Solubilization of Liposomes Containing Cholesterol by \(N\)-dodecyl-\(N\),\(N\)-dimethylamine-\(N\)-oxide

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Abstract. \(N\)-dodecyl-\(N\),\(N\)-dimethylamine-\(N\)-oxide (C\(_{12}\)NO) is a very potent bactericidal agent. It is supposed, that this is related to the solubilization of membranes. For this reason we study the effect of the C\(_{12}\)NO on solubilization of the model membrane system – bilayers in multilamellar liposomes from egg yolk phosphatidylcholine (EYPC). The solubilization process causes a decrease in particle size, therefore a suitable method for its monitoring is turbidimetry. In this study we investigated the effect of the addition of cholesterol (CHOL) on the solubilization of EYPC liposomes, caused by C\(_{12}\)NO, at different CHOL:EYPC molar ratios. The solubilizing concentration (c\(_S\)) of C\(_{12}\)NO was determined as the C\(_{12}\)NO concentration causing the half-maximum decrease in the turbidance. The solubilizing concentration value increases linearly with the CHOL:EYPC molar ratio. We conclude that the inclusion of cholesterol in EYPC bilayers makes the bilayers more resistant to solubilization.

Introduction

Biological membrane consists mainly of lipids, proteins and saccharides molecules and represents the phase boundary between the cell and its surrounding. Basic structural units of membranes are bilayers consisting of phospholipids with hydrophilic heads (cholin, serin, ethanolamine, inositol), glycerol or sphingosine connecting chain and lipophilic tails (fatty acids with 14-24 carbons). Therefore, the general feature of membrane lipids is their amphiphilic nature and spontaneous tendency to form organized structure in the aqueous environment. These structures are used for the preparation of model membranes which are (in the present study) phospholipid bilayers enclosed in multilamellar liposomes from EYPC (egg yolk phosphatidylcholine). \(N\)-alkyl-\(N\),\(N\)-dimethylamine-\(N\)-oxides (C\(_n\)NO, \(n\) is the number of carbon atoms in the alkyl substituent) are non-ionic surfactants at physiological values of pH, with a strong polar N–O bond and a high electron density on the oxygen [1]. C\(_n\)NOs have been employed as bactericidal and algicidal agents. Also they are widely used as indispensable agents for isolation, purification and crystallization of membrane proteins [2]. In the C\(_n\)NO homologous series, solubilizing properties of \(N\)-dodecyl-\(N\),\(N\)-dimethylamine-\(N\)-oxides (C\(_{12}\)NO) were intensively studied. The physico-chemical and biological properties of C\(_{12}\)NO result from its amphiphilic structure [3]. Therefore they are predetermined to affect the phospholipid bilayer, the structural matrix of biological membrane [4]. The polar fragment (N-oxide group) interacts with polar fragments of phospholipid and the non-polar fragment (dodecyl chain) interacts with the lipophilic part of phospholipid. However, the dodecyl chain of C\(_{12}\)NO is shorter than phospholipids chains. Therefore, due to the unequal length of chains, the free volume in bilayer is formed. The free volume theory predicts, that the size of a structural defect should be a non-linear quasi-parabolic function of the length of amphiphile hydrocarbon chain [5]. Devinsky et al. [6] found, that increasing of alkyl chain in N-oxides, the biological activity increases, but it starts to decrease after reaching the maximum value at a certain chain length. This dependence in homologous series of N-oxides as well as of other amphiphiles is called the cut-off effect. Consequences of the free volume formation are conformation changes of acyl and alkyl chains – trans-gauche isomerisation, or their interdigitation. Therefore the changes of physical parameters in biomembranes occur [7]. These changes can lead to the membrane solubilization. This process starts with a gradual incorporation of amphiphile into the lipid membrane. Thus, the lipid membrane attacked by amphiphile is weakened with structural defects, and finally this process results in its fragmentation and solubilization. Cholesterol (CHOL) as the main sterol plays an important role in mammalian and fungal cell membranes as a modulator of membrane structure, dynamics, and/or function through three major molecular mechanism. CHOL 1. modulates membrane proteins function through sterol-protein interaction, 2.modulates the internal properties of
the lipid bilayer of the cell membrane, and alters the lateral distribution of components in the cell membrane [8].

In the present paper we study C_{12}NO induced solubilization of liposomes containing cholesterol at different CHOL:EYPC molar ratios. The effect of C_{12}NO on multilamellar EYPC is studied by turbidimetry. In the previous work [4] the solubilization of multilamellar EYPC liposomes was studied. In our contribution we extend these measurements by incorporating cholesterol into the multilamellar liposomes.

