state of the molecule, which is accompanied by significant changes in the IR spectrum of the molecule when pH is increased from 2 to 4 and further to pH 7 (Fig. 3a). Significant changes are observed in the 1640–1600 (the vibration region of carbonyl and carboxyl groups) and 1590–1400 cm^{-1} regions (the vibration region of the aromatic structure of quinolone) (Fig. 3b). An increase in pH leads to the appearance of a band at 1580 cm^{-1} corresponding to the deprotonated carboxyl group, as well as to significant changes in structure of bands at 1470–1450 cm^{-1} , i.e., a band with the main contribution from the stretching vibrations of the C–C aromatic ring and bands at 1400–1339 cm^{-1} related to the C–N bond vibrations (aromatic tertiary amine and amine piperazine ring). A significant change is also observed for the absorption region associated with the C=O bond of carboxyl group (1270 cm^{-1}): an

increase in pH leads to appearance of this absorption band (Fig. 3a).

The analysis of IR spectra acquired at different pH values (Fig. 3) made it possible to correlate the main absorption bands of LF using literature data [18, 19]. Thus, the bands at 1730–1720 and 1622 cm^{-1} correspond to the stretching vibrations of the C=O bond in carboxyl and carbonyl groups, respectively, 1580 cm^{-1} is the band of the deprotonated carboxyl group, absorption bands at 1606–1549 cm^{-1} correspond to the vibrations of C=C bonds in the quinolone structure, a group of bands at 1500–1400 cm^{-1} belongs to the vibrations of C–C bonds in the aromatic backbone, which may overlap with vibrations of the methyl group of the $\text{CH}_3\text{--N}$ bond; absorption bands at 1390–1350 and 1208 cm^{-1} , most likely, belong to vibrations of the C–N bond (aromatic tertiary amine), the absorption band at 1339 cm^{-1} is sensitive to protonation of the piperazine ring and corresponds to the C–N bond of the piperazine group, the band at 1283–1270 cm^{-1} is attributed to vibrations of the C–O bond of carboxyl group, the band at 1100 cm^{-1} corresponds to the vibrations of the C–O–C bond; the bands in the region of 1053–1028 cm^{-1} correspond to the C–F bond vibrations; the band at 979 cm^{-1} corresponds to vibrations of the C–N and C–H bonds in the piperazine backbone.

When LF is included in liposomes, mild changes are observed in the IR spectrum of the fluoroquinolone (Fig. 4). The shape of the absorption band at 1474 cm^{-1} corresponding to absorption of the aromatic backbone changes uniformly. The absorption band at 1458 cm^{-1} characteristic of free LF spectrum disappears when LF is included in liposomes, which indicates a change in the fluoroquinolone microenvironment due to interaction with a bilayer. A decrease in the intensity of the absorption band at 1580 cm^{-1}

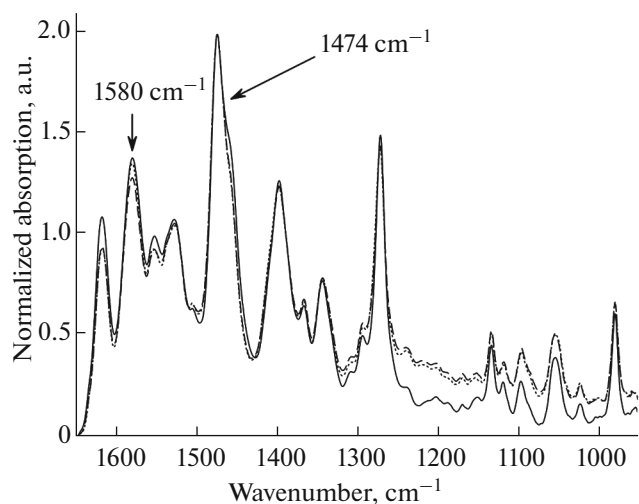


Fig. 4. Normalized IR spectra of free levofloxacin (full line), levofloxacin in DPPC liposomes (dashed line) and DPPC : CL (dotted line). Conditions: 0.02-M sodium phosphate buffer solution (pH 7.4), 22°C.

corresponding to vibrations of the deprotonated carboxyl group is notable, and this effect is most marked for anionic liposomes. This is in good agreement with the data presented above: interaction of LF with DPPC : CL liposomes evidently occurs in the near-surface layer.

