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EFFECT OF *N*-DODECYL-*N*,*N*-DIMETHYLAMINE *N*-OXIDE ON UNILAMELLAR LIPOSOMES

ÚČINOK *N*-DODECYL-*N*,*N*-DIMETYLAMÍN *N*-OXIDU NA UNILAMELÁRNE LIPOZÓMY

Original research article

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Abstract The effect of *N*-dodecyl-*N*,*N*-dimethylamine *N*-oxide (C12NO) on the unilamellar liposomes prepared from egg yolk phosphatidylcholine (EYPC) was studied using turbidimetric and fluorescence spectroscopic methods. The concentrations of C12NO causing saturation and total solubilization of membrane were evaluated turbidimetrically. Liposomes filled with hydrophilic fluorescent probe calcein were used for the fluorescence leakage measurements. Dependence of fluorescence intensity on C12NO concentration can be divided into three linear sections. The turning points between individual sections represent two concentrations of C12NO which induce formation of small defects, specifically large pores in liposomal membrane. These pores are wide enough to enable calcein to be washed out from liposomes completely. We compared results obtained by the two methods used. Measurements of fluorescent probe leakage showed that free diffusion of calcein through pores in EYPC bilayer was observed at such C12NO concentrations when the form of liposome is still preserved and the saturation of bilayer is not yet finished. Higher C12NO concentrations are needed for solubilization, a phase transition from bilayers to micelles.

Slovak abstract Efekt *N*-dodecyl-*N*,*N*-dimetylamín *N*-oxidu (C12NO) na unilamelárne lipozómy (ULL) pripravené z vaječného fosfatidylcholínu (EYPC) bol študovaný metódou turbidimetrie a fluorescenčnej spektroskopie. Koncentrácia C12NO vyvolávajúca saturáciu a úplnú solubilizáciu membrány bola vyhodnotená turbidimetricky. Pre fluorescenčné merania boli použité lipozómy naplnené hydrofilnou fluorescenčnou sondou kalceínom. Závislosť intenzity fluorescencie od koncentrácie C12NO môže byť rozdelená na tri priamkové sekcie. Zlomové body, získané z priesečníkov priamok, zodpovedajú dvom koncentráciám C12NO, pri ktorých nastáva vznik malých štrukturálnych defektov resp. veľkých perforácií v membráne lipozómov. Tieto defekty sú natoľko veľké, že dochádza k úplnému vyplaveniu kalceínu z lipozómov. Výsledky získané z dvoch metód sme následne porovnali. Meranie fluorescenčnou metódou ukázalo, že voľná difúzia kalceínu cez póry v EYPC dvojvrstvách prebieha pri koncentráciách C12NO, kedy saturácia membrány nie je ukončená a tvar lipozómov zostáva zachovaný. Ku solubilizácii, teda k fázovej premene z dvojvrstiev na micely sú potrebné vyššie koncentrácie C12NO.

Keywords *unilamellar liposomes – solubilization – calcein – N-dodecyl-N,N-dimethylamine N-oxide*

Kľúčové slová: *unilamelárne lipozómy – solubilizácia – kalceín – N-dodecyl-N,N-dimetylamín N-oxid*

INTRODUCTION

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.

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

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result from their amphiphilic structure. Therefore, they are predestined to affect the phospholipid bilayer resulting in antimicrobial (Balgavý and Devínsky, 1996, Singh et al., 2006), phytotoxic (Murín et al., 1990), antiphotosynthetic (Šeršeň et al., 1992) and immunomodulatory effects (Bukovský et al., 1996). CnNO (n=10–16) is the most commercially used form from all CnNO (Sanderson, 2009). The homologue with dodecyl substituent (*N*-dodecyl-*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 multi- and unilamellar 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). K_2HPO_4 was obtained from Lachema (Czech Republic) and NaCl and K_2HPO_4 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/dm^3) was prepared from K_2HPO_4, K_2HPO_4, NaCl (0.15 mol/dm^3) and redistilled water. The required amount of calcein was dissolved in the adequate amount of NaOH solution (1 mol/dm^3). 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/dm^3.

