 DOI: 10.17344/acsi.2014.750

Scientific paper

Cholesterol Protects Phosphatidylcholine Liposomes from *N,N*-dimethyl-1-dodecanamine *N*-oxide Influence

Silvia Huláková,[1,*] Jana Gallová[1] and Ferdinand Devínsky[2]

[1] *Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University in Bratislava, Bratislava, Slovakia*

[2] *Department of Chemical Theory of Drugs, Faculty of Pharmacy, Comenius University in Bratislava, Bratislava, Slovakia*

** Corresponding author: E-mail: hulakova@fpharm.uniba.sk*
Tel.: +421 250117289

Received: 16-06-2014

Abstract

The interaction of *N,N*-dimethyl-1-dodecanamine *N*-oxide ($C_{12}NO$) with egg yolk phosphatidylcholine (EYPC) liposomes containing cholesterol (CHOL) was studied. The perturbation of CHOL-EYPC bilayers in unilamellar liposomes (ULL) was observed by the leakage of fluorescence probe calcein. Weak leakage is observed at low surfactant concentration c_{C12NO} (minimal perturbation of the bilayer) followed by an intensive leakage at a middle c_{C12NO} (creation of pores). No change in fluorescence intensity was measured at high c_{C12NO} (calcein totally released from liposomes). The higher CHOL amount in the bilayer, the more surfactant is needed to create pores in the bilayer.
Solubilization of CHOL-EYPC ULL induced by $C_{12}NO$ was studied turbidimetrically. The solubilization curve consists of three parts: saturation of bilayer at low c_{C12NO} (liposomes are preserved), followed by solubilization (liposome – mixed micelle transition) and post-solubilization. The $C_{12}NO$ concentration needed for the onset of the solubilization raise with the increase of n_{CHOL}:n_{EYPC}. The structure of liposomes is still preserved at total calcein release for all n_{CHOL}:n_{EYPC}.

Keywords: Unilamellar liposome; solubilization; cholesterol; calcein; leakage; *N,N*-dimethyl-1-dodecanamine *N*-oxide

1. Introduction

N,N-dimethyl-1-alkanamine *N*-oxides (C_nNOs, where n is the number of carbon atoms in the alkyl substituent) exert a wide range of biological effects, among them antiphotosynthetic,[1] phytotoxic,[2] immunomodulatory[3] and antimicrobial effect,[4–6] where *N,N*-dimethyl-1-dodecanamine *N*-oxide ($C_{12}NO$) is one of the most efficient homolog. C_nNOs (n = 10–16) are surfactants of everyday contact because they are widely commercially used as a component in home cleaning products, shampoos, conditioners and pharmaceutical formulations. $C_{12}NO$ is also used as a mild biological surfactant in membrane studies for purification, reconstitution and crystallization of membrane proteins and solubilization of membranes. pKa for C_nNOs is 4,9 which implies a non-ionic character at physiological pH.[7,8] In this paper, the influence of surfactant $C_{12}NO$ on a model membrane is studied in a broad concentration range.

Perturbations in the phospholipid bilayer arise as a consequence of surfactant partitioning in biological or model membrane.[4,9–12] These perturbations can lead to a breakdown of the hydrophobic barrier and its permeabilization to solutes. Creation of pores by stabilizing the hydrophobic edges with surfactant-rich rims is supposed.[10] If liposomes are filled with water-soluble fluorescence probe at high, self-quenching concentration, the probe leaks through these pores and dilutes to a detectable concentration in bulk solution.[13] Fluorescence method is hence a valuable tool sensitive to initial destruction of membrane. Calcein[13–15] and carboxyfluorescein[9,16–18] are often used fluorescence probes in leakage experiments.

Solubilization of phospholipid bilayers can occur at high surfactant concentration where liposomes are transformed to small mixed micelles.[10,19–24] The process of solubilization can be followed using several experimental methods.[25] Light scattering suffices for accurate and fast detection of a decrease in particle size during lamellar –

micellar phase transition. It is often substituted by turbidance (apparent absorbance) measurement around 400 nm using UV-VIS spectrophotometer. Turbidance proportionally depends on the particle radius[26,27] and its measurement is therefore a reliable tool to study a solubilization process, especially completion of solubilization.[24]

Solubilization of one-component phosphatidylcholine membranes by $C_{12}NO$ was already investigated in our earlier papers by turbidimetry[11,28] and small angle neutron scattering.[29] Unilamellar liposomes (ULL) from egg yolk phosphatidylcholine (EYPC) with increasing amount of cholesterol (CHOL) are used as model membrane in the present study. CHOL (or its analogues) together with phosphatidylcholine is an inevitable constituent of eukaryotic biological plasma membranes. A huge number of papers (see[30–32] for a review) have studied the influence of CHOL on the physicochemical properties of biological and model membranes, nevertheless not all aspects of CHOL function in membrane have been elucidated. The role of CHOL in the solubilization of different model membranes has been studied mostly using surfactant Triton X-100.[19,33,34]

In this paper, turbidimetry and fluorescence probe leakage are used to a systematic investigation of the effect of increasing amount of CHOL on the solubilization of EYPC bilayers in ULL by the non-ionic surfactant $C_{12}NO$. The highest used molar ratio of CHOL:EYPC was 0,8, CHOL was therefore fully solubilized in EYPC bilayer.[35]

2. Materials and Methods

2. 1. Chemicals

Chromatographically pure EYPC was isolated and purified from hen eggs according to[36] as modified in.[37] ULL were prepared by extrusion using LiposoFast Basic Extruder and 100 nm polycarbonate filter purchased from Avestin Europe (Germany) as described in.[38] CHOL and $C_{12}NO$ were purchased from Sigma Aldrich (Germany). NaCl, NaOH and KH_2PO_4 were obtained from Centralchem (Slovakia) and K_2HPO_4 from Lachema (Czech Republic). Calcein, also known as fluorexon, was purchased from Acros Organics (USA). Redistilled water was prepared before use. All chemicals used, except of EYPC, were of the analytical grade. Sephadex™ G-50 (fine) (Pharmacia, Fine Chemicals AB, Sweden), Whatman GF/B glass microfiber filter (GE Healthcare, UK), 5 ml disposable syringes and 15 ml disposable polypropylene centrifuge tubes were used for column preparation. Quartz cells were purchased from Hellma Müllheim (Germany).

