The Charge and Phase State of Liposomes Dramatically Affects the Binding of Mannosylated Chitosan

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Abstract: Liposomal complexes with mucoadhesive polymers, e.g., mannosylated chitosan, are considered as prospective antituberculosis drug delivery systems. The properties of such complexes can be critically affected by the charge and phase state of liposomes. The aim of our work was to study the interaction of mannosylated chitosan with liposomes of various compositions and to identify the key patterns of this process. We tracked the interaction by titrating the liposomes with an increasing base-molar excess using the DLS method and ATR-FTIR spectroscopy. Sorption isotherms were obtained using ATR-FTIR spectroscopy and linearized in the Scatchard coordinates to evaluate the dissociation constant ($K_{diss}$). The inclusion of cardiolipin (CL) in the lipid composition helps to reduce the $K_{diss}$ of the complexes by an order of magnitude for dipalmitoylphosphatidylcholine (DPPC) and DPPC:CL 80:20 (weight ratio), respectively. Pre-heating at 37 °C of gel-like anionic liposomes helps to reduce the $K_{diss}$ to $3.5 \times 10^{-5}$ M. Anionic liposomes, both in liquid crystal and in the gel-like state, form multipoint non-covalent complexes with chitosan–mannose conjugates due to the partial neutralization of the charges on the surface of the vesicles. Meanwhile, neutral liposomes in both states form unstable heterogeneous complexes, probably due to the predominant sorption of the polymer on the vesicles. Complex formation provides preferable binding with the model mannoside-binding receptor concanavalin A and sustained pH-sensitive release of the antituberculosis drug moxifloxacin.

Keywords: chitosan; liposome; noncovalent complex; drug delivery; ATR-FTIR spectroscopy

1. Introduction

*Mycobacterium tuberculosis* is one of the most insidious pathogens: tuberculosis has swept not only developing countries, but also the ten countries offering golden visas [1]. The development of multi-resistant strains of Koch’s bacillus requires long-term combination therapy and, if indicated, surgery. Many drugs that are used to treat multidrug-resistant forms of tuberculosis have severe side effects [2]. For example, according to the Vidal database, aminoglycosides and capreomycin can cause damage to the kidneys, hearing organs and vestibular apparatus. Isoniazid, ethambutol, prothionamide, cycloserine and fluoroquinolones can cause damage to the central and peripheral nervous system and lead to neuropsychiatric disorders. Rifampicin, pyrazinamide and ethionamide can cause liver damage. Moreover aminoglycosides, capreomycin, cycloserine and fluoroquinolones cause damage to the cardiovascular system, while rifampicin and prothionamide treatment leads to changes in the hemogram. Thus, an acute problem in biomedicine is the design of new dosage forms for the treatment of severe infections such as tuberculosis.

Inhalation drug delivery systems targeting the host cells of *Mycobacterium tuberculosis*, namely the alveolar macrophages of the lung, could be considered as a prospective approach to overcome these drawbacks [3]. The surface of these cells is decorated with a...
large amount of the mannose-binding receptor CD 206; thus, mannose derivatives are promising target labels for the delivery of antituberculosis drugs [4].

In order to design a novel drug delivery system with active targeting of the alveolar macrophages, we have considered liposomes as a carrier for antituberculosis drugs [5]. These vesicles are suitable for both intravenous and inhaled administration; however, their low thermodynamic stability and tendency to aggregate significantly limit their application in clinical practice. The majority of FDA-approved liposomal drugs possess extra functionalizing agents, e.g., modified lipids, as is the case with Doxil, where PEGylated lipids are included in the composition.

Another approach is based on the design of non-covalent complexes. As a functionalizing agent, here, one can apply polyelectrolytes with variable modifications. Chitosan is a perspective biopolymer originating from chitin, the major component of shrimp and crab shell. Chitosan possesses several key advantages for biomedical purposes [6]: biocompatibility, a variety of properties depending on the molecular weight and degree of deacetylation, and a variety of chemical modifications of the amino group, and its mucoadhesive properties [7]. Chitosan can be used to create drugs for inhalation [8] or transdermal administration [9]. Chitosan is able to influence the liposomal properties and lipid composition, and the phase state is one of the key factors in this process [10]. However, low solubility and a tendency towards gel formation still limit chitosan application in the design of liposomal formulations. Our laboratory has proposed an approach to engineering the surface of liposomes through the formation of a multipoint non-covalent complex of vesicles with chitosan derivatives. Chitosan derivatives, for example, PEG-chitosan [11], glycol-chitosan [12], etc., are described in the literature as reliable functionalizing agents for liposomes.

Mannose-modified chitosan derivatives (ChitMan) are considered as promising polymers for the design of lipid–polymer delivery systems for antituberculosis drugs. We have previously reported on the synthesis of such derivatives (Figure 1a) and demonstrated that their affinity for the model mannose-binding receptor concanavalin A is close to that of trimanoside [6]. Concanavalin A is considered as a relevant model to investigate ligand binding with mannose-binding receptors close to CD206, as revealed by molecular modeling [13].

**Figure 1.** Chitosan–mannose conjugates: (a) Synthesis (b) ATR-FTIR spectrum of ChitMan. 0.02 M Na-phosphate buffer solution, pH 7.4, 22°. (c) Calibration curves for 1078 cm\(^{-1}\) and 990 cm\(^{-1}\) bands in ATR-FTIR spectrum of ChitMan (SD, \(n = 3\)). 0.02 M Na-phosphate buffer solution, pH 7.4, 22°.