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 sniped. 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 SephadexTM G-50 gel was poured into the syringe directly on the filter membrane. After centrifugation at 1000 g (3400 rpm) for 5 min., SephadexTM G-50 column was created. The syringe filled with the SephadexTM G-50 column was then transferred from the centrifuge tube with residual solvent to the new clean tube. We added EYPC + calcein solution drop-wise to the Sephadex™ G-50 column. The drops had to fall directly on the top of the column. It was very important to ensure the drops not fall in the void space between the column and syringe walls. Loaded column was let sit for 5 min. prior to centrifugation and then centrifuged for 7 min. at 500 g (2400 rpm) and for 3 min. at 1000 g (3400 rpm). The bulk calcein solution stayed in the column, while liposomes filled with calcein were at the bottom of the tube.

Method

Turbidimetry

In all samples, there was a constant concentration of EYPC 0.4 mmol/dm^3 and increasing concentration of C12NO (0–2.83 mmol/dm^3). The turbidance of the samples was measured 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/dm^3) 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/dm^3) and post-solubilizing (2.83 mmol/dm^3) 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/dm^3). In every set, the concentration of EYPC + calcein was the same and increasing concentration of C12NO (0–1.83 mmol/dm^3) 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/dm^3 and 1.96±0.04 mmol/dm^3 for 0.4 mmol/dm^3 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/dm^3 and this concentration range represents a linear detection regime of calcein. Self-quenching starts to prevail at 20 μmol/dm^3, 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/dm^3. According to Hrubšová et al. (2003) and Karlovská et al. (2004), 0.3 mmol/dm^3 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/dm^3 C12NO. Calcein fluorescence intensity was measured within concentration interval corresponding to the calibration curve (0–100 μmol/dm^3). 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.