2. 2. Preparation of Liposomes

Weighted amount of CHOL was dissolved in chloroform. Appropriate volumes of CHOL solution were added to weighted amounts of dry lipid in glass tubes. CHOL and EYPC were co-solubilized. Solvent was evaporated under a stream of gaseous nitrogen followed by evacuation using a vacuum chamber to complete dryness. Glass tubes with dry CHOL-EYPC mixtures were stored in nitrogen atmosphere in a freezer and heated to room temperature before each measurement. A PBS (pH 7,4; 0,05 M) was prepared from K_2HPO_4, KH_2PO_4, NaCl (0,15 M) and redistilled water.

The required amount of calcein was dissolved in the adequate amount of NaOH solution. Calcein solution was very well stirred for at least 20 min and diluted in the excess of PBS to final concentration of 10 mM and pH 7,45. The multilamellar liposomes (MLL) were prepared by hydrating of dry CHOL-EYPC mixtures with 1 ml of calcein solution in PBS and mixed in a vortex for few minutes. The MLL dispersion was slowly extruded through a 100 nm polycarbonate filter 51 times yielding ULL.[38] ULL containing calcein were freed of unencapsulated fluorescence dye by passage through Sephadex™ G-50 by column chromatography.[13,14]

2. 3. Fluorescence Measurement

Fluorescence measurements were performed by spectrofluorometer Fluoromax 4 (Horiba Jobin Yvon, USA). Emission spectra of the calcein were measured at the wavelenght interval 490–580 nm and the fluorescence intensity was evaluated at 514 nm. The fluorescence intensity was measured in CPS units (counts per second). Excitation wavelength of calcein was 485 nm. Samples were prepared and measured at room temperature. It was crucial to cover the samples with aluminium foil during the whole preparation time to avoid an effect of the sunlight.

Increasing concentration of calcein causes a linear increase in fluorescence till some maximum fluorescence intensity at a threshold calcein concentration (20 μM), beyond which the probe self-quenches. With further calcein concentration increase, fluorescence signal gradually decreases and diminishes completely at ~100 μM. The linear part of the dependence is called linear detection regime and is needed for determination of the extraliposomal probe´s concentration. To ensure, that the concentration of calcein will fall to the linear detection regime after leakage we diluted the final CHOL-EYPC-calcein solution by a factor of 100.

Calcein leakage measurement was performed with 5 sets of samples with different n_{CHOL}:n_{EYPC} molar ratios. Each set contained 27 vials. The concentration of EYPC (0,4 mM) was equal in all vials while the concentration of $C_{12}NO$ increases from 0 to 2 mM. Solution of $C_{12}NO$ was added to the vial just before the measurement, sample was than mixed and filled into 10 mm quartz cell.

The time period between calcein liposomes preparation and fluorescence measurement was not longer than 24 hours.

2. 4. Turbidimetry Measurement

The analysis of turbidance spectrum allows us to evaluate changes in particle size, which are a significant consequence of the solubilization process. For turbidance measurements, samples with molar ratios n_{CHOL}:n_{EYPC} = 0; 0,2 and 0,6 were prepared. EYPC and CHOL were mixed in an organic solvent and dried using gaseous nitrogen and a vacuum chamber. Dry lipid film was hydrated with redistilled water and the MLL dispersion was slowly extruded 51 times through a 100 nm polycarbonate filter. Resulting ULL dispersion was divided into 25 vials. Final samples of the same volume, 3 ml, contained EYPC at equal concentration (0,4 mM) and increasing concentration of C_{12}NO. The measurement was carried out at room temperature in the spectrophotometric 10 mm quartz cell using the Hewlett Packard 8452 spectrophotometer (Palo Alto, USA). The turbidance was evaluated at 400 nm.

3. Results and Discussion

The interaction of the non-ionic surfactant C_{12}NO with ULL prepared from EYPC and CHOL at different molar ratios n_{CHOL}:n_{EYPC} was studied by fluorescence probe leakage and turbidimetry at room temperature. The main phase transition of EYPC is below 0 °C,[39] so the bilayers are in a liquid-disordered state which can be transformed to liquid-ordered state with increasing CHOL content. The same concentration of c_{EYPC} = 0,4 mM was used in both experimental methods.

The process of bilayer perturbation by C_{12}NO was manifested by leakage of fluorescent probe calcein. Traditional leakage assay represents liposomes loaded with fluorescence dye at a concentration, at which its quantum yield is strongly reduced by self-quenching.[14] Self-quenching of calcein is caused by forming of non-fluorescence dimers. The total dye concentration in the sample should fall into the range where the fluorescence intensity increases linearly with the dye concentration. To find both the self-quenching concentration and the linear detection regime, dependence of fluorescence intensity on the calcein concentration in PBS was measured (Fig. 1, inset). The concentration range 0–7 μM was estimated as the appropriate linear detection regime and 10 mM was chosen as a self-quenching concentration, similarly to.[15,40] Further measurements showed that these values were not influenced by the presence of C_{12}NO either below or above CMC (results not shown).

