The general feature of membrane lipids is their amphiphilic nature and spontaneous tendency to form organised structures in an aqueous environment. Phosphatidylcholines aggregate into bilayers in a wide region of concentration, temperature and pH. Phospholipid bilayer structure is characteristic for different types of biological membranes. The tendency of phosphatidylcholines to form bilayers is also used for the preparation of model membranes. In the present study, egg yolk phosphatidylcholine (EYPC) bilayers forming unilamellar liposomes are used as a model system to study the influence of surfactant.

\[ \text{N-alkyl-N,N-dimethylamine N-oxides (CnNO, where n is the number of carbon atoms in the alkyl substituent) are non-ionic surfactants at physiological pH with a strong polar N–O bond and a high electron density on the oxygen.} \]

CnNO are widely used as a component in home cleaning products, shampoos, conditioners and pharmaceutical formulations. The physico-chemical and biological properties of CnNO...
result from their amphiphilic structure. Therefore, they are predestined to affect the phospholipid bilayer resulting in antimicrobial (Balgaš and Devinsky, 1996, Singh et al., 2006), phytotoxic (Murín et al., 1990), antiphotosynthetic (Seršěh et al., 1992) and immunomodulatory effects (Bukovský et al., 1996). CnNO (n=10–16) is the most commonly used form from all CnNO (Sanderson, 2009). The homologue with docyl substituent (N-docetyl-N,N-dimethylamine N-oxide; C12NO) is also used as a mild biological surfactant in membrane studies for purification, reconstitution and crystallisation of membrane proteins and solubilization of membranes. The phenomena associated with surfactant–membrane interaction are described through a three-stage model (Helenius & Simons, 1975, Paternostre et al., 1988, Lichtenberg, 1985). In the initial stage, surfactant monomers are incorporated within the bilayer according to the partition equilibrium between lipid and aqueous phases. This stage is called surfactant binding and corresponds to the low surfactant concentration. The second stage called lamellar-micellar phase transition starts after exceeding of the saturation value of the surfactant. Phospholipids are gradually solubilized into the mixed micelles that coexist with surfactant saturated vesicles. The system enters the third stage, with further surfactant concentration increase, when only micelles exist. Complete solubilization of liposomes occurs and no lamellar structures are left (Lichtenberg, 1985, Memoli et al., 1999a, Heerklotz, 2008). This phase transformation is accompanied by change of particle size. Liposome size modification can be very well observed by turbidimetric method. The main parameter evaluated from turbidimetric measurement is turbidance, which proportionally depends on the particle radius. Therefore, turbidimetry is a suitable method for investigation of the liposome solubilization. This is confirmed by many reviews (Lichtenberg, 1985, Lichtenberg et al., 2000, Schurtenberger et al., 1985, de la Maza et al., 1997). Turbidimetry has already been used to investigate interaction of C12NO with multilamellar liposomes of EYPC (Hrubšová et al., 2003, Karlovská et al., 2004).

Fluorescence spectroscopy is another valuable tool for detecting the solubilization process. The low concentration of surfactant causes defects of membrane and increases its permeability. Therefore, a hydrophilic fluorescent probe calcein, which can leak through these structural defects, appears to be a valuable substance. Moreover, calcein possesses a self-quenching feature. With increasing concentration, calcein monomers bind to the non-fluorescent dimers. Liposomes filled with highly concentrated probe become “invisible” to the detector. After leaking to the bulk solution, calcein dilute to the detectable concentration. This method can provide interesting information about vesicle structure, behaviour and stability (Memoli et al., 1999a).

In the present paper, we report the results of the effect of C12NO on the EYPC unilamellar liposomes using turbidimetric and fluorospectroscopic method and finally compare the two methods used for investigation of solubilization. Turbidimetry and fluorescence spectroscopy are optimal for determination of onset and completion of solubilization. Each of these methods can demonstrate different abilities of C12NO as a solubilizing agent.

MATERIAL AND METHODS/EXPERIMENTAL PART

2.1 Chemicals
Phospholipid EYPC was isolated and purified from hen eggs according to Singleton et al. (1965). Unilamellar liposomes were prepared by extrusion using Liposofast basic Extruder and 100 nm polycarbonate filter purchased from Avestin Europe (Germany). C12NO was purchased from Sigma Aldrich Chemie (Germany). K2HPO4 was obtained from Lachema (Czech Republic) and NaCl and K2HPO4 from Centralchem (Slovakia). Redistilled water was prepared before use. Calcein, also known as fluorexon, was purchased from Acros Organics (USA) and dissolved in NaOH (CentralChem, Slovakia). All chemicals used 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).