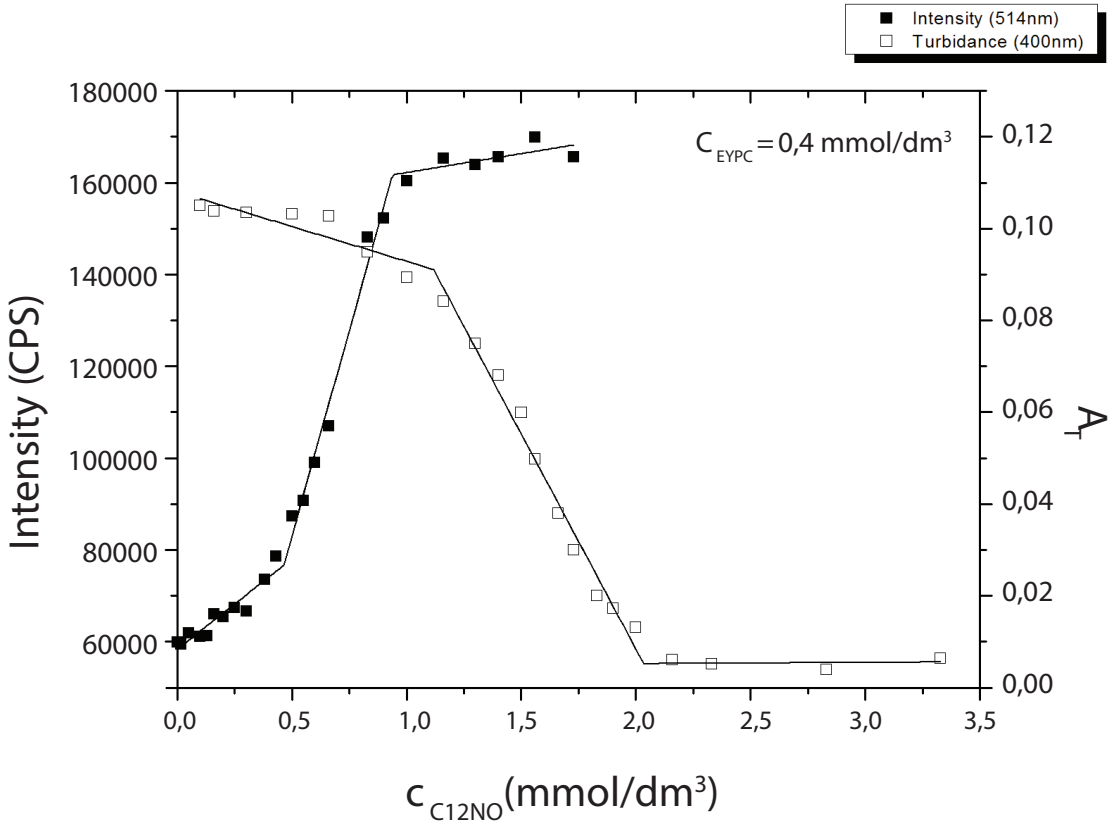

Figure 1. Dependence of the fluorescence intensity in counts per second (left axis, full symbols) and turbidance (right axis, open symbols) on the concentration of *N*-dodecyl-*N*,*N*-dimethylamine *N*-oxide (C12NO). Concentration of egg yolk phosphatydilcholine (EYPC) = 0,4 mmol/dm³

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 turning points between individual sections were again evaluated according to Gallová and Szalayová (2004). The total concentrations of C12NO in the sample at the first and second turning points are 0.41±0.03 mmol/dm³ and 0.96±0.03 mmol/dm³ for 0.4 mmol/dm³ EYPC.

Some volume of buffer was added to the sample at the end of the experiment at cC12NO around 1.7 mmol/dm³. The intensity of calcein decreased after the dilution. This confirms that the concentration of calcein in the bulk solution was in the linear detection regime during the whole experiment. Otherwise, the intensity would increase after dilution due to the decomposition of dimers to monomers and restoration of the fluorescent activity.

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 AT 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 cS

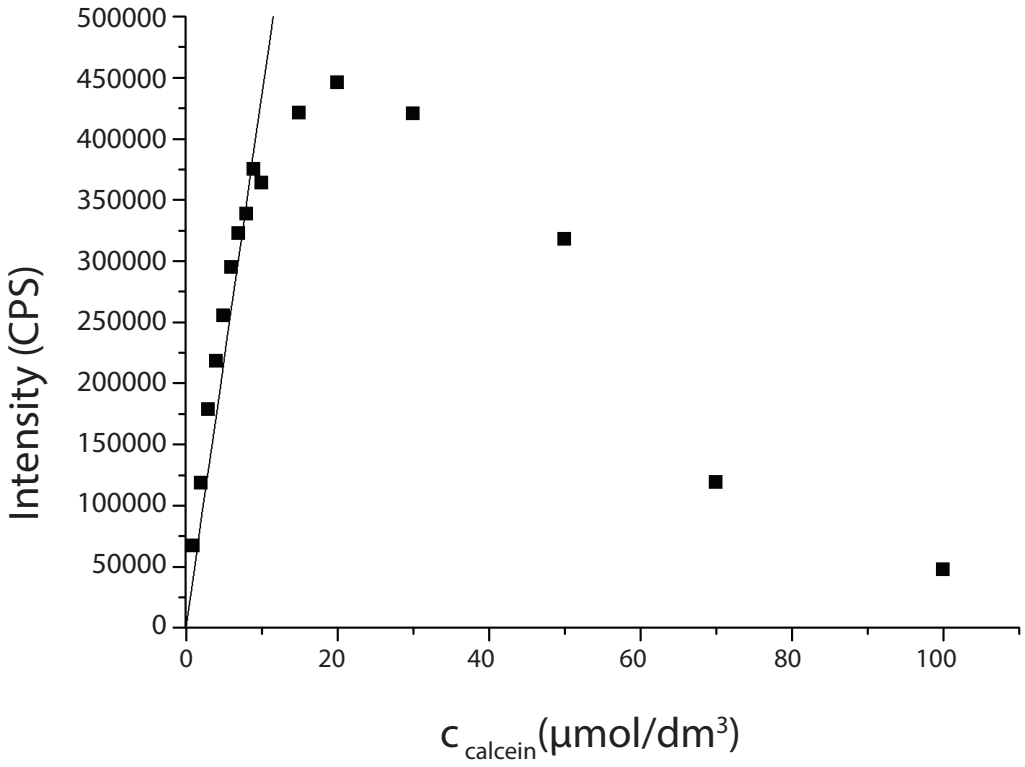

Figure 2. Calibration curve of calcein solution. Concentration interval contains linear part, which represents linear detection regime of the calcein and nonlinear part, which represents the self-quenching process.