Liposomes loaded with quenched calcein (10 mM) were prepared according to the Section 2.2 and exposed to the increasing concentration of C_{12}NO. Molecules of C_{12}NO incorporate into model membrane and destabilize it. As a result, the normalized fluorescence intensity depended on the C_{12}NO concentration as can be seen in the Fig. 1. The release of the fluorescence probe through the liposomal membrane pores was manifested by a gradual increase in fluorescence intensity reflecting the incorporation of the surfactant into the lipid bilayer. Three stages of this process are clearly visible in the dependences in the Fig. 1. There is a minimal perturbation in the bilayer within the first stage characterised by a low level of fluorescence intensity. Steep increase of the intensity in the second stage starts at D_T^{PERT}, the total concentration of surfactant, at which the bilayer is perturbed in such an extent that the fluorescence probe can leak intensively. This is a consequence of growing number of pores and/or increasing diameters of pores present in bilayer in the second stage. D_T^{PERT} was evaluated by a bi-linear function used earlier in.[41] The maximum of fluorescence intensity is achieved at D_T^{REL}, the total concentration of surfactant which causes a complete probe release. The damage of the bilayer enables equilibration of fluorescence probe concentration inside and outside of liposomes. Fluorescence intensity is constant in the third stage and the emission spectrum is no more sensitive to changes caused by further addition of surfactant.

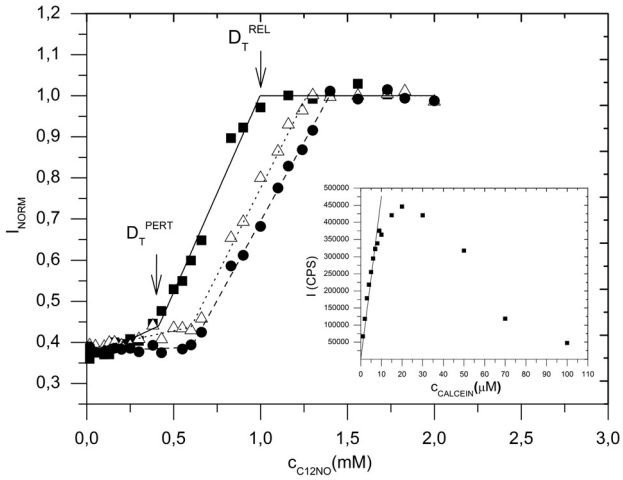

Figure 1. Dependence of the normalized fluorescence intensity, I_{NORM}, on the increasing concentration of C_{12}NO in the sample. Comparison of the three different molar ratios n_{CHOL}:n_{EYPC} = 0 (■), 0,4 (△), 0,6 (●).The arrows indicate the position of the intersection points D_T^{PERT} and D_T^{REL} for the dependence n_{CHOL}:n_{EYPC} = 0. Inset: Dependence of the fluorescence intensity on the calcein concentration.

The fluorescence intensity does not originate from 0 at c_{C12NO} = 0 mM (Fig. 1). This effect can be caused by incomplete separation of calcein during the gel filtration. The initial concentration can be different in various samples.

Liposomes with encapsulated calcein were stored different time periods (less than 24 h) before fluorescence measurement. The stability of these samples was tested by measurement of fluorescence intensity at zero concentra-

tion of $C_{12}NO$ at different time interval after preparation. A small increase in fluorescence intensity was observed. This indicates that some pores exist in the EYPC bilayers at zero $C_{12}NO$ concentration. The change in intensity during 24 h storage of $C_{12}NO$-free sample was less than 1% of the intensity increase caused by $C_{12}NO$ at $c_{C12NO} \geq D_T^{REL}$. The influence of different storage time periods on the results of our calcein leakage experiment is therefore negligible.

To study the interaction of $C_{12}NO$ with EYPC, calcein loaded liposomes were treated with different $C_{12}NO$ concentrations and changes in fluorescent intensity were studied in time. Resulting curves are shown in the Fig. 2.

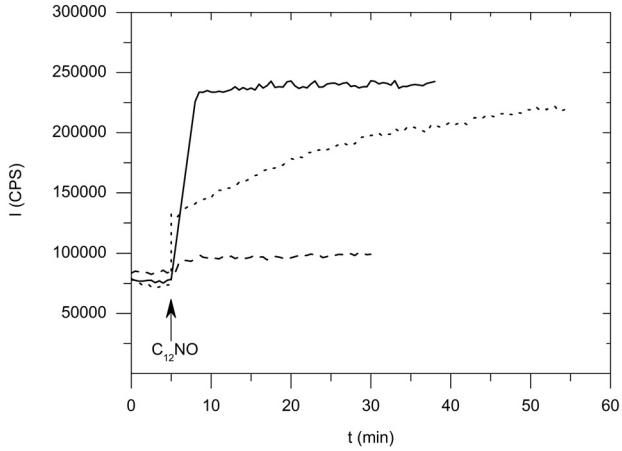

Fig. 2. Time curves of the intensity increase after $C_{12}NO$ addition to calcein loaded EYPC liposomes. Three different $C_{12}NO$ concentrations were used: 0,2 mM (dashed line), 0,68 mM (dotted line) and 1 mM (full line). The arrow indicates the addition of $C_{12}NO$.