Preparation of liposomes
The multilamellar liposomes (MLLs) were prepared by dispersing EYPC in the redistilled water by shaking with hand until the opalescent dispersion is formed. The MLL solution was slowly extruded through a 100 nm polycarbonate filter 51 times.

Preparation of loaded liposomes
A phosphate-buffered saline (PBS) (pH 7.4; 0.05 mol/dm3) was prepared from K2HPO4, K2HPO4, NaCl (0.15 mol/dm3) and redistilled water. The required amount of calcein was dissolved in the adequate amount of NaOH solution (1 mol/dm3). Calcein solution was stirred very well for at least 15 min. and diluted in the excess of PBS to a final concentration of 10 mM and pH 7.45. The weighted amount of dried EYPC was hydrated with 1 ml of calcein solution and mixed in a vortex mixer for few minutes. EYPC MLLs filled with calcein were present in the solution after complete homogenisation. The MLL solution was slowly extruded through a 100 nm polycarbonate filter 51 times. The extruder’s glass syringes were covered with aluminium foil to protect the solution from sun light. EYPC + calcein solutions were stored in amber glass vials and protected by aluminium foil. Required concentrations of EYPC stock solutions were: 40, 25, 10 and 5 mmol/dm3.

Separation of the bulk calcein solution
Calcein was removed from extraliposomal solution according to the procedure of Kapoor et al. (2009). We weighted 0.5 g of Sephadex™ G-50 which was moisturised by 6.5 ml PBS. This gel was allowed to soak for 24 hours in the refrigerator.
For separation of the bulk calcein solution, we prepared small column in 5 ml disposable syringe. The plunger from the syringe was removed. Then, we folded the glass microfiber filter Whatman GF/B and both sharp corners were snipped. The microfiber filter adapted in this manner was carefully placed at the bottom of the syringe using spatula. Syringe outlet had to be completely covered by filter membrane. Syringe was placed to the centrifuge tube and the Sephadex™ G-50 gel was poured into the syringe directly on the filter membrane. After centrifugation at 1000 g (3400 rpm) for 5 min., Sephadex™ G-50 column was created. The syrup filled with the Sephadex™ G-50 column was then transferred from the centrifuge tube to the new clean tube. For separation of the bulk calcein solution, we prepared small column in 5 ml disposable syringe. The plunger from the syringe was removed. Then, we folded the glass microfiber filter Whatman GF/B and both sharp corners were snipped. The microfiber filter adapted in this manner was carefully placed at the bottom of the syringe using spatula. Syringe outlet had to be completely covered by filter membrane. Syringe was placed to the centrifuge tube and the Sephadex™ G-50 gel was poured into the syringe directly on the filter membrane. After centrifugation at 1000 g (3400 rpm) for 5 min., Sephadex™ G-50 column was created. The syrup filled with the Sephadex™ G-50 column was then transferred from the centrifuge tube to the new clean tube.

Method

Turbidimetry

In all samples, there was a constant concentration of EYPC 0.4 mmol/dm³ and increasing concentration of C12NO (0–2.83 mmol/dm³). The turbidity of the samples was determined immediately after preparing mixture of EYPC + redistilled water + C12NO at room temperature in the spectrophotometric 10 mm quartz cell using the Hewlett Packard 8452 spectrophotometer (Palo Alto, USA). Turbidance was evaluated at 400 nm.

Fluorescence spectroscopy

Calibration curve for calcein fluorescence was measured first. The samples with increasing concentration of calcein (0–100 μmol/dm³) were prepared in PBS buffer. The aim was to determine the concentration region of calcein with a linear detection regime (see Results section). The measurement of the calibration curve was also repeated in the presence of C12NO at pre-solubilizing (0.3 mmol/dm³) and post-solubilizing (2.83 mmol/dm³) concentrations. Before each liposome leakage experiment, EYPC + calcein solution was diluted with factor 100 to get the proper concentration of calcein in the samples (within the linear detection regime). Solubilization measurement was performed with four sets of samples distinguished by EYPC concentrations (0.4; 0.25; 0.1; 0.05 mmol/dm³). In every set, the concentration of EYPC + calcein was the same and increasing concentration of C12NO (0–1.83 mmol/dm³) was used. Samples were filled into 10 mm quartz cells and measured using a Spectrofluorometer Fluoromax 4 (Horiba Jobin Yvon, USA) with excitation and emission wavelengths 485 and 515 nm, respectively. Samples were measured at room temperature and the intensity was evaluated at 514 nm. The period between the sample preparation and its measurement was a few seconds.