was evaluated as a centre of the reverse sigmoid. The value obtained in this work, cS = 1.71 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 C12NO between EYPC bilayers and water phase, Kp = 740 (Hrubšová, 2003, Karlovská, 2004). Our results show that C12NO molecules can be incorporated into EYPC bilayers and this process proceeds without a marked change in liposome's dimension until the molar ratio C12NO 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 C12NO 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 C12NO between lipid and water phase is the same for liposomes and micelles. According to Hrubšová et al. (2003) and Karlovská (2004), CMC of C12NO at 30°C is 1.96 mmol/dm^3. This concentration of C12NO is higher than both turning points for fluorescence measurements and first turning point for turbidimetry and even higher than cs. Therefore, we suppose that C12NO interacts and solubilizes membrane by monomers.

For fluorescent probe leakage experiments, EYPC liposomes were filled with calcein at the self-quenching concentration (10 mmol/dm^3) 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 C12NO 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 C12NO could be divided into three sections. The increase of fluorescence, though slow, with increasing C12NO 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 C12NO 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/dm^3 of C12NO and cause a steep increase of intensity. Fluorescent intensity nearly does not change when C12NO concentration

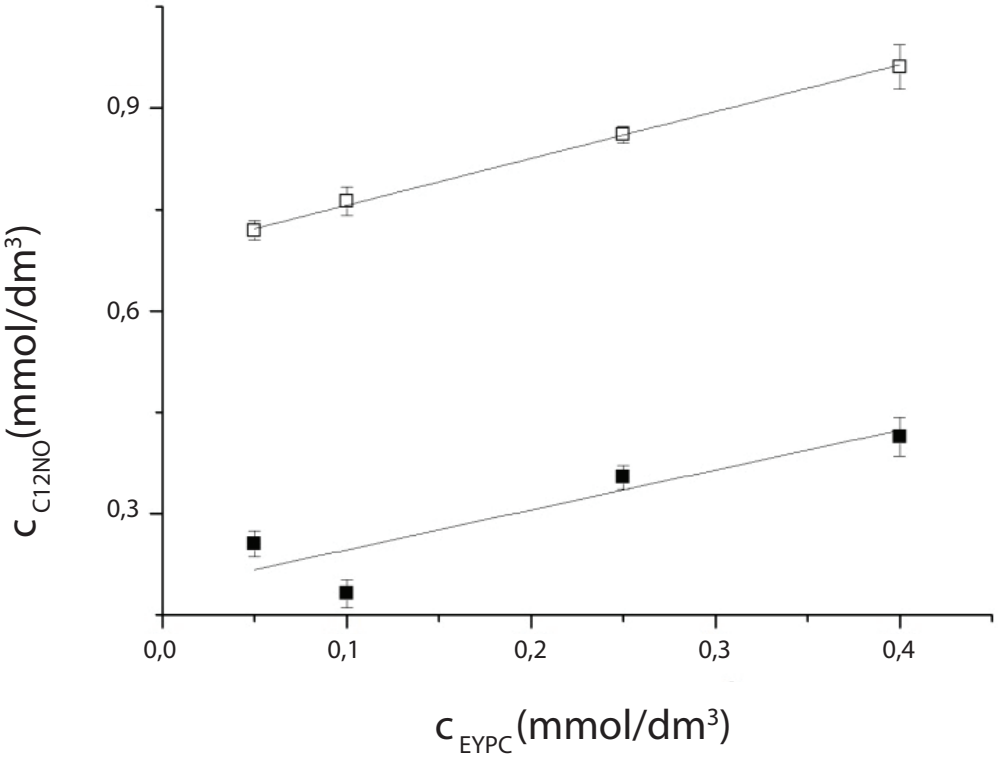

Figure 3. Dependence of the concentration of *N*-dodecyl-*N*,*N*-dimethylamine *N*-oxide (C12NO) evaluated from turning points on the concentrations of egg yolk phosphatydilcholine (EYPC).

is higher than 0.96 mmol/dm^3. 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/dm^3.

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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