A sudden increase in fluorescence intensity is observed short time after the addition of $C_{12}NO$ solution. If the concentration of $C_{12}NO$ (0,2 mM) is smaller than D_T^{PERT}, the increase of the intensity proceeds very slowly after the initial jump, this small change is not visible within the scale of the y-axis in the Fig. 2. Fluorescence intensity increases gradually after the jump for $D_T^{PERT} < 0{,}68$ mM $C_{12}NO < D_T^{REL}$. If the concentration of $C_{12}NO$ (1 mM) is higher than D_T^{REL}, the intensity remains constant after the jump increase. It is seen that the effect of $C_{12}NO$ on the calcein-loaded liposome dispersion is manifested soon after surfactant addition. It means that our measurements realized several minutes after $C_{12}NO$ addition to calcein-liposome dispersion are relevant.

The dependence of D_T^{PERT} and D_T^{REL} on the n_{CHOL}:n_{EYPC} molar ratio is shown in Fig. 3. Both dependencies increase approximately linearly. More surfactant is needed to perturb EYPC bilayer when n_{CHOL}:n_{EYPC} ratio rises.

A slight intensity increase in the Fig. 1 is seen already in the first stage ($c_{C12NO} < D_T^{PERT}$) of the leakage curve. This indicates that the number of pores in the membrane or their diameter increase already within the first stage, but this process is much more moderate than in the second stage. The Fig. 4 shows that the slope, k_I, of the intensity increase within the first stage depends on the n_{CHOL}:n_{EYPC} molar ratio. The membrane becomes more resistant to $C_{12}NO$ with increasing content of CHOL and nearly no calcein leakage is observed at n_{CHOL}:$n_{EYPC} \geq 0{,}6$.

The results of our experiments (Fig.3 and 4) show that the EYPC-CHOL model membrane becomes less permeable with increasing n_{CHOL}:n_{EYPC} molar ratio.

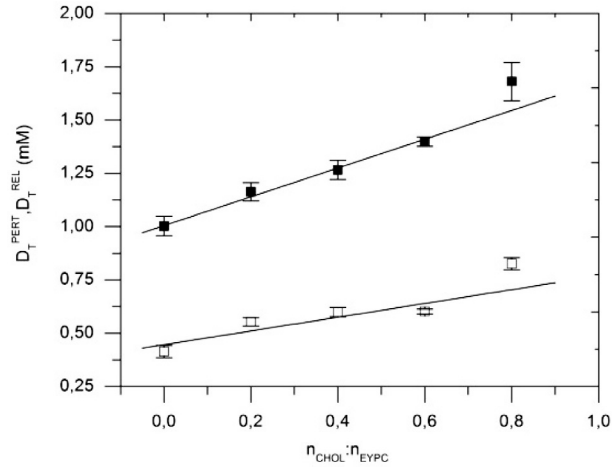

Figure 3. Dependences of D_T^{PERT} (□) and D_T^{REL} (■) on the increasing n_{CHOL}:n_{EYPC} molar ratio. D_T^{PERT} and D_T^{REL} were evaluated as the total $C_{12}NO$ concentrations, causing the onset of the intensive leakage of calcein and the total release of calcein, respectively.

The protective influence of CHOL against sodium dodecyl sulphate (SDS) induced probe leakage from EYPC liposomes was observed in.[43] The authors supposed that a reduction of the surfactant partition coefficient and an increased mechanical stability of membrane in the presence of cholesterol are responsible for the observed effect. The increase in EYPC liposomes integrity in the presence of CHOL was seen also by[18] by the leakage of encapsulated 5,6-carboxyfluorescein.

The study of the calcein leakage through CHOL-EYPC was complemented by turbidimetry experiment. Turbidance (A_T) of ULL was measured as $C_{12}NO$ concentration increased. The example of the solubilization curve is depicted in the Fig. 5 (open symbols) for n_{CHOL}:n_{EYPC} = 0,6. It can be seen that the solubilization curve can be described by the well-known "three-stage model".[12,21,22,24] Surfactant monomers partition into the membrane during the stage I until the saturation is reached at D_T^{SAT} (nomenclature taken from[24]). When the total surfactant concentration exceeds D_T^{SAT}, lamellar – micellar phase transition starts and lipid-saturated micelles coexist with surfactant saturated bilayers (stage II). Proportion of bilayers to

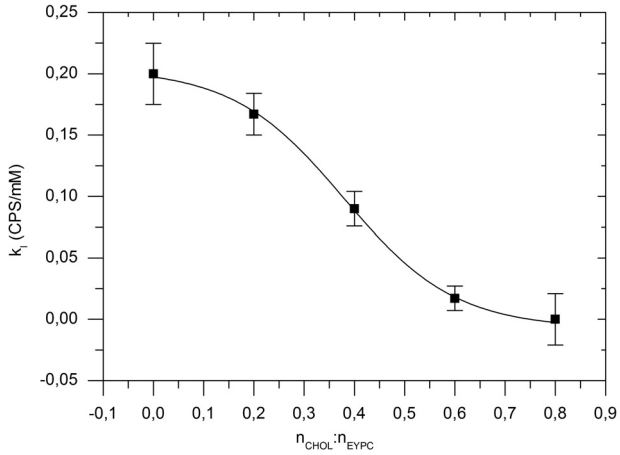

Figure 4. Dependence of k_I on the n_{CHOL}:n_{EYPC} molar ratio (k_I - the slope of $I_{NORM} = f(c_{C12NO})$ at $c_{C12NO} < D_T^{PERT}$ in the Fig.1).

micelles decreases and the liposomal bilayer is fully solubilized at the total surfactant concentration D_T^{SOL}.[24] The system enters the stage III with further increase of surfactant concentration.