Results

The process of solubilization of EYPC unilamellar liposomes induced by C12NO was studied by two methods, turbidimetry and a leakage of a fluorescent dye. Solubilization curve measured turbidimetrically is shown in Fig. 1 as a dependence of turbidance AT on the C12NO concentration (open symbols). It shows a typical three stage course described in Section 1. According to Lichtenberg (1985), C12NO molecules incorporate into EYPC bilayer of liposomes at low C12NO concentration (part I). The decrease of turbidance in the second stage (part II) is caused by a gradual transition from lamellar phase to mixed micelles. Liposomes are transformed to micelles with much smaller size. Only small mixed micelles are present in the third region (part III) characterised by a low value of turbidance. The turning points between individual stages were evaluated by a sum of linear functions according to Gallová and Szalayová (2004). The concentrations of C12NO at the boundary between parts I and II, resp. parts II and III are 1.12±0.05 mmol/dm³ and 1.96±0.04 mmol/dm³ for 0.4 mmol/dm³ EYPC.

Filled symbols (Fig. 1) show a dependence of fluorescence intensity of calcein on the C12NO concentration. The method of leakage of fluorescent probe is based on the fluorescence quenching. At high concentration of calcein, quenching is a result of the formation of non-fluorescent dimers in ground-state (Lakowitz, 2006). To find a linear detection regime, the calibration curve as a concentration dependence of fluorescence intensity of calcein in buffer was measured first (Fig. 2). Calcein is present in monomers at low concentrations. The intensity of fluorescence increases with increasing calcein concentration in the buffer solution until a maximal intensity is reached at ≈20 μM. The increase is linear for 0–10 μmol/dm³ and this concentration range represents a linear detection regime of calcein. Self-quenching starts to prevail at 20 μmol/dm³, which is manifested by a decrease of intensity in the last part of the calibration curve (Fig. 2). It is also very important to verify that C12NO does not affect the spectral behaviour of calcein (Memoli et al., 1999b). We repeated therefore the measurement of the calibration curve in the presence of C12NO at concentrations 0.3 and 2.82 mmol/dm³. According to Hrubšová et al. (2003) and Karlovská et al. (2004), 0.3 mmol/dm³ C12NO is not able to solubilize EYPC liposomes at the used EYPC concentration, while liposomes are fully transformed to mixed micelles at 2.82 mmol/dm³ C12NO. Calcein fluorescence intensity was measured within concentration interval corresponding to the calibration curve (0–100 μmol/dm³). There was no difference between emission spectra of calcein in the presence and absence of C12NO. From these results, we assume that C12NO does not affect spectral behaviour of calcein.
Liposomes for fluorescent probe leakage experiments were prepared according to the Sections 2.3 and 2.4. Liposomes were filled with calcein at the self-quenching concentration (10 mmol/dm³) and the fluorescent probe became "invisible" to the detector. EYPC + calcein liposomes were then exposed to the increasing concentration of the C12NO (Fig. 1, filled symbols). As a consequence, calcein was gradually released from the liposome interior and its concentration in bulk water phase increased and the intensity grew. The dependence of intensity on the C12NO concentration can be again divided into three linear sections. We can observe only slow ascent of fluorescence intensity in the first section. The second section is characterised by steep increase of the fluorescence intensity. Very small changes in fluorescence intensity can be seen in the last part of the solubilization curve.

The experiment with fluorescence leakage was repeated for four different EYPC concentrations $c = 0.05; 0.1; 0.25; 0.4$ mmol/dm³. The dependence of the C12NO concentration in turning points on the amount of EYPC in the solution is shown in Fig. 3. It can be seen that both dependencies are increasing, approximately linear, and their slope is similar.