Study of levofloxacin state in liposomes by CD spectroscopy. Circular dichroism (CD) spectroscopy is a highly informative technique for analyzing the micro-environment and ionogenic form of optically active molecules, including fluoroquinolones, in achiral matrices, such as liposomes. Studying the state of chiral molecules, such as LF, in lipid vesicles is a conve-

nient way to determine the nature of the drug-bilayer interaction.

In the CD spectrum of LF, the most informative region is in the range of 220–310 nm with characteristic ellipticity maxima or minima [20]. In order to analyze the changes in the CD spectra when LF is included in a lipid bilayer, it is necessary to trace in independent experiments how the LF spectrum changes when the ionic state changes.

Depending on the pH of a buffer solution, LF exists in various ionogenic forms (Fig. 1), which is reflected in the CD spectrum (Fig. 5a) [21]. Two minima are observed in the CD spectrum of LF: a peak in the region of 287–300 nm corresponds to a conjugated aromatic system of LF (the first minimum) and the peak at 222–231 nm mainly corresponds to carbonyl and carboxyl groups of LF (the second minimum). In addition to the two minima, it is possible to observe a maximum in the range of 268–285 nm on the spectrum, but its analysis is difficult since the peak is very wide.

At pH 4.0, the main form is H_2X^+ with a characteristic appearance of the spectrum (the first minimum is 297 ± 0.5 nm, the second minimum is 222 ± 0.5 nm). In a solution with a physiologic pH value of 7.4, an equilibrium is established between several forms, namely HX , HX^{+-} , X^- , which leads to significant changes in the spectrum, primarily to a 16-nm shift of the maximum position to the blue region (268 ± 0.5 nm) and a 6-nm shift of the second minimum to the red region (226 ± 0.5 nm). In an alkaline medium (pH 9.2), the X^- form prevails, shift of the first minimum to the blue region is completed and the total shift upon change of pH 4.0 (fully protonated form, 287 ± 0.5 nm) to pH 9.2 is 10 nm (297 ± 0.5 nm).

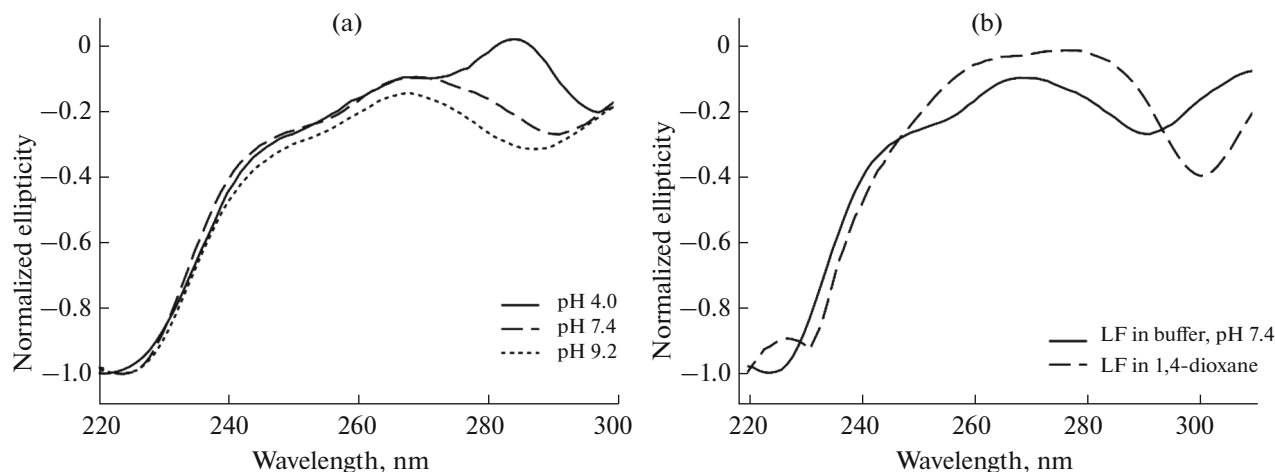


Fig. 5. Normalized CD spectra of levofloxacin: (a) in different buffer solutions: pH 4.0 (0.1-mM solution of hydrochloric acid), pH 7.4 (0.02-M sodium phosphate buffer), pH 9.2 (0.02-M borate buffer); (b) in different media: pH 7.4 (0.02-M sodium phosphate buffer, 1,4-dioxane), 25°C.