Obtained turbidimetric data (open symbols) are compared to the results of a leakage experiment (full symbols) in the Fig. 5. It is clearly seen that the concentration of $C_{12}NO$ causing the total calcein release, D_T^{REL}, is smaller than the saturation concentration D_T^{SAT}. This implies that monomers of $C_{12}NO$ incorporate into EYPC bilayers and cause a serious perturbation of the bilayer without a marked change in liposome's dimension until D_T^{SAT} is achieved. This fact is important for antimicrobial activity of $C_{12}NO$ – the leakage of the internal content of a microbial cell can occur at smaller than saturation and solubilization concentration.

According to minimal inhibition concentration (MIC) of $C_{12}NO$ for *Staphylococcus aureus* and *Escherichia coli* corresponds to the 0,34 mM concentration. Kopecká-Leitmannová et al. [45] found that MIC of $C_{12}NO$ for *Escherichia coli* was 0,29 mM. The range of $C_{12}NO$ concentrations, where the membrane perturbation and solubilization was observed for the EYPC model membrane, correspond surprisingly well to these MIC values.

D_T^{SAT} and D_T^{SOL} obtained for different n_{CHOL}:n_{EYPC} molar ratios are summarized in the Table 1. The values of D_T^{REL} are smaller than D_T^{SAT} for all model membranes studied here.

Table 1. Total surfactant concentrations D_T^{SAT} and D_T^{SOL} measured at different n_{CHOL}:n_{EYPC} molar ratios.

n_{CHOL}:n_{EYPC}	D_T^{SAT} [mM]	D_T^{SOL} [mM]
0	1,12 ± 0,05	1,96 ± 0,04
0,2	1,42 ± 0,05	2,21 ± 0,04
0,6	1,66 ± 0,02	2,34 ± 0,04

Smaller value of D_T^{REL} than D_T^{SAT} was observed for Triton X-100 in EYPC and soya phosphatidylcholine bilayers[14,46] and cationic surfactant SDS in EYPC bilayers.[43] CHOL protects EYPC liposomes from solubilization and caused a decrease of SDS partition coefficient.[43] The authors assume, that the effect of cholesterol may make difficult the formation of hydrophilic pores, which lead to the restrictions of membrane permeability caused by SDS.

The effect of cholesterol on the solubilization process is temperature-dependent as was shown by Schnitzer et al.[34] and Lichtenberg.[24] More Triton X-100 was needed to solubilize DPPC/CHOL bilayers in a liquid-crystalline state than the "pure" DPPC bilayers. On the other hand, D_T^{SOL} was higher for POPC/CHOL bilayers compared to single component POPC bilayer only at low temperatures. The difference decreased as the temperature rise and at 15–35 °C the solubilization concentrations were comparable for POPC and POPC/CHOL bilayers. Schnitzer et al.[34] supposed that D_T^{SOL} is determined by two thermodynamic factors – the bending energy and the interstice energy of bilayers which depend oppositely on the temperature.

Our study clearly shows that D_T^{SOL} increases with increasing CHOL content in the EYPC bilayer at room temperature. As the spontaneous curvature of CHOL is more negative than that of phospholipids,[47] the solubilization of CHOL containing bilayers can be expected to require more surfactant with positive spontaneous curvature. According to previous studies, CHOL causes the ordering of EYPC acyl chains[48,49] and thickening of the bilayer[50] in its fluid state. This is associated with the increase of EYPC bilayer integrity and leads to higher D_T^{SOL} values.

The negative spontaneous curvature of liposomes with CHOL can be associated with the increase in liposome radius and therefore the change in entrapped volume. The entrapped volume was calculated as the difference between the calcein concentration obtained after complete disruption of the liposome induced by $C_{12}NO$, and the calcein concentration in the external solution of intact liposome dispersion.[14] Trapped volumes of samples with different n_{CHOL}:n_{EYPC} are reported in Table 2.

Table 2. Entrapped volumes of different n_{CHOL}:n_{EYPC} molar ratios

n_{CHOL}:n_{EYPC}	entrapped volume (µl/mg)
0	0,2381
0,2	0,31517
0,4	0,27127
0,6	0,2826
0,8	0,373

Entrapped volume slightly increases with CHOL content in EYPC liposomes. A moderate increase of radius and therefore increase of entrapped volume of DOPC

liposomes after cholesterol addition was detected using small angle neutron scattering.[51]

Solubilization of a similar model membrane, dioleoylphosphatidylcholine (DOPC) in ULL, induced by $C_{12}NO$ was studied using small angle neutron scattering.[29] Bilayers or/and bilayer fragments were observed up to the molar ratio $n_{C12NO}:n_{DOPC} = 1,5$, rod-like particles (tubular, cylindric micelles) at $2,5 < n_{C12NO}:n_{DOPC} < 3,5$, and transition to globular particles (spheroid micelles) at $n_{C12NO}:n_{DOPC} > 4$. Our results show that the liposomes with bilayer structure are preserved up to $C_{12}NO$ concentration D_T^{SAT} which corresponds to molar ratio $n_{C12NO}:n_{EYPC} = 2,8$. The difference in D_T^{SAT} concentration between[29] and our results could be attributed to different procedure of sample preparation. Lipid was mixed with $C_{12}NO$ in organic solvent before unilamellar liposome preparation in,[29] it means that equilibrium distribution of $C_{12}NO$ in DOPC aggregates is achieved. In this paper, we are interested in the surfactant – lipid interaction from the first moment of the surfactant addition, when the equilibrium has not yet been achieved.