**DISCUSSION**

We have studied the process of the solubilization of unilamellar EYPC liposomes by detergent C12NO. Turbidimetry and the method of leakage of fluorescent probe calcein were used. The dependence of turbidance $A$ on the detergent concentration was measured at EYPC concentration $0.4$ mmol/dm³ (Fig. 1). Different methods are described in the literature to characterise a solubilization curve. This type of dependence was fitted by a reverse sigmoid in Hrubšová et al. (2003) and Karlovská et al. (2004) and the solubilization concentration $c_S$
was evaluated as a centre of the reverse sigmoid. The value obtained in this work, $c_S = 1.71 \text{ mmol/dm}^3$, is in good agreement with results of Hrubšová et al. (2003) and Karlovská et al. (2004). Lichtenberg (1985) usually determines $RSAT$ resp. $RSOL$, the molar ratios of (detergent in bilayer)/(lipid in the bilayer) in the turning points between the I and II part, resp. the II and III part of the solubilization curve. We calculated $RSAT = 0.19$ and $RSOL = 0.45$ using a partition coefficient of $C_{12NO}$ between EYPC bilayers and water phase, $K_p = 740$ (Hrubšová, 2003, Karlovská, 2004). Our results show that $C_{12NO}$ molecules can be incorporated into EYPC bilayers and this process proceeds without a marked change in liposome's dimension until the molar ratio $C_{12NO}$ in bilayer/EYPC is 0.19. The creation of mixed micelles starts at molar ratios equal or higher than 0.19. This process is fulfilled at $C_{12NO}$ in bilayer/EYPC = 0.45. The destruction of liposomes is completed at this molar ratio. To obtain these results, we supposed that the partition coefficient of $C_{12NO}$ between lipid and water phase is the same for liposomes and micelles. According to Hrubšová et al. (2003) and Karlovská (2004), CMC of $C_{12NO}$ at 30°C is 1.96 mmol/dm³. This concentration of $C_{12NO}$ is higher than both turning points for fluorescence measurements and first turning point for turbidimetry and even higher than $c_S$. Therefore, we suppose that $C_{12NO}$ interacts and solubilizes membrane by monomers.

For fluorescent probe leakage experiments, EYPC liposomes were filled with calcein at the self-quenching concentration (10 mmol/dm³) at which the fluorescent probe is not detectable. Calcein was removed from the extraliposomal area. We suppose that a low level of fluorescence observed before $C_{12NO}$ addition is caused by traces of probe that stayed out of the liposomes after the passage through the Sephadex™G-50 column. The dependence of fluorescence of calcein on the $C_{12NO}$ could be divided into three sections. The increase of fluorescence, though slow, with increasing $C_{12NO}$ concentration means that some molecules of calcein permeate from the inside of liposome to the bulk water phase in part I of the solubilization curve. Because of hydrophilic character of calcein, some defects have to arise in EYPC bilayers under the influence of $C_{12NO}$ and enable the permeation of calcein. According to Heerklotz (2008), even small concentrations of detergent can cause a formation of membrane leaks or pores by stabilizing the hydrophobic edges with a highly curved detergent-rich rim. Calcein can leak through these defects to the bulk solution and be diluted to the concentration corresponding to the linear detection regime. We assume that the number and size of pores rise sharply in the concentration region 0.41–0.96 mmol/dm³ of $C_{12NO}$ and cause a steep increase of intensity. Fluorescent intensity nearly does not change when $C_{12NO}$ concentration

![Figure 2. Calibration curve of calcein solution. Concentration interval contains linear part, which represents linear detection regime of the calcein and nonlinear part, which represents the self-quenching process.](image-url)
Effect of N-dodecyl-N,N-dimethylamine N-oxide on unilamellar liposomes

is higher than 0.96 mmol/dm³. It means that concentration of calcein inside the liposome and in the bulk water phase is the same and the diffusion of calcein is therefore stopped at cC12NO > 0.96 mmol/dm³. The method of leakage of fluorescent probe calcein shows that the creation of large pores through which the concentration of intra- and extraliposomal solutions could be equilibrated is completed when turbidimetry still detects a presence of large particles – liposomes – in the solution. From the biological point of view, the death of a cell under the influence of C12NO can take place at a molar ratio lower than RSAT. Our results show that the method of leakage of fluorescent probe from liposome is more sensitive to changes of lipid bilayer which precedes the solubilization while turbidimetry better characterises the destruction of liposome to much smaller lipid aggregates.