**Material and methods**

**Chemicals**

EYPC was isolated and purified according to [9]. Egg yolks from fresh hen eggs (cca 500 g) were separated from the whites. Than yolks were multiple blended with acetone at 25 °C and filtered. The acetone mixture containing most of the neutral lipids and pigments was discarded. Filtered solids were twice suspended in 96 % ethanol and dried on a rotary evaporator. The raw phosphatides were extracted with petroleum ether and the extract was poured into acetone (cooled to 15 °C) until the supernatant cleared. Acetone extraction step was repeated with the precipitate. The solvent was removed using rotary evaporator. The raw phosphatides were fractionated using alumina column chromatography. The purity of EYPC in collected fractions from chromatography was checked by a two-dimensional TLC. The first dimension of developing system was chloroform:methanol:ammonia (25% aqueous solution) = 65:30:4. The system chloroform:methanol:acetic acid:water = 170:25:25:6 was applied in the second dimension. Ammonia vapour and bromthymol blue were used for detection. Only one spot corresponding to phosphatidylcholine was observed. The solvent was removed from collected fractions on the rotary evaporator and its traces by a two-stage vacuum oil rotary pump. Dry EYPC was stored under nitrogen atmosphere at –40 °C. The molecular weight of EYPC estimated from the composition of its acyl chains according to [10] was 779.7 g/mol.

The chemicals used as solvents for preparation, purification and chromatographic analysis of EYPC and also for sample preparation were obtained from Slavus (Bratislava, Slovakia). Organic solvents and water were redistilled before use. C_{12}NO and CHOL were from Sigma–Aldrich (Germany).

**Sample preparation**

Weighted amounts of EYPC and CHOL in EYPC:CHOL= 0; 0,1; 0,2; 0,3; 0,4; 0,7 ;1 molar ratios were dissolved in required volumes of chloroform and were mixed in glass tubes. The solvent was removed with a stream of gaseous nitrogen and its traces by evacuation in a vacuum chamber. The multilamellar liposomes were prepared by dispersing EYPC+CHOL in the redistilled water by hand shaking until the opalescent dispersion formed. Before the experiment, the samples containing dispersion of EYPC:CHOL, redistilled water and C_{12}NO solution with increasing concentration were prepared. The turbidance of the samples was measured 30 min after sample preparation at 25 °C and 400 nm in the spectrophotometric 1 cm quartz cell using the Hewlett Packard 8452 spectrophotometer (Palo Alto, USA).

**Methods**

The solubilization process causes a decrease in size of particle, therefore a suitable method for its monitoring is the turbidimetry. Turbidimetry as analytical method is based on measuring the Rayleigh scattering of attenuated radiation. The turbidimetric measurement can be performed on a conventional spectrophotometer if the attenuation of primary radiation flow is measured in the direction of impact,. Attenuation, after passing dissipating environment, is characterized as turbidance. Turbidance \( A_T \) is defined as:

\[
A_T = \tau \cdot d \cdot \log e.
\]

where \( \tau \) is the turbidity coefficient, \( d \) is the optical path of light in the sample and \( e \) is the base of natural logarithms. According to the Rayleigh law, the turbidity coefficient is proportional to deformability of particles dissipating light \( \alpha \) and their number in unit volume \( N \), and inversely
proportional to the fourth square of the wavelength of incident light as follows:

\[ \tau = \frac{8\pi}{3} \left( \frac{2\pi}{\lambda} \right)^2 N\alpha^2. \]

In the case of spherical particles of radius \( r \) it is valid:

\[ \alpha = 4\pi\varepsilon_0 r^3, \]

where \( \varepsilon_0 \) is vacuum permittivity.

**Results**

The \( A_T \) values of liposomes increase with the EYPC concentration \( c_{EYPC} \) in the liposome dispersion as expected (Fig. 1.). Safely linear is in the range \( c_{EYPC} < 0.5 \text{ mmol/dm}^3 \). The correlation coefficient for samples without cholesterol was \( r^2 = 0.998 \) and for samples with cholesterol \( r^2 = 0.996 \) in this region. For next experiments we use \( c_{EYPC} = 0.4 \text{ mmol/l} \) in all samples.