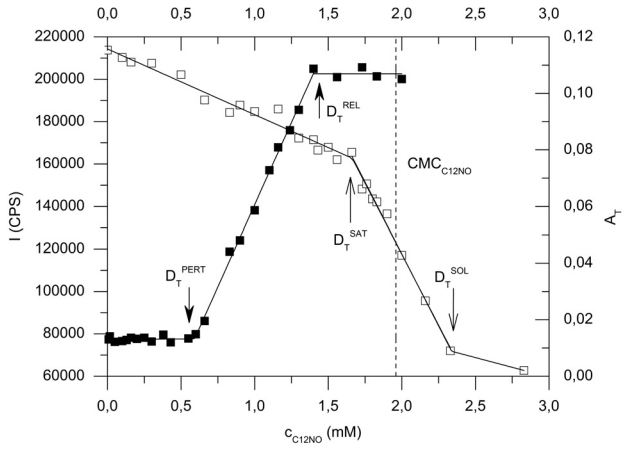

Figure 5. Dependence of the fluorescence intensity, I, in counts per second (left axis, full symbols) and turbidance, A_T, (right axis, open symbols) on the $C_{12}NO$ concentration at $n_{CHOL}:n_{EYPC} = 0,6$ molar ratio and EYPC concentration $c_{EYPC} = 0,4$ mM.

The saturation of membrane by surfactant can proceed by two different mechanisms.[12] The trans-membrane mechanism is characterized by a non-cooperatively surfactant monomers insertion into the membrane. This process is accompanied by fast flip-flop, what leads to the surfactant accumulation and formation of pores stabilized by surfactant rich rims. The micellar mechanism involves a cooperative interaction of surfactant micelles with membrane on the outside of liposomes. The surfactant interacts with the outer monolayer which leads to a partial depletion of phospholipid, resulting in liposome redistribution and reorganization of phospholipid molecules from the inner to the outer monolayer. As a consequence, liposomes are slowly opened up, fragmented and finally solubilized by the surfactant micelles. This results either in the formation of pores in the membrane structure or in the formation of bilayer discs sealed at the edges by surfactant molecules.[12]

According to[52] the critical micelle concentration (CMC) of $C_{12}NO$ at 30 °C is 1,96 mM. It can be seen from the Fig. 5 that D_T^{REL} is lower than CMC for all $n_{CHOL}:n_{EYPC}$ molar ratios. The formation of pores enabling calcein leakage occurs thus via trans-membrane mechanisms of $C_{12}NO$ interaction with EYPC membranes. Results in Table 1 also indicate that CHOL-EYPC bilayers are saturated by $C_{12}NO$ at $D_{SAT} < CMC$. It means that, the saturation process is performed by monomers, not micelles. $C_{12}NO$ micelles are present in the second stage of turbidity measurement, together with mixed micelles.

4. Conclusions

The perturbation of EYPC bilayers in ULL containing different amounts of CHOL ($n_{CHOL}:n_{EYPC} = 0–0,8$) induced by $C_{12}NO$ was studied by the leakage of fluorescence probe calcein. D_T^{PERT} and D_T^{REL} were evaluated as the total $C_{12}NO$ concentrations, causing the onset of the intensive leakage of calcein and the total release of calcein, respectively. More surfactant is needed to perturb the phospholipid bilayer when the amount of CHOL in bilayer increases.

D_T^{SAT} and D_T^{SOL} were determined turbidimetrically as the total $C_{12}NO$ concentrations, causing the onset of bilayer – micellar phase transition and the completion of bilayer solubilization, respectively. Both D_T^{SAT} and D_T^{SOL} increase with the increasing $n_{CHOL}:n_{EYPC}$ molar ratio. The structure of liposomes is still preserved at total calcein release (D_T^{REL}) for all $n_{CHOL}:n_{EYPC}$ molar ratios.

5. Acknowledgement

We would like to thank Janka Karlovská, PhD. for valuable advice and help during experiments and prof. Pavol Balgavý for his advices, ideas and fruitful discussions. This work was supported by UK/496/2012, VEGA 1/1224/12 grants, the Dubna JINR 04-4-1121-2015/2017 project and by research project Center of Excelence in Security Research ITMS code: 26240120034 supported by the Research & Development Operational Programme funded by the European Regional Development Fund (ERDF) and by the Slovak Research and Development Agency under the contract No. APVV-0516-12.

6. Abbreviations

$C_{12}NO$, *N,N*-dimethyl-1-dodecanamine *N*-oxide; C_nNO, *N,N*-dimethyl-1-alkanamine *N*-oxide; EYPC, egg

yolk phosphatidylcholine; CHOL, cholesterol; POPC, palmitoyloleoylphosphatidylcholine; DOPC,dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine, MLL, multilamellar liposomes; ULL, unilamellar liposomes; PBS, phosphate buffer saline; CMC, critical micelle concentration; SDS, sodium dodecyl sulphate.