The fluorescent measurement of the effect of C12NO on the EYPC liposomes was repeated for several lower EYPC concentrations. The typical three stage dependences of fluorescent intensity on the C12NO concentration were obtained. The turning points between the individual stages were found at lower C12NO concentrations. Similar result was obtained for the dependence of solubilization concentration cs = f(cEYPC) for MLLs (Karlovská et al., 2004).

CONCLUSION

The solubilization of EYPC unilamellar liposomes induced by C12NO was observed turbidimetrically and expected results were obtained. The study of fluorescent measurement of leakage of calcein showed that relatively low C12NO concentration, when the structure of liposome is still preserved, is able to cause formation of pores in EYPC bilayer. Calcein can freely diffuse through these pores from the inside of liposome into the bulk water phase.

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REFERENCES

[1] Balgavý P., Devínsky F. Cut-off effects in biological activities of surfactants. Adv Colloid Interface Sci. 1996;66:23–63.

[2] Bukovský M., Mlynarčík D., Ondráčková V. Immunomodulatory activity of amphiphilic antimicrobials on mouse macrophages. Int J Immunopharmacol. 1996;18(6-I):423–426.

[3] de la Maza A., PARRA JL. Solubilizing effects caused by the nonionic surfactant dodecylmaltoside in phosphatidylcholine liposomes. Biophys J. 1997; 72:1668–1675.

[4] Gallová J., Szalayová S. The Effect of Stobadine on the Copper-Induced Peroxidation of Egg Yolk Phosphatidylcholine in Multilamellar Liposomes. Gen Physiol Biophys. 2003;23:297–306.

[5] Heerklotz H. Interactions of surfactants with lipid membranes. Q Rev Biophys. 2008;41(3/4):205–264.

[6] Helenius A., Simons K. Solubilization of membranes by detergents. Biochim Biophys Acta. 1975; 415:29–79.

[7] Karlovská J., Devínsky F., Lacko I., Balgavý P. Solubilization of unilamellar liposomes by N-alkyl-N,N-dimethylamine N-oxide. Czech Farm. 2003;52(6):299–305.

[8] Kapoor Y., Howell BA., Chauhan A. Liposome Assay for Evaluating Ocular Toxicity of Surfactants. Invest Ophthalmol Vis Sci. 2009;50(6):2727–2735.

[9] Lichtenberg D. Interactions of surfactants with lipid membranes. Q Rev Biophys. 2008;41(3/4):205–264.

[10] Lichtenberg D., Opatowski E., Kozlov MM. Phase boundaries in mixtures of membrane-forming amphiphiles and micelle-forming amphiphiles. Biochim Biophys Acta. 2000; 1508:1–19.

[11] Memoli A., Annesini MC., Petralito S. Surfactant-induced leakage from liposomes: a comparison among different lecithin vesicles. Int J Pharm. 1999a; 184:227–235.

[12] Memoli A., Palermiti GL., Travagli V., Alhaique F. Effects of surfactants on the spectral behaviour of calcein (II): a method of evaluation. J Pharm Biomed Anal. 1999b; 19:627–632.

[13] Murín A., Devínsky F., Koleková A., Lacko I. Relation between chemical structure and biological activity of N-alkyl dimethylamineoxides series and some other related compounds. Biol Brat. 1990;45:521–531.

[14] Paternostre MT., Roux M., Rigaud JL. Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by triton X-100, octyl glucoside, and sodium cholate. Biochemistry. 1988;27:2668–2677.

[15] Sanderson H., Tibazarwa C., Greggs W. High Production Volume Chemical Amine Oxides [C8–C20] Category Environmental Risk Assessment Risk Anal. 2009; 29(6):857–867.

[16] Schurtenberger P., Mazer N., Känzig W. Micelle to vesicle transition in aqueous solution of bile salt and lecithin. J Phys Chem. 1985;89:1042–1049.

[17] Singleton WS., Gray MS., Brown ML., White JL. Chromatographically homogeneous lecithin from egg phospholipids. J Amer Oil Chem Soc. 1965; 42:53–56.

[18] Šeršeň F., Gabunia G., Krejčírová E., Kráľová K. The relationship between lipophilicity of N-alkyl-N,N-dimethylamine oxides and their effect on the thylakoid membranes of chloroplasts. Photosynthetica. 1992; 26:205–212.

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