Table 4. Position of main bands in CD spectrum of levofloxacin in different media

Peak position, nm	Functional group	pH 4.0	pH 7.4	pH 9.2	1,4-Dioxane	DPPC 100% LFL	DPPC : CL 80 : 20 LFL
The first minimum	Conjugated aromatic system	297 ± 0.5	291 ± 0.5	287 ± 0.5	300 ± 0.5	296 ± 0.5	294 ± 0.5
The second minimum	Carbonyl and carboxyl groups + aromatic backbone	222 ± 0.5	224 ± 0.5	223 ± 0.5	231 ± 0.5	229 ± 0.5	224 ± 0.5
Forms of LF	H ₂ X ⁺		HX, HX ^{+−} , X [−]		HX	Interaction with a bilayer at the lipid-water surface	Interaction mainly on the surface of a bilayer

The experiment was conducted in the media: 0.1-mM solution of hydrochloric acid (pH 4.0); 0.02-M sodium phosphate buffer solution (pH 7.4); 0.01-M sodium borate buffer solution (pH 9.2). Levofloxacin concentration for all media was 2 mM, 22°C.

CD spectra of LF are very sensitive to changes in the ionic state of a molecule. In addition, polarity of the LF microenvironment also affects a spectrum. For example, an aprotic polar solvent 1,4-dioxane, which to a first approximation corresponds to the polar part of the lipid bilayer, significantly changes the CD spectrum. Compared to the aqueous medium, in 1,4-dioxane, transition between different ionogenic forms is suppressed, and the observed spectrum corresponds to the HX form (Fig. 5b). Compared to a spectrum of LF solution in physiologic pH, spectrum of LF in 1,4-dioxane is shifted to the red region, and the first minimum is most marked (Table 4). This is in good agreement with the literature data: as hydrophobicity of media increases, CD spectra of tyrosine and tryptophan demonstrate a shift to the red region [22].

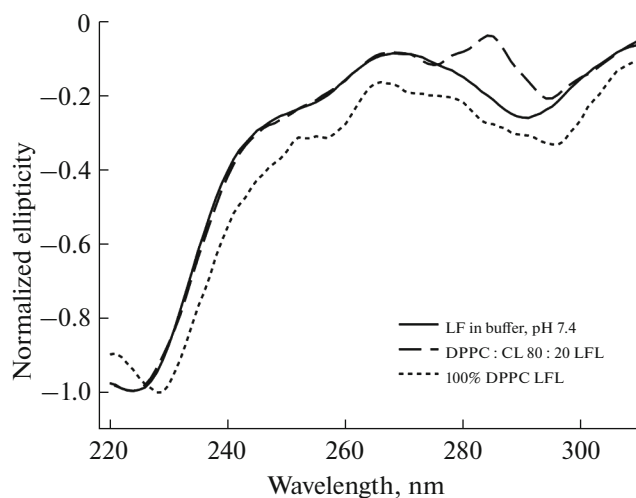


Fig. 6. Normalized CD spectra of levofloxacin in buffer solution (0.02-M sodium phosphate buffer, pH 7.4) and in liposomal forms (DPPC : CL 80 : 20% and DPPC 100%), 25°C.

The first minimum in the near UV region changes similarly to the absorption minimum in tryptophan and shifts to the red region with decreasing solvent polarity [22]. The second minimum shifts more noticeably when an organic solvent is used, i.e., by 9 nm to the red region, which is predictable for a peak corresponding to the carbonyl and carboxyl groups of LF.

The first minimum is convenient for studying the state of LF depending on pH of media: a higher pH shifts the peak to the blue region. In addition, the first minimum is sensitive to hydrophilicity of media. The second minimum is convenient for assessing a hydrophilic-hydrophobic balance of the LF microenvironment: a replacement of aqueous medium with organic medium shifts the peak to the red region.

The effect of levofloxacin loading in liposomes on the CD spectrum. Next, we will consider the changes observed in the CD spectrum of LF when LF is included in liposomes (Table 4, Fig. 6).

For DPPC liposomes, encapsulation of LF leads to a shift of the first minimum in the CD spectrum by 5 nm to the red region (from 291 to 296) and the second minimum by 5 nm (from 224 to 229 nm). These changes indicate a possible protonation of carboxyl groups and a change in the LF microenvironment to more hydrophobic. These data support the assumption made on the basis of Fourier infrared spectroscopy: LF can penetrate into the DPPC bilayer by interacting with the lipid–water phase interface.