6. References

1. F. Šeršeň, G. Gabunia, E. Krejčírová, and K. Král'ová, *Photosynthetica*, **1992,** 26, 205–212.
2. A. Murín, F. Devínsky, A. Koleková, and I. Lacko, *Biológia*, **1990,** *45*, 521–531.
3. M. Bukovský, D. Mlynarčík, and V. Ondráčková, *Int. J. Immunopharmacol.*, **1996,** *18*, 423–426. http://dx.doi.org/10.1016/S0192-0561(96)00040-9
4. F. Devínsky, A. Kopecká-Leitmanová, F. Šeršeň, and P. Balgavý, *J. Pharm. Pharmacol.*, **1990***, 42,* 790–794. http://dx.doi.org/10.1111/j.2042-7158.1990.tb07022.x
5. S. K. Singh, M. Bajpai, and V. K. Tyagi, *J. Oleo Sci.*, **2006,** *55,* 99–119. http://dx.doi.org/10.5650/jos.55.99
6. J. Šubík, G. Takácsová, M. Pšenák, and F. Devínsky, *Antimicrob. Agents Chemother.*, **1977,** *12,* 139–146. http://dx.doi.org/10.1128/AAC.12.2.139
7. A. Búcsi, J. Karlovská, M. Chovan, F. Devínsky, and D. Uhríková, *Chem. Pap.*, **2014,** *68*, 842–846. http://dx.doi.org/10.2478/s11696-013-0517-3
8. K. W. Herrmann, *J. Phys. Chem.*, **1964,** 68, 1540–1546. http://dx.doi.org/10.1021/j100788a047
9. A. de la Maza and J. L. Parra, *Biophys. J.*, **1997,** *72,* 1668–1675. http://dx.doi.org/10.1016/S0006-3495(97)78812-X
10. H. Heerklotz, *Q. Rev. Biophys.*, **2008,** *41*, 205–264. http://dx.doi.org/10.1017/S0033583508004721
11. J. Karlovská, F. Devínsky, I. Lacko, J. Gallová, and P. Balgavý, *ACTA Fac. Pharm. Univ. Comen.*, **2004,** *51*, 119–128.
12. U. Kragh-Hansen, M. le Maire, and J. V. Møller, *Biophys. J.*, **1998,** *75,* 2932–2946. http://dx.doi.org/10.1016/S0006-3495(98)77735-5
13. Y. Kapoor, B. A. Howell, and A. Chauhan, *Invest. Ophthalmol. Vis. Sci.*, **2009,** *50*, 2727–2735. http://dx.doi.org/10.1167/iovs.08-2980
14. A. Memoli, M. C. Annesini, and S. Petralito, *Int. J. Pharm.*, **1999,** *184,* 227–235. http://dx.doi.org/10.1016/S0378-5173(99)00105-2
15. A. Memoli, L. G. Palermiti, V. Travagli, and F. Alhaique, *J. Pharm. Biomed. Anal.*, **1999,** *19*, 627–632. http://dx.doi.org/10.1016/S0731-7085(98)00229-5
16. A. de la Maza, O. Lopez, L. Coderch, and J. L. Parra, *Chem. Phys. Lipids*, **1998,** *94*, 71–79. http://dx.doi.org/10.1016/S0009-3084(98)00045-0
17. A. de la Maza, L. Coderch, P. Gonzalez, and J. L. Parra, *J. Controlled Release*, **1998,** *52,* 159–168. http://dx.doi.org/10.1016/S0168-3659(97)00205-8
18. D. G. Fatouros, K. Hatzidimitriou, and S. G. Antimisiaris, *Eur. J. Pharm. Sci.*, **2001,** *13,* 287–296. http://dx.doi.org/10.1016/S0928-0987(01)00114-2
19. H. Ahyayauch, M. I. Collado, F. M. Goñi, and D. Lichtenberg, *FEBS Lett.*, **2009,** *583,* 2859–2864. http://dx.doi.org/10.1016/j.febslet.2009.07.046
20. M. Almgren, *Biochim. Biophys. Acta*, **2000,** *1508,* 146–163. http://dx.doi.org/10.1016/S0005-2736(00)00309-6
21. A. Helenius and K. Simons, *Biochim. Biophys. Acta*, **1975,** *415*, 29–79. http://dx.doi.org/10.1016/0304-4157(75)90016-7
22. M. le Maire, P. Champeil, and J. V. Moller, *Biochim. Biophys. Acta*, **2000,** *1508*, 86–111. http://dx.doi.org/10.1016/S0304-4157(00)00010-1
23. D. Lichtenberg, *Biochim. Biophys. Acta*, **1985,** *821,* 470–478. http://dx.doi.org/10.1016/0005-2736(85)90052-5
24. D. Lichtenberg, H. Ahyayauch, A. Alonso, and F. M. Goñi, *Trends Biochem. Sci.*, **2013,** *38*, 85–93. http://dx.doi.org/10.1016/j.tibs.2012.11.005
25. F. M. Goñi and A. Alonso, *Biochim. Biophys. Acta*, **2000,** *1508*, 51–68. http://dx.doi.org/10.1016/S0304-4157(00)00011-3
26. M. M. A. Elsayed and G. Cevc, *Pharm. Res.*, **2011,** *28*, 2204–2222. http://dx.doi.org/10.1007/s11095-011-0448-z
27. F. M. Goñi and A. Alonso, *Biochim. Biophys. Acta*, **2000,** *1508,* 51–68. http://dx.doi.org/10.1016/S0304-4157(00)00011-3
28. A. Hrubšová, J. Karlovská, F. Devínsky, I. Lacko, and P. Balgavý, *čes. Slov. Farm.*, **2003,** *52*, 299–305.
29. D. Uhríková, N. Kučerka, A. Islamov, V. Gordeliy, and P. Balgavý, *Gen. Physiol. Biophys.*, **2001,** *20*, 183–189.
30. F. M. Goñi, A. Alonso, L. A. Bagatolli, R. E. Brown, D. Marsh, M. Prieto, and J. L. Thewalt, *Biochim. Biophys. Acta*, **2008,** *1781*, 665–684.
31. H. Ohvo-Rekilä, B. Ramstedt, P. Leppimäki, and J. Peter Slotte, *Prog. Lipid Res.*, **2002,** *41*, 66–97. http://dx.doi.org/10.1016/S0163-7827(01)00020-0
32. J. R. Silvius, *Biochim. Biophys. Acta*, **2003,** *1610*, 174–183. http://dx.doi.org/10.1016/S0005-2736(03)00016-6
33. M. Castaing, A. Loiseau, and L. Djoudi, *Eur. J. Pharm. Sci.*, **2003,** *18*, 81–88. http://dx.doi.org/10.1016/S0928-0987(02)00243-9
34. E. Schnitzer, M. M. Kozlov, and D. Lichtenberg, *Chem. Phys. Lipids*, **2005,** *135*, 69–82. http://dx.doi.org/10.1016/j.chemphyslip.2005.02.002
35. J. Huang, J. T. Buboltz, and G. W. Feigenson, *Biochim. Biophys. Acta - Biomembr.*, **1999,** *1417,* 89–100.
36. W. S. Singleton, M. S. Gray, M. L. Brown, and J. L. White, *J. Am. Oil Chem. Soc.*, **1965,** *42*, 53–56. http://dx.doi.org/10.1007/BF02558256
37. E. Švajdlenka, V. Kvaka, B. Kvasničková, E. Riečanská, and P. Balgavý, *českoslov. Farm.*, **1987,** *36*, 368–373.
38. J. Gallová, D. Uhríková, N. Kučerka, M. Svorková, S. S. Funari, T. N. Murugova, L. Almásy, M. Mazúr, and P. Balgavý, *J. Membr. Biol.*, **2011,** *243,* 1–13. http://dx.doi.org/10.1007/s00232-011-9387-1
39. D. M. Small, *J. Lipid Res.*, **1967,** *8*, 551–557.
40. S. Huláková, B. Fulier, J. Gallová, and P. Balgavý, *Acta Fac. Pharm. Univ. Comen.*, **2013,** *LX*, 7–13.