Increasing concentration of C12NO in the liposome dispersion leads to a decrease of \( A_T \) values, which represents the solubilizing curve. Its course was explained by Lichtenberg et al. [11]. They describe a three-phase model of membrane interaction with a detergent. With an increase of detergent concentration there are assumed following transformation phases of bilayers. In the I. phase the concentration of detergent in solution is low, less than solubilizing concentration. The incorporation of detergent molecules into the lipid bilayer occurs. Formed mixed bilayers are in equilibrium with monomers of detergent in the solution. The physico-chemical properties of bilayer are changing. In the II. phase, a bilayer phase transition occurs. After saturating bilayers with detergent mixed micelles of lipid-detergent begin to form. The system is characterized by a significant heterogeneity of particle dimensions. The liposomes bilayers saturated with detergent coexist with mixed lipid - detergent micelles and with detergent micelles. The size of mixed micelles is smaller than the original phospholipid liposomes. Further increasing of detergent concentration leads to the III.phase, where almost all bilayer components are fully solubilized and incorporated in mixed micelles. All phospholipid bilayer phases disappear and only mixed micelles are present. After complete phase transition, the ratio detergent : phospholipid in mixed micelles increases.

In the following experiment we observed the dependence of turbidance on increasing concentration of C12NO at a constant concentration of EYPC (0.4 mmol/dm³). Different solubilization curves were obtained for samples with increasing CHOL:EYPC molar ratio. Typical results are shown on Fig. 2. In a CHOL-free samples (●), the turbidance remains unchanged at lower C12NO concentrations which represents the I. phase (detergent incorporates into liposomes). In the II. stage, the turbidance decreases sharply, liposomes transform into mixed-micelles and coexistence with detergent micelles. Finally, turbidance reaches the zero value (III. phase, presence of only mixed micelles). In the presence of CHOL, already small concentration of C12NO (up to 0.5 mmol/dm³)
causes a slight decrease of turbidance values. We have no explanation for this effect. Therefore,
further experiments will be needed to specify the structure of resulting aggregates in this stage. We
also observed an increase of $A_T$ values during the I. and II. phase with increasing CHOL:EYPC molar
ratio. It indicates probably an increase of multilamellar liposome diameter.

The experimental $A_T$ values were evaluated in range of C$_{12}$NO concentrations from 0.5–
5 mmol/dm$^3$ and data were fitted with the reverse sigmoidal function which simulates the sharp
decrease of $A_T$ in the II. phase:

$$
A_T = A_T(0) \left(1 - \frac{1}{1 + \exp \left(\frac{c_{\text{C12NO}} - c_S}{\Delta c_{\text{C12NO}}} \right)}\right),
$$

where the constant $A_T(0)$ is the value of $A_T$ before solubilization i.e. at a C$_{12}$NO concentration, which
assumes the existence of liposomes only. The parameter $c_S$ (solubilizing concentration) represents the
concentration of C$_{12}$NO, which causes a half-decrease of $A_T(0)$. The parameter $\Delta c_{\text{C12NO}}$ characterises
the width of II. phase, the coexistence of liposomes and mixed-micelles.

Fig. 3 shows that $c_s$ for the CHOL-free samples is 1.57 mmol/dm$^3$ of C$_{12}$NO. Similar result was
presented by Karlovská et al. [4]. It can be seen that the $c_s$ increases approximately linearly ($r^2 =
0.986$) depending on increasing CHOL:EYPC molar ratio. Recent papers from our group [13–16]
demonstrated that cholesterol increases the lipid bilayer thickness. This is connected with an increase
of the order of lipid acyl chains in the liquid-crystalline state. We suppose therefore that higher
concentration of C$_{12}$NO is needed for solubilization of ordered CHOL+EYPC bilayers.

![Figure 2](image_url)

**Figure 2.** Dependence of turbidance $A_T$ of EYPC liposomes on C$_{12}$NO concentration $c_{\text{C12NO}}$ at 400 nm
at CHOL:EYPC molar ratios ● 0; ■ 0.2; ▲0.7; ▼1. Concentration of EYPC $c_{\text{EYPC}}$= 0.4 mmol/l.

![Figure 3](image_url)

**Figure 3.** Dependence of solubilizing concentration of C$_{12}$NO on increasing molar ratio of CHOL:EYPC
at a constant concentration $c_{\text{EYPC}} = 0.4$ mmol/dm$^3$. 

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The parameter $\Delta c_{C12NO}$ (Fig.4) characterize the width of the II. phase in which liposomes bilayers coexist with mixed micelles and detergent micelles. With the increasing of CHOL:EYPC molar ratio was seen only a slight increase of $\Delta c_{C12NO}$. Large experimental errors of measuring are mainly caused by small number of experimental points (?). Our results show that the amount of CHOL in EYPC bilayer do not influence the width of the II. phase of solubilization process.

**Conclusion**

In conclusion, the influence of CHOL on the solubilization of EYPC liposomes caused by C12NO was studied by turbidimetry. We observed an increase of $A_T$ values during the I. and II. phase with increasing CHOL:EYPC molar ratio. We showed that higher concentration of C12NO is needed to solubilize EYPC liposomes in the presence of CHOL. $c_s$ increases when CHOL:EYPC molar ratio rises.

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**References**