Study of the solubilisation process of bacterial model membranes induced by DDAO

Original Paper

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Abstract Solubilisation of two bacterial model membranes induced by *N,N*-dimethyl-1-dodecanamine-*N*-oxide (DDAO) was studied. The first model membrane consisted of a mixture of palmitoyl-oleoylphosphatidylethanolamine (POPE) and palmitoyl-oleoylphosphatidylglycerol (POPG) in a molar ratio 0.6:0.4 mol/mol, and a second model membrane was enriched with tetraoleoylcardiolipin (TOCL) with a composition POPE-POPG-TOCL = 0.67:0.23:0.1 mol/mol/mol. Solubilisation of these model membranes was studied by static light scattering (nephelometry). Effective ratio R_e (the amount of DDAO integrated into the bilayer to the amount of lipid) at different steps of the solubilisation process was determined. The molar partition coefficient of DDAO was calculated – in case of the POPE-POPG membrane, $K_p = 5,300 \pm 400$, for the POPE-POPG-TOCL membrane, $K_p = 6,500 \pm 500$.

Keywords *N,N*-dimethyl-1-dodecanamine-*N*-oxide – solubilisation – bacterial model membranes – cardiolipin

INTRODUCTION

Increasing number of bacteria resistant against conventionally used antibiotics poses a serious health threat for the society. Conventional antibiotics have a specific target within a bacterial cell. Many bacteria have developed resistance to these specific mechanisms of action, rendering the antibiotics no longer effective. Particularly dangerous are the bacteria that are resistant to several conventional antibiotics (Pontali et al., 2013; Xu et al., 2014). In order to overcome multi-drug-resistant bacterial infections, research of substances targeting bacterial cells non-specifically is essential. An example of such substances are long-chain amphiphilic surfactants, such as the one used in the presented study.

N,N-dimethyl-1-dodecanamine-*N*-oxide (DDAO) is a surfactant with 12 carbon hydrophobic chains and a highly polar N-O group. It is widely commercially used in home cleaning detergents, personal hygiene products and pharmaceutical formulations as well (Singh et al., 2006). Within the research applications, DDAO is used to reconstitute, crystallise and purify membrane proteins, as well as to solubilise membranes, which is the focus of this study. DDAO is an amphiphilic molecule with $pK \sim 5$, meaning

that at physiological pH, DDAO is in a non-ionic form (Búcsi et al., 2014; Herrmann, 1962). DDAO manifests various biological effects, such as antimicrobial (Devínsky et al., 1990) and antiphotosynthetic (Murín et al., 1990) activity or phytotoxic (Šeršeň et al., 1992) and immunomodulatory (Bukovský et al., 1996) effect. In contact with the biological membrane, DDAO interacts mostly with its phospholipids (Kragh-Hansen et al., 1998). DDAO molecules incorporate themselves into the bilayer, influence physical properties of bilayer, and induce formation of defects and pores (Memoli et al., 1999; Ruiz et al., 1988), followed by complete disintegration of the bilayer after a certain concentration of surfactant is reached (Lichtenberg et al., 2013a; Lichtenberg et al., 2013b). For the interaction studies, a binary and ternary mixture of phospholipids was used. Interaction of DDAO with model membranes of various phosphatidylcholines (PCs) or PCs mixed with cholesterol (lipids typical for the mammalian model membranes) was studied in several papers (Belička et al., 2014a; Belička et al., 2014b; Hrubšová et al., 2003; Huláková et al., 2013, 2015; Karlovská et al., 2004a; Karlovská et al., 2004b; Uhríková et al., 2001). The aim of this study was to determine how different

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is the interaction of DDAO with bacterial model membranes. For most bacteria, phosphatidylethanolamines (PEs) are the most predominant phospholipids. All bacteria have at least 15% of anionic phospholipids such as phosphatidylglycerols (PGs) or cardiolipins (CLs) (Epand & Epand, 2009). PEs are zwitterionic phospholipids with a molecular shape of a truncated cone. Their polar headgroup is smaller in diameter in comparison with its hydrocarbon chain area. Therefore, they tend to form structures with negative curvature, which leads to the formation of non-lamellar phases at higher temperatures. PGs at physiological conditions are negatively charged, and because of their cylindrical molecular shape, they tend to form non-curved bilayers, even at higher temperatures. CLs have a double negative charge at physiological pH and relatively rigid (regarding mobility of the molecule) cylindrical molecular shape (Lopes et al., 2012). As a model organism for our experiments, G^- bacteria *Escherichia coli* (*E. coli*) were selected. Membrane lipids of *E. coli* are a mixture of PEs (57%), PGs (15%), CLs (10%) and other lipids (18%) (such mixture of lipids is commercially available as *E. coli* Total Lipid Extract). Frequently used models are two-component PE and PG mixtures (Lopes et al., 2010). The fatty acids found in the *E. coli* membrane phospholipids are predominantly 16–18 carbon long, with a substantial portion of them being unsaturated (Meister et al., 2014). For this reason, we chose to use a mixture of palmitoyl-oleoylphosphatidylethanolamine (POPE) and palmitoyl-oleoylphosphatidylglycerol (POPG