An inclusion of anionic CL lipid in liposomes leads to less marked changes on the CD spectrum. The first minimum similarly shifts by 3 nm to 294 nm, while the second remains unchanged. The interaction of LF with DPPC : CL 80 : 20 liposomes does not change hydrophilicity of the LF microenvironment and the interaction may occur on the surface due to displacement of water from the microenvironment of phosphate groups of lipids.

The data suggest that in neutral liposomes, LF moves deeper into the bilayer compared to anionic liposomes. The bilayer containing CL may hold LF at the surface due to interaction of phosphate groups of CL with a protonated heterocycle and thus interfere with LF penetrating into the membrane.

CONCLUSIONS

Liposomal forms of levofloxacin and their properties have been studied. It was determined that composition of liposomes (anionic or neutral) does not affect loading of the fluoroquinolone. We obtained liposomal forms with a relatively high encapsulation efficiency, i.e., with the drug/lipid mass percentage ratio of 4%. It was found using the Fourier transform infrared spectroscopy that in DPPC liposomes, LF moves deep into the subpolar region at the interface of the lipid–water phases. For mixed anionic liposomes, the role of anionic lipid in the interaction of an active molecule with a bilayer has been established: LF enters the microenvironment of the phosphate group, displacing water, and does not penetrate into the hydrophobic part of the bilayer.

The proposed process is confirmed by CD spectroscopy. It was found that the CD spectrum of LF in the wavelength range of 220–350 nm contains three peaks corresponding to the microenvironment of functional groups of LF: a minimum in the range of 285–305 nm corresponding to the state of the conjugated nitrogen-containing aromatic structure, which shifts to the short-wavelength region with increasing pH and to the long-wavelength region when an aprotic organic solvent is used (upon a decrease in hydrophilicity of the medium); a minimum in the range of 220–235 nm corresponding to the state of carboxyl and carbonyl groups, as well as double bonds, which slightly shifts with a change in pH and shifts to the long-wavelength region with a decrease in hydrophilicity of the medium; a minimum in the range of 265–285 nm corresponding to the state of the aromatic structure, which shifts to the short-wavelength region with an increase in pH and shifts to the long-wavelength region with a decrease in hydrophilicity of the medium.

The experimental data allow us to draw conclusions on how small organic molecules, primarily antibacterial drugs, interact with a bilayer. Such an approach based on a combination of three spectral methods opens up new prospects for the creation of new medicinal properties and the possibility of predicting the nature of the interaction of active molecules with biomembranes in order to predict their efficacy and potential side effects.

FUNDING

This study was supported by the Russian Foundation for Basic Research and the Government of Moscow, project No. 21-33-70035.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals or human participants performed by any of the authors.

Conflict of Interests

The authors declare that they have no conflict of interest.

REFERENCES

- Weers, J., *Adv. Drug Deliv. Rev.*, 2015, vol. 85, pp. 24–43.
<https://doi.org/10.1016/j.addr.2014.08.013>
- Geller, D.E., Flume, P.A., Staab, D., Fischer, R., Loutit, J.S., and Conrad, D.J., *Am. J. Respir. Crit. Care Med.*, 2011, vol. 183, pp. 1510–1516.
<https://doi.org/10.1164/rccm.201008-1293OC>
- Andrade, S., Ramalho, M.J., Loureiro, J.A., and Pereira, M.C., *J. Mol. Liq.*, 2021, vol. 334, p. 116141.
<https://doi.org/10.1016/j.molliq.2021.116141>
- Le-Deygen, I.M., Skuredina, A.A., Safronova, A.S., Yakimov, I.D., Kolmogorov, I.M., Deygen, D.M., Burdova, T.V., Grinberg, N.V., Grinberg, V.Y., and Kudryashova, E.V., *Chem. Phys. Lipids*, 2020, vol. 228, p. 104891.
<https://doi.org/10.1016/j.chemphyslip.2020.104891>
- Matos, C., Moutinho, C., and Lobao, P., *J. Membr. Biol.*, 2012, vol. 245, pp. 69–75.
<https://doi.org/10.1007/s00232-011-9414-2>
- Kłosińska-Szmurlo, E.K., Grudzień, M., Betlejewska-Kielak, K., Pluciński, F., Biernacka, J., and Mazurek, A.P., *Acta Chim. Slov.*, 2014, vol. 61, pp. 827–834.
<https://journals.matheo.si/index.php/ACSi/article/view/562>
- Ross, D.L. and Riley, C.M., *Int. J. Pharm.*, 1992, vol. 83, pp. 267–272.
[https://doi.org/10.1016/0378-5173\(82\)90032-1](https://doi.org/10.1016/0378-5173(82)90032-1)
- Yacoub, T.J., Reddy, A.S., and Szleifer, I., *Biophys. J.*, 2011, vol. 101, pp. 378–385.
<https://doi.org/10.1016/j.bpj.2011.06.015>
- Mohammed, A.R., Weston, N., Coombes, A.G.A., Fitzgerald, M., and Perrie, Y., *Int. J. Pharm.*, 2004, vol. 285, pp. 23–34.
<https://doi.org/10.1016/j.ijpharm.2004.07.010>
- Sorokoumova, G.M., Yasin, Ya.O., Mikulovich, Ju.L., Smirnova, T.G., Andreevskaya, S.N., Selischeva, A.A., Chernousova, L.N., and Shvets, V.I., *Fine Chemical Technologies*, 2013 vol. 8, pp. 72–76 (in Russ.).
<https://www.finechem-mirea.ru/jour/article/view/531>
- Barenholz, Y., *J. Control. Release*, 2012, vol. 160, pp. 117–134.
<https://doi.org/10.1016/j.jconrel.2012.03.020>
- Bensikaddour, H., Fa, N., Burton, I., Deleu, M., Lins, L., Schanck, A., Brasseur, R., Dufrene, Y.F., Goorma-