Thus, ChitMan is a perspective functionalizing coating for liposomes as a carrier for antituberculosis drugs with active targeting of alveolar macrophages. For the precise design of delivery systems for antituberculosis drugs based on liposome complexes with...
chitosan–mannose conjugates, a deep understanding of the mechanism of this interaction is required. Thus, despite the advantages of non-covalent liposomal complexes as drug delivery systems, careful selection of the lipid composition of vesicles and optimization of complexation are required. It is important not only to formally characterize the resulting complexes, but also to identify the main driving forces of complex formation.

The aim of this study is to study the interaction of chitosan–mannose conjugates with liposomes of various lipid compositions and to identify the key patterns of this process.

2. Materials and Methods

2.1. Materials

DPPC, cardiolipin, egg PC and moxifloxacin were obtained from Avanti Polar Lipids, Alabaster, AL, USA. Sodium phosphate and sodium acetate buffer tablets for solution preparation, CaCl$_2$, MnCl$_2$, concanavalin A were obtained from Pan-Eco, Moscow, Russia. ChitMan (chitosan $M_w$ 90–120 kDa, mannosylation degree 25%) was synthetized and characterized as described [6]. Briefly, chitosan was dissolved in 5% acetic acid. Next, $\alpha$-D-mannose solution in 15 M excess was added to the chitosan solution and the pH was adjusted to 9.0 with 1 M NaOH. The mixture was agitated for 10 min, 0.1 mg NaBH$_3$CN was added, and the agitation was continued for 40 min. The product was purified in dialysis bags (12 kDa cut-off; Orange Scientific, Braine-l’Alleud, Belgium) against a sodium phosphate buffer solution (pH 7.4). The degree of chitosan modification with mannose was determined by means of a trinitrobenzene sulfonic acid assay [6].

2.2. Liposome Preparation

Solutions of lipids in chloroform 25 mg/mL were taken in the desired ratio. CHCl$_3$ solvent was carefully removed on a vacuum rotary evaporator at a temperature not exceeding 55 °C. The resulting thin film of lipids was dispersed in a 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 on a 4710 “Cole-Parmer Instrument” disperser.

2.3. Complex Preparation

To obtain complexes, a solution of ChitMan (5 mg/mL) was added to the suspension of liposomes (5 mg/mL) in a base-molar ratio of 1:0.5 to 1:10.

In order to study the role of preheating, we incubated complexes on a shaker at a temperature of 37 °C. The temperature is close to the phase transition temperature of DPPC and DPPC-CL liposomes. The duration of preheating varied from 0 min to 60 min in increments of 15 min.

2.4. Moxifloxacin Liposomal Form Preparation

Moxifloxacin liposomal form (LMox) was prepared as the plain liposomes, but the buffer solution for dispersing the thin lipid film contained 4 mg/mL moxifloxacin, according to the procedure described in [14]. After sonication LMox suspension was separated from the free drug by dialysis against a sodium phosphate buffer solution pH 7.4 (Serva MW cut-off 3500). To the LMox, the ChitMan solution was added in a base-molar ratio of 1:5.

2.5. DLS Measurements

Determination of the size and zeta-potential of vesicles was carried out using a Zetasizer Nano S Malvern (Manchester, UK) (4 mW He—Ne laser, 633 nm) in a thermostated cell at 22 °C. The Malvern software was used.

2.6. NTA Measurements
Measurements of the concentration of particles were performed using the Nanosight LM10-HS instrument (UK) based on the nanoparticle tracking analysis (NTA). Solutions were diluted with Milli Q water until the optimum concentration for the analysis was reached (10⁷–10⁸ particles/mL). Each sample was measured three times.

2.7. ATR-FTIR Spectroscopy

The spectra were recorded using a Tensor 27 ATR-FTIR Fourier spectrometer (Bruker, Ettlingen, Germany) equipped with an MCT detector cooled with liquid nitrogen and a thermostat (Huber, Raleigh, NC, USA). The measurements were carried out in a BioATR 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; performing 70-fold scanning and averaging. 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, Ettlingen, Germany.

Carbonyl group spectral region deconvolution was conducted as described [15]. Curve-fitting was performed using the Bruker Opus 7.5 software. The center positions of the band components were found by the second-derivative production. Bands were fitted by components of the Gauss shape, with a correlation of at least 0.995. For DPPC:CL 80:20 (weight %) liposomes and its complex with ChitMan (base-molar excess 5), four components were observed: 1723 cm⁻¹, 1730 cm⁻¹, 1741 cm⁻¹, and 1748 cm⁻¹.

2.8. Kin Evaluation

Liposome–ChitMan complexes with different lipid/polymer ratios were prepared as described in Section 2.3. The liposome–ChitMan base-molar ratio was varied from 1:0.5 to 1:10. To determine the complex composition, we applied the procedure described in [16]. Briefly, unbounded ChitMan was separated from the complex by centrifugation with Eppendorf MiniSpin, 5000 rpm for 10 min, and then 10,000 rpm for 4 min. Under these conditions, ChitMan remains in the supernatant, while liposomes and complexes precipitate. After centrifugation, both supernatant and precipitate compositions were analyzed by ATR-FTIR spectroscopy using 1078 cm⁻¹ and 990 cm⁻¹ for ChitMan.