41. J. Gallová and S. Szalayová, *Gen. Physiol. Biophys.*, **2004,** *23*, 297–306.
42. O. G. Mouritsen and M. J. Zuckermann, *Lipids*, **2004,** *39,* 1101–1113. http://dx.doi.org/10.1007/s11745-004-1336-x
43. M. Cócera, O. López, L. Coderch, J. L. Parra, and A. de la Maza, *Colloids Surf. Physicochem. Eng. Asp.*, **2003,** *221*, 9–17. http://dx.doi.org/10.1016/S0927-7757(03)00104-3
44. C. R. Birnie, D. Malamud, and R. L. Schnaare, *Antimicrob. Agents Chemother.*, **2000,** *44*, 2514–2517. http://dx.doi.org/10.1128/AAC.44.9.2514-2517.2000
45. A. Kopecká-Leitmanová, F. Devínsky, D. Mlynarcík, and I. Lacko, *Drug Metabol. Drug Interact.*, **1989,** *7*, 29–51. http://dx.doi.org/10.1515/DMDI.1989.7.1.29
46. M. A. Partearroyo, M. A. Urbaneja, and F. M. Goñi, *FEBS Lett.*, **1992,** *302***,** 138–140. http://dx.doi.org/10.1016/0014-5793(92)80424-F
47. Z. Chen and R. P. Rand, *Biophys. J.*, **1997,** *73*, 267–276. http://dx.doi.org/10.1016/S0006-3495(97)78067-6
48. A. Kusumi, W. K. Subczynski, M. Pasenkiewicz-Gierula, J. S. Hyde, and H. Merkle, *Biochim. Biophys. Acta*, **1986,** *854*, 307–317. http://dx.doi.org/10.1016/0005-2736(86)90124-0
49. M. Svorková, J. Annus, and J. Gallová, *Acta Fac. Pharm. Univ. Comen.*, **2006,** *53***,** 238–244.
50. J. Gallová, D. Uhríková, N. Kučerka, S. Doktorovová, S. S. Funari, J. Teixeira, and P. Balgavý, *Eur. Biophys. J. EBJ*, **2011,** *40*, 153–163. http://dx.doi.org/10.1007/s00249-010-0635-6
51. N. Kučerka, J. Pencer, J. N. Sachs, J. F. Nagle, and J. Katsaras, *Langmuir ACS J. Surf. Colloids*, **2007,** *23*, 1292–1299. http://dx.doi.org/10.1021/la062455t
52. L. Benjamin, *J. Phys. Chem.*, **1964,** *68*, 3575–3581. http://dx.doi.org/10.1021/j100794a023

Povzetek

Proučevali smo interakcije *N,N*-dimethil-1-dodekanamin *N*-oksida ($C_{12}NO$) z liposomi fosfatidilholina (EYPC) iz jajčnega rumenjaka, vsebujočega holesterol (CHOL). Sproščanje fluorescenčnega barvila kalcein je povzročilo perturbacijo dvoplasti EYPC-CHOL v enolamelarne liposome (ULL). Pri nizkih koncentracijah surfaktanta smo opazili šibkejše sproščanje (minimalna perturbacija dvoplasti), ki pa se je intenzivirala s povečevanjem koncentracije c_{C12NO} (nastanek por). Pri visokih koncentracijah c_{C12NO} nismo opazili spremembe v intenziteti fluorescence (kalcein se je popolnoma sprostil iz liposomov). Pri večji koncentraciji CHOL v dvoplasti je za nastanek por v dvoplasti potrebno več surfaktanta. Solubilizacijo EYPC-CHOL, inducirano z $C_{12}NO$, smo raziskovali s turbimetrijo. Izkazalo se je, da potek solublizacije lahko razdelimo v tri procese: a) nasičenje dvoplasti pri nizki c_{C12NO} (liposomi so ohranjeni), b) solubilizacija (prehod liposomi-micele) in c) post-solubilizacija. Pri večjem razmerju n_{CHOL}:n_{EYPC} je za solubilizacijo potrebna višja koncentracija $C_{12}NO$. Pri popolni sprostitvi kalceina iz liposomov se struktura le teh ohranja pri vseh razmerjih n_{CHOL}:n_{EYPC}.