- ghtigh, E., and Mingeot-Leclercq, M.P., *Biophys. J.*, 2008, vol. 94, pp. 3035–3046.
<https://doi.org/10.1016/j.bbmem.2008.08.015>
13. Deygen, I.M. and Kudryashova, E.V., *Colloids Surf. B*, 2016, vol. 141, pp. 36–43.
<https://doi.org/10.1016/j.colsurfb.2016.01.030>
14. Manrique-Moreno, M., Howe, J., Suwalsky, M., Garidel, P., and Brandenburg, K., *Lett. Drug Des. Discov.*, 2009, vol. 7, pp. 50–56.
<https://doi.org/10.2174/157018010789869280>
15. Tretiakova, D., Le-Deigen, I., Onishchenko, N., Kuntsche, J., Kudryashova, E., and Vodovozova, E., *Pharmaceutics*, 2021, vol. 13, p. 473.
<https://doi.org/10.3390/pharmaceutics13040473>
16. Deygen, I.M., Seidl, C., Kölmel, D.K., Bednarek, C., Heissler, S., Kudryashova, E.V., Bräse, S., and Schepers, U., *Langmuir*, 2016, vol. 32, pp. 10861–10869.
<https://doi.org/10.1021/acs.langmuir.6b01023>
17. Deygen, I.M. and Kudryashova, E.V., *Russ. J. Bioorg. Chem.*, 2014, vol. 40, pp. 547–557.
<https://doi.org/10.1134/S1068162014050057>
18. Nisar, J., Iqbal, M., Iqbal, M., Shah, A., Akhter, M.S., Uddin, S., Khan, R., Uddin, I., Shah, L.A., and Khan, M.S., *Z. Phys. Chem.*, 2020, vol. 234, pp. 117–128.
<https://doi.org/10.1515/zpch-2018-1273>
19. Bhardwaj, V., Bhardwaj, T., Sharma, K., Gupta, A., Chauhan, S., Cameotra, S.S., Sharma, S., Gupta, R., and Sharma, P., *RSC Adv.*, 2014, vol. 4, pp. 24935–24943.
<https://doi.org/10.1039/c4ra02177k>
20. Chamseddin, C. and Jira, T., *Curr. Pharm. Anal.*, 2013, vol. 9, pp. 121–129.
<https://doi.org/10.2174/1573412911309010016>
21. Ranjbar, B. and Gill, P., *Chem. Biol. Drug Des.*, 2009, vol. 74, pp. 101–120.
<https://doi.org/10.1111/j.1747-0285.2009.00847.x>
22. Li, R., Nagai, Y., and Nagai, M., *J. Inorg. Biochem.*, 2000, vol. 82, pp. 93–101.
[https://doi.org/10.1016/S0162-0134\(00\)00151-3](https://doi.org/10.1016/S0162-0134(00)00151-3)

Translated by M. Novikova