Initial concentrations of ChitMan ([ChitMan]₀) and unbounded ([ChitMan]ₜ) in supernatant were determined by ATR-FTIR spectroscopy using a calibration curve (Figure 1c). According to the experimental setup, the supernatant was out of liposomes, so typical liposomal bands did not contribute to the spectra of [ChitMan]₀ and [ChitMan]ₜ. The amount of bounded ChitMan ([ChitMan]ₖ) was determined using the equation:

\[
[\text{ChitMan}]ₖ = [\text{ChitMan}]₀ - [\text{ChitMan}]ₜ
\]

To determine the thermodynamic dissociation constant (Kₖₒ), the analysis of ATR-FTIR data was performed with Scatchard coordinates. The equilibrium in the system containing liposomes and polymer can be described by the following scheme:

\[
[\text{Liposome}] + [\text{ChitMan}] \leftrightarrow [\text{liposome − ChitMan}]
\]

This scheme corresponds to the following equation for Kₖₒ:

\[
K_{\text{dis}} = \frac{[\text{Liposome}]ₜ \times [\text{ChitMan}]ₜ}{[\text{Liposome} − \text{ChitMan}]}
\]

The material balance equations:

\[
[\text{Liposome}]₀ = [\text{Liposome}]ₜ + [\text{Liposome}]ₖ
\]

\[
[\text{ChitMan}]₀ = [\text{ChitMan}]ₜ + [\text{ChitMan}]ₖ
\]
\[ \text{Liposome} - \text{ChitMan} = [\text{ChitMan}]_b \]

Scatchard transformation of \(K_{dis}\) equation leads to the following:

\[
1 \div K_{dis} = \frac{[\text{ChitMan}]}{[\text{Liposome}]_b} \times ([\text{Liposome}]_b - [\text{ChitMan}])_f
\]

or

\[
\frac{[\text{ChitMan}]}{[\text{ChitMan}]}_f = \frac{1}{K_{dis}} \times [\text{Liposome}]_b - \frac{1}{K_{dis}} \times [\text{ChitMan}]_b
\]

The values of \([\text{ChitMan}]_b\) and \([\text{ChitMan}]_f\), both in molar concentrations, were plotted in Scatchard coordinates: \([\text{ChitMan}]_b\) versus \([\text{ChitMan}]_f\), and the dissociation constant \(K_{dis}\) was calculated by an approximation of the data by line.

2.9. Determination of Moxifloxacin Encapsulation Efficacy via UV-Vis Spectroscopy

UV-Vis spectroscopy was performed with Amersham Biosciences Ultraspec 2100 pro. An aliquot of an outer buffer solution after dialysis was transferred into quartz cells 108-QS Hellma Analytics, and the UV spectra of moxifloxacin were recorded, 210–380 nm at 22 °C.

2.10. Fluorescence Analysis

The emission spectra of moxifloxacin during drug release studies were recorded in the range from 400 nm to 550 nm at an excitation wavelength of 290 nm using a SpectraMax M5 (New York, USA). Experiments were performed in a temperature-controlled cell at 25 °C in sodium phosphate buffer solution pH 7.4 or sodium acetate buffer solution pH 5.5. A peak of 467 nm was determined. The excitation spectra were recorded at the range from 260 nm to 320 nm with an emission maximum of 467 nm with the same device. Spectra were analyzed in SoftMax software.

2.11. Moxifloxacin Release Kinetics Study

Studies were carried out in 0.02 M sodium phosphate (pH 7.4) and 0.02 M sodium acetate buffer solution (pH 5.5). The liposomal suspension was transferred into a dialysis capsule and placed on a shaker at 37 °C. During a period of 25 h, probes of external solution were analyzed with fluorescence to evaluate moxifloxacin concentration.

2.12. Studies of the Interaction of the Complex Liposomes—ChitMan with Concanavalin A

A complex of DPPC:CL 80:20 (weight %) with ChitMan in a base-molar excess of 5 was prepared as described in Section 2.3; however, the buffer solution also contained CaCl2 and MnCl2, both \(3.6 \times 10^{-5}\) M. The complex was added to a concanavalin A solution in a similar buffer solution to achieve the final protein concentration of 5.4 mg/mL. ChitMan excess was calculated as the ratio between the concentration of mannose units in ChitMan and mannose-binding sites in ConA (each protein contains 4 of them), which varied between 0.1 and 2. After 15 min of incubation at room temperature, the ATR-FTIR spectra were recorded. Spectra of liposomes in the corresponding concentrations were subtracted as a background. Spectra were min–max normalized on the Amide I band. \(K_{dis}\) values were calculated via linearization in the Scatchard coordinates, according to the procedure described in Section 2.8, considering the interaction of mannose-binding sites in the protein and mannose units in ChitMan.

3. Results and Discussion

3.1. Objects of Study

In order to control the ChitMan concentration in heterogeneous systems, e.g., liposomal systems, we have analyzed the ATR-FTIR spectra of polymer solutions with variable
concentrations. On the polymer ATR-FTIR spectrum, several bands were presented, and bands of 1078 cm\(^{-1}\) (C-O-C valence oscillations [17]) and 990 cm\(^{-1}\) (skeletal vibrations of C-O stretching [18]) are the most informative (Figure 1b). On the basis of these bands, we have obtained calibration curves with a clear linear approximation (Figure 1c).

As a model vesicle, we have considered four types of vesicles with variable phase state and surface charge (Table 1). As major lipids, we have chosen DPPC and egg PC, which are typical matrices for liposomal drug delivery systems. The addition of cardiolipin (20% weight) to the vesicle composition provides a negative ζ-potential caused by extra phosphate groups on the membrane surface.

Table 1 provides information about the lipid formulations under consideration and their physicochemical characteristics. One could note the gap between the DLS and NTA data caused by differences in physical approaches. This gap is typical, and for liposomes, Kim et al., in a recent paper, recommended that DLS be preferred over NTA [19]. Regardless, the obtained data are in suitable agreement and would be used further to investigate how ChitMan sorption on the liposomal surface influences the size and charge of vesicles.

### Table 1. Liposomes under consideration and their physicochemical characteristics. SD (n = 3). 0.02 M Na-phosphate buffer solution, pH 7.4, 22 °C.

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>Phase</th>
<th>Dh, nm (DLS)</th>
<th>Dh, nm (NTA)</th>
<th>ζ-Potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>Gel-like</td>
<td>57 ± 2</td>
<td>70 ± 3</td>
<td>-1.5 ± 0.5</td>
</tr>
<tr>
<td>DPPC:CL 80:20</td>
<td>Gel-like</td>
<td>80 ± 3</td>
<td>89 ± 3</td>
<td>-12 ± 1</td>
</tr>
<tr>
<td>Egg PC</td>
<td>Liquid crystal</td>
<td>112 ± 4</td>
<td>115 ± 3</td>
<td>-2.5 ± 1</td>
</tr>
<tr>
<td>Egg PC:CL 80:20</td>
<td>Liquid crystal</td>
<td>74 ± 5</td>
<td>80 ± 4</td>
<td>-16.0 ± 1.5</td>
</tr>
</tbody>
</table>

3.2. Effect of Complex Formation on the Size and ζ-Potential of Complexes

3.2.1. Liquid-Crystalline Liposomes: Role of Cardiolipin in Complex Formation

Mixed liposomes containing CL interact with polycations mainly by an electrostatic mechanism [20]. However, not only the charge, but the phase state of liposomes plays an important role affecting the nature of the interaction with the polymer. Here, we compared the interaction of liquid-crystalline (LC) neutral and anionic liposomes based on egg PC and egg PC:CL 80:20 (weight %) with a high M\(_w\) mannosylated chitosan derivative (molecular weight 90–120 kDa, mannosylation degree 25%).

The interaction of LC anionic liposomes (egg PC:CL 80:20) with the ChitMan results in the growth of the hydrodynamic diameter (Dh) from 74 nm to 110 nm with the formation of a polymer coat, up to 20 nm (Figure 2a), and partial neutralization of the ζ potential from -16 mV to -11 mV (Figure 2b), which is in good agreement with the data described in [11,21].
Figure 2. Dependence of main physicochemical properties of liposomal complex with ChitMan: egg PC: CL 80:20 liposomes (Dh, nm (a) and ζ-potential, mV (b)) and egg PC liposomes (Dh, nm (c) and ζ-potential, mV (d)). SD, n = 3. 0.02 M Na-phosphate buffer solution, pH 7.4, 22 °C.

Unexpectedly, the Dh curve for anionic LC liposomes (Figure 2a) is bell-shaped with a maximum base-molar excess (BME) of 2. The return of Dh to the initial values at a high BME is accompanied by the formation of a visible precipitate: apparently, only a small number of free vesicles remain in the solution. Thus, it is advisable not to exceed a BME of 3 for the formation of stable complexes in the case of anionic LC liposomes.

For LC neutral liposomes, no significant trend in changes in size (Figure 2c) or charge was observed (Figure 2d). However, fluctuations in these parameters may indicate partial sorption of the polymer on the surface of the vesicle.

Thus, egg PC:CL liposomes form a complex with the polymer according to the electrostatic mechanism, while the interaction of egg PC liposomes with the ChitMan is characterized by adsorption, probably with the formation of hydrogen bonds.

3.2.2. Gel-Like Liposomes: The role of Cardiolipin in Complex Formation

Comparing DPPC and DPPC:CL 80:20 (weight %) vesicle interaction with ChitMan, we have found a clear difference. Similar to the neutral LC liposomes, the interaction of neutral gel-like DPPC liposomes with ChitMan is not accompanied by either the hydrodynamic diameter or the ζ-potential significant changing (Figure 3, red dots).
Figure 3. Dependence of main physicochemical properties of liposomal complex with ChitMan: DPPC: CL 80:20 liposomes (black) and DPPC liposomes (red): Dh, nm (a) and ζ-potential, mV (b). SD, n = 3. 0.02 M Na-phosphate buffer solution, pH 7.4, 22 °C.

For anionic gel-like liposomes (DPPC:CL 80:20), an almost twofold increase in hydrodynamic diameter from 80 nm to 170 nm is observed with a BME of 5 (Figure 3a). The results differ from those obtained in previous studies [16], where the size of the complex of similar liposomes with PEGylated chitosan increased from 80 nm to 105 nm, which corresponded to a polymer coating size of approximately 25 nm. Apparently, the complexes of liposomes with PEGylated chitosan described in the literature are characterized by the exposure of long PEG chains (5 kDa) to the solution, while ChitMan, modified with short mannose units, forms a more compact, dense complex, which is detected by the DLS method. This is also confirmed by the data on the change in the ζ-potential of vesicles during complex formation: for liposomes with PEG-chitosan, the ζ-potential increased from −20 mV to −12 mV, whereas with ChitMan the charge on the vesicle surface changes from −12 mV to −5 mV (Figure 3b).

The maximum size is reached at a BME of 5, in contrast to egg PC:CL liposomes, in which the maximum is already observed at a two-fold excess. It is interesting to note that, for PC-CL liposomes, the maximum is reached at a BME two times lower, and the increase in the size of vesicles is also approximately two times lower (110 nm for PC:CL instead of 170 nm for DPPC:CL) than for the liposome DPPC:CL. The most pronounced effect for PC:CL liposomes with a lower BME than the polymer can be explained by the possible induction of a flip-flop of CL molecules from the inner side of the liposomal membrane to the outer one. However, if the maximum is clear for PC:CL liposomes, accompanied by a further decrease in the hydrodynamic radius, then for DPPC:CL liposomes, a further increase in the content of the polymer in the complex contributes to a decrease in the average diameter by about 40% (up to 110 nm). It is possible that the polycation loosens the gel-like membrane pointwise, and the dense packing of hydrophobic chains prevents the polymer from incorporating into the membrane over the entire surface of the liposome.

3.3. Discovering the Main Binding Sites of ChitMan via ATR-FTIR Spectroscopy

To discover the main binding sites of ChitMan on the vesicle surface we have applied ATR-FTIR spectroscopy, which is suitable for studying colloidal systems such as the
liposome–polymer complex [22]. This method provides detailed information on the state of the lipid functional groups; thus, one can obtain data on each part of bilayer: surface, subpolar region and hydrophobic area. The typical ATR-FTIR spectra of liposomes (Figure 4a) contain few absorption bands, which are informative in the analysis of the interaction of liposomes with polymers. Symmetric and asymmetric stretching vibrations of the CH₂ group correspond to bands in the region of 2850 ± 1 cm⁻¹ and 2919 ± 1 cm⁻¹. These absorption bands are sensitive to changes in liposome acyl chain packing [23]. The absorption band of the carbonyl group is located in the region of 1715–1750 cm⁻¹ [24] 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: νPO₂⁻_{s} 1088 cm⁻¹ and νPO₂⁻_{as} 1250–1220 cm⁻¹ [24]. The analytically significant νPO₂⁻_{as} band is of greatest interest as it is sensitive to the interaction of cationic ligands, e.g., polycations with the polar head of liposomes [15]. Changes in the position and shape of these bands usually indicate a change in the microenvironment of the corresponding functional groups; thus, the analysis of ATR-FTIR spectra makes it possible to identify the main binding sites of ligands, including polymers.

Figure 4. (a) Typical ATR-FTIR spectrum of liposomes DPPC:CL 80:20 (weight %). (b) Dependence of νPO₂⁻_{as} peak position on the base-molar excess of ChitMan for DPPC:CL 80:20 (weight %) SD, n = 3. (c,d) ATR-FTIR spectra of liposomes DPPC:CL 80:20 (weight %) and its complex with ChitMan (base-molar excess 5): carbonyl group area. Deconvolution was conducted with Gaussian function. Black line is initial spectrum, red line is component of low hydrated carbonyl groups, green line is component of moderately hydrated carbonyl groups and blue line is component of high hydrated carbonyl groups. 0.02 M Na-phosphate buffer solution, pH 7.4, 22 °C. Polymer spectrum was subtracted as a background in (a) proper concentration of (b–d).

As we proposed that phosphate groups on the liposomal surface are the main binding sites of the polycation, we have studied changes in this region cause by interaction with ChitMan with a varied BME (Figure 4b). With an increase in the BME, the absorption band νPO₂⁻_{as} shifts to the region of higher wavenumbers. This is a typical change that has been described for various electrostatic liposome complexes [15,24,25]. The high-
frequency shift is associated with a decrease in the degree of hydration of the phosphate groups due to the breaking of hydrogen bonds with water and the formation of electrostatic bonds with the polymer. The inflection in the curve is reached at a BME of about 5–6, which is in good agreement with the DLS data. Thus, phosphate groups are an important binding site of ChitMan.

On the other hand, the carbonyl group valence oscillation \( \nu_{CO} \) band is very sensitive to the changes in the subpolar area of the bilayer. How does complex formation influence this region? To address this question, we have applied a band deconvolution procedure for spectra obtained from a complex with a BME of 5. The \( \nu_{CO} \) band usually consists of several components [26] associated with high, medium and low state of hydration carbonyl groups [15]. The position and number of components depends on the composition of the liposomes and microenvironment of the carbonyl groups [26,27]. The ratio of the integral fractions of the components is associated with the redistribution of carbonyl groups according to the degrees of hydration.

The \( \nu_{CO} \) band of DPPC:CL 80:20 (weight %) consists of four components corresponding to low, medium and high state of hydration groups. The majority of carbonyl groups are in a low state of hydration (integral ratio is around 75% (Figure 4c), which is typical for gel-like vesicles with a density of lipids package. Complex formation leads to significant and unexpected changes: the integral ratio of moderately hydrated groups clearly increases (from 20% to 44%). How is it possible that the formation of an electrostatic complex leads to the binding of more water molecules with carbonyl groups? It is likely that this area of the bilayer does not interact with the polymer directly, but binds water molecules from the hydration shell of the polymer. Comparing the obtained results with previously published data [15,16,28–31], we would like to emphasize that this pattern is typical for “soft” polymers with a low density of charge such as chitosan and its derivatives.

When it comes to CH\(_2\) group bands, no significant changes were observed; thus, complex formation of gel-like anionic liposomes with ChitMan is driven by the electrostatic interaction with the liposomal surface (namely phosphate groups), while carbonyl groups “sense” only the hydration shell of polymer.

Is it possible to enhance this interaction? To address this question, we have studied the role of preheating in complex formation.

3.4. Influence of Preheating on the Complexation of Gel-Like Liposomes with ChitMan

DPPC liposomes undergo a main phase transition to gel-like LC at around 42 °C, with a preliminary transition at approximately 36 °C, corresponding to the gel-like ripple phase [32]. The addition of CL to the DPPC matrix, in a mass ratio of 80 to 20, contributes to a decrease in the phase transition temperature to 33 °C with a low-temperature shoulder (in the temperature range of 23–25 °C) [14]. Thus, incubation of liposome complexes near the phase transition temperature (37 °C) promotes an increase in the mobility of hydrophobic lipid chains and, as a result, could facilitate the incorporation of the polymer into the bilayer, which can potentially increase the stability of the liposome–polymer complexes.

Let us compare the size and charge dependences for DPPC and DPPC:CL 80:20 liposomes with and without preheating of the complex for 45 min. Incubation of the polymer with gel-like liposomes for 45 min at 37 °C accelerates lateral lipid segregation and enables flip–flops [33]. This leads to more pronounced trends in the change in the charge and size of anionic gel-like liposomes (Figure 5a,b). In the case of neutral DPPC liposomes (Figure 5c,d), heating did not contribute to a significant change in the \( \zeta \)-potential of the vesicles; however, the size increased with an increase in the molar excess, which indicates an increase in sorption interactions.
Figure 5. Dependance of main physicochemical properties of liposomal complex with ChitMan with preheating at 37 °C for 45 min: DPPC: CL 80:20 liposomes (Dh, nm (a) and ζ-potential, mV (b)) and DPPC liposomes (Dh, nm (c) and ζ-potential, mV (d)). SD, n = 3. 0.02 M Na phosphate buffer solution, pH 7.4, 22 °C.

Preheating of the complexes promotes a tighter interaction for both anionic and neutral gel-like liposomes.

Interestingly, the maximum size for DPPC:CL liposomes during preheating shifts from a BME of 5 to a BME of 2, as in case of the egg PC:CL system, but the course of the curve is smoother. Obviously, the mobility of hydrophobic chains plays a key role in the process of complex formation, but even incubation does not make the DPPC:CL and PC:CL complexes the same, since preheating increases not only the mobility of lipids, but also the mobility of chitosan chains.

3.5. \( K_{\text{dis}} \) Evaluation of Liposome Complexes with ChitMan

For engineering the surface of liposomes, it is necessary to select stable complexes with polyelectrolytes, so knowledge of the values of the dissociation constants is key. Depending on the composition of the lipid matrix and on the conditions of complex formation, it seems possible to control the stability of the resulting complex.

To calculate the dissociation constants, we used the standard method detailed in [16]. Briefly, the suspension of the complex was centrifuged under conditions whereby the complex and free liposomes precipitated and the free polymer remained in solution. Its content was determined using ATR-FTIR spectroscopy from the intensity of the absorption bands at 990 cm\(^{-1}\) and 1078 cm\(^{-1}\), for which linear calibration dependences were obtained (Figure 1b,c). Sorption isotherms were linearized in the Scatchard coordinates.

Previously we have shown that preheating significantly affects the \( K_{\text{dis}} \) values for the DPPC:CL liposome-glycol-chitosan complex [31]. When liposomes are close to phase transition, the anionic groups of lipids are more accessible for complexation.

Let us firstly compare \( K_{\text{dis}} \) for neutral and anionic gel-like liposomes (Table 2). The DPPC:CL 80:20–ChitMan complex turned out to be approximately three times more stable, which correlates well with previously obtained data. However, the order of the constant \( (10^{−4}) \) makes it impossible to recommend these complexes for use in dilute systems. For biomedical applications, it is desirable to use non-covalent complexes with a dissociation constant of no more than \( 10^{−5} \) M.
For more efficient binding of the polymer on the surface of anionic gel-like liposomes, it is advisable to preheat the mixture. On the one hand, preheating accelerates the lateral segregation and flip–flop effect for CL. On the other hand, preheating leads to greater mobility of the rigid chains of high molecular weight chitosan [34]. However, prolonged heating may lead to the complete release of the liposome content, so it is necessary to set the minimum allowable preheating time to obtain stable complexes.

Obtained data (Table 2) indicate a positive effect of prolonged heating on complex stability: the longer the preheating, the lower the dissociation constant. Even 15 min of preheating leads to a lower $K_{\text{dis}}$, but a clearer effect is observed with longer preheating (45 min and 60 min). Thus, long preheating leads to a lower dissociation constant, reducing it by an order of magnitude.

The obtained data correlates with the stability of the liposomal complexes: during 3 weeks of storage under 4 °C, samples were stable according to DLS data, except for the egg PC liposomes complexed with a high BME of ChitMan (9–10), which precipitated during one day, and thus are not suitable for further drug delivery system design.

Table 2. $K_{\text{dis}}$ of complexes of gel-like liposomes and ChitMan with and without preheating. 0.02 M Na-phosphate buffer solution, pH 7.4, 22 °C. SD, $n = 3$.

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>Preheating 37 °C</th>
<th>$K_{\text{dis}}$, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>0 min</td>
<td>$(3.8 \pm 0.1) \times 10^4$</td>
</tr>
<tr>
<td>DPPC:CL 80:20</td>
<td>0 min</td>
<td>$(1.2 \pm 0.1) \times 10^4$</td>
</tr>
<tr>
<td>DPPC:CL 80:20</td>
<td>15 min</td>
<td>$(9.3 \pm 0.1) \times 10^5$</td>
</tr>
<tr>
<td>DPPC:CL 80:20</td>
<td>30 min</td>
<td>$(9.5 \pm 0.1) \times 10^5$</td>
</tr>
<tr>
<td>DPPC:CL 80:20</td>
<td>45 min</td>
<td>$(6.4 \pm 0.1) \times 10^5$</td>
</tr>
<tr>
<td>DPPC:CL 80:20</td>
<td>60 min</td>
<td>$(3.5 \pm 0.1) \times 10^5$</td>
</tr>
</tbody>
</table>

3.6. Complex DPPC:CL Liposomes with ChitMan Binds with Model Mannose Receptor Concanavalin A

We consider the complex of liposomes DPPC:CL 80:20 (weight %) with ChitMan as suitable for further tailoring to an antituberculosis drug delivery system. However, will this complex be able to bind with mannose-binding receptors? Concanavalin A (ConA) is a suitable protein for screening studies, as proved by molecular modeling [13] and experiments [35,36]. These experiments show clear correlation between effective ligand binding with ConA and a high affinity for mannose-binding receptors of macrophages. In previous experiments, we have studied the interaction of ChitMan and arabinomannan with ConA, and ChitMan has shown a clear affinity for the lectin [6]. Here, we apply the same technique to study the interaction of DPPC:CL 80:20 (weight %) liposomes–ChitMan complex with ConA.

The most informative area on the ATR-FTIR spectrum of proteins such as ConA is the amide I and II region, corresponding to the valence oscillation of peptide bonds [37]. While spectra are normalized on the amide I band, changes in amide II intensity indicate changes in the protein microenvironment and binding process [38,39].

When even a small concentration of the complex of liposomes with ChitMan appears in ConA solution, the normalized intensity of amide II bands increases significantly (Figure 6a).
Dependance of the amide II normalized intensity on the ChitMan (complexed with liposomes) excess, presented in Figure 6b, can be approximated with hyperbolic shape with a leap around the equimolar ratio. On the other hand, titration of ConA with a liposomal suspension without ChitMan leads to an immediate leap and then plateau, indicating nonspecific interactions. Here we would like to underline that, for better representation of the data, we have calculated ChitMan excess (Figure 6b X axes) as the ratio between the concentration of mannose units in ChitMan and mannose-binding sites in ConA (each protein contains 4 of it [35]).

Linearization in the Scatchard coordinates displays two clear regions indicating two populations of sorption centers (Figure 7). When, for the first region (blue line on Figure 7), tga leads to a $K_{diss}$ value $(6.7 \pm 0.1) \times 10^{-6}$ M, this indicates strong binding with ConA, and the second region (red line on Figure 7) corresponds to nonspecific binding with a $K_{diss}$ value $(8.0 \pm 0.1) \times 10^{-5}$ M. The obtained $K_{diss}$ value for the complex is better than that for ChitMan separately $1.6 \times 10^{-5}$ M [6].
Figure 7. Linearization of the sorption isotherms of the complex of DPPC:CL 80:20 (weight %) liposomes with ChitMan (BME 5) in Scatchard coordinates. SD, \( n = 3 \). 0.02 M Na-phosphate buffer solution, pH 7.4, 22 °C. ConA concentration 5.4 mg/mL. The concentration of the Ca\(^{2+}\) and Mn\(^{2+}\) ions is \( 3.6 \times 10^{-5} \) M.

What is the nature of the observed pattern? ConA obviously binds with the mannose-modified object, however, is it unbounded polymer, which could be present in liposomal complexes, or the exact complex of ChitMan with liposomes? Let us have a look at the shape of the amide I band in the ConA spectra (Figure 6a). The main peak becomes narrower as the complex is added, but typical shoulders appear around 1680–1690 cm\(^{-1}\), indicating changes in β-sheet organization [38]. Interestingly, when ConA binds with ChitMan separately [6], the amide II normalized intensity decreases in contrast with the data under discussion (Figure 6b). According to Van De Weert [40], changes in the amide II normalized intensity could still correlate with protein structure as a function of its surroundings (e.g., pH, ionic strength, denaturants, elevated temperature). It is probable that binding with a large liposome–ChitMan complex changes the microenvironment of ConA in another manner than just ConA. Nonspecific binding that is uncovered is also observed, which supports the hypothesis that the observed pattern is caused by binding between protein and complex.

Obtained results are in very good agreement with previously published studies on the mannosylated DPPC liposome interaction with ConA, as revealed by the time resolved fluorescence methods and surface plasmon resonance kinetic measurements [41]. Sandoval-Altamirano et al. have also described two types of binding (mono and multivalence), that are in good agreement with the data presented here.

Thus, complex formation provides the liposomes with preferable binding with the model mannose-binding receptor ConA.

3.7. Moxifloxacin Release Kinetics Study

Complex formation could significantly decrease the rate of drug release and it is desirable to create a pH-sensitive drug delivery system. It is well known that inflammation usually leads to lower pH values ca. 5.5. [42]; thus, we have tried to find out if ChitMan is able to form a “smart” drug delivery system. As a model antituberculosis drug, we consider the fourth generation fluoroquinolone moxifloxacin. Its liposomal form was previously described [14] when included in the DPPC:CL 80:20 liposomes; Mox, located in the
inner aquas space of the liposome, binds with the bilayer inner surface by means of electrostatic interactions.

LMox liposomes have a Dh (according to DLS) of 82 ± 3 nm, ζ-potential of −11.5 ± 1.5 mV, and encapsulation efficiency of 72 ± 4%, which corresponds to 5.6% of the drug:lipid mass ratio. The obtained data are in a good agreement with the previously published literature [14].

A coating with ChitMan with a BME of 5 led to formation complexes with a Dh of 150 ± 4 nm and ζ-potential of −9.2 ± 1.5 mV. These values are close to those for “empty” liposomes (Figure 3); thus, drug loading at the first glance does not significantly influence the complex formation.

We have studied moxifloxacin release under two model conditions: the neutral buffer solution pH 7.4 represents the simplest model of healthy tissues (Figure 8a), while the acidic buffer solution pH 5.5 represent inflammation (Figure 8b).

In neutral media, complex formation has significantly slowed down the drug release (tgα decreases from 1.01 to 0.16); the polymer coating prevents burst release, which is observed for LMox. On the other hand, in acidic media, drug release curves are much closer to each other’s release (tgα decreases from 1.02 to 0.27). This significant difference is probably caused by the influence of H+ on the amino groups of ChitMan. For DPPC:CL liposomes, electrostatic forces are the main course of complex formation, mainly because of binding between the amino groups of ChitMan and the phosphate groups of lipids. Thus, the higher concentration of hydrogen ions weakens this interaction. In a neutral solution, the complex remains more stable and the polymer coating slows down moxifloxacin release. Thus, the ChitMan coating provides obvious benefits for liposomal moxifloxacin, namely, pH-sensitive drug release.

How does the chitosan derivative nature affect the release of contents from liposomes? Let us compare the data obtained here with the previously obtained results on the release of doxorubicin from glycol–chitosan (Mw 72,000)-coated liposomes. According to [31], at pH 7.4, doxorubicin release is significantly slowed down, while at pH 5.5, almost all doxorubicin is released after 25 h. Thus, the low molecular weight derivative of chitosan acts more severe, preventing the release of the drug in a neutral environment and promoting it in an acidic one. High molecular weight mannosylated chitosan acts more gently, only limiting the release rate of the content in a neutral medium. The selection of a chitosan derivative allows tailoring of the properties of the delivery system depending on the target organs and tissues.

Figure 8. Moxifloxacin release kinetics from DPPC:CL 80:20 liposomes (blue line) and complex of DPPC:CL 80:20 liposomes in complex with ChitMan, BME 5 (red line). (a) 0.02 M Na-phosphate buffer solution, pH 7.4, 37°. (b) 0.02 M Na-acetate buffer solution, pH 5.5, 37°.
4. Conclusions

The development of delivery systems based on functionalized liposomal constructs remains an urgent task for biomedicine and bionanotechnology. The choice of the optimal functionalizing agent, the selection of the lipid composition and the influence of conditions is crucial when it comes to the design of a new generation of drug delivery.

The aim of this work was to study the interaction of mannosylated chitosan with liposomes of various compositions and to identify the key patterns of this process.

Anionic liposomes, both in LC and in the gel-like state, form multipooint non-covalent complexes with ChitMan due to partial neutralization of charges on the surface of vesicles, while neutral liposomes in both phase states form unstable heterogeneous complexes with ChitMan, probably due to the predominant sorption of the polymer on the vesicles.

During the titration of liposomes by ChitMan, an atypical bell-shaped course of the curves was found with a characteristic maximum at a BME of 2 for egg PC:CL liposomes and at a BME of 5 for DPPC:CL liposomes. PC:CL liposomes increased in size at a BME of 2 to 110 nm, then the hydrodynamic diameter returned to its initial values. DPPC:CL liposomes increased in size at a BME of 5 by almost two-fold (up to 160 nm), and then their size decreased to 40%.

According to the ATR-FTIR data, complex formation of gel-like anionic liposomes with ChitMan is driven by the electrostatic interaction with the liposomal surface (namely phosphate groups), while carbonyl groups “sense” only the hydration shell of polymer.

A comprehensive study of the influence of the lipid matrix composition and complex formation conditions on the value of the dissociation constant was carried out. The inclusion of cardiolipin in the lipid composition helps to reduce the dissociation constant of the complexes by an order of magnitude, with the best incubation time at 37 °C being 45 min (from $1.2 \times 10^{-4}$ M to $3.5 \times 10^{-5}$ M for DPPC:CL liposomes). Optimal binding was found for the system DPPC:CL liposomes by preheating for 45 min at 37 °C: $K_{\text{dis}}$ was $6.4 \times 10^{-5}$ M.

Complex formation provides preferable binding with the model mannose-binding receptor ConA with a $K_{\text{dis}}$ value $(6.7 \pm 0.1) \times 10^{-5}$ M; this is promising for further drug delivery system design. Moreover, the polymer coating provides the liposomal formulation of moxifloxacin sustained and pH-sensitive drug release, which is very promising in design, not only for antituberculosis therapeutics, but also anti-inflammatory drugs, where fast drug release in an acidic media is desired.

The results obtained in the course of the work contribute to a deeper understanding of the fundamental principles of the interaction mechanism of vesicles with polymers of various molecular architectures and further development of liposomal delivery systems.

Author Contributions: Conceptualization, I.M.L.-D., . . .; Methodology, data curation V.V.R., I.D.Y. and I.M.L.-D.; Original draft preparation, E.V.K. and A.A.S.; Writing – review and editing., E.V.K. and A.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: The research was funded by RFBR and Moscow city Government, project number 21-33-70035.

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Acknowledgments: The work was performed using equipment (FTIR spectrometer Bruker Tensor 27 and Jasco J-815 CD Spectrometer) of the program for the development of Moscow State University. We thank Anna Berkovich for the constructive discussions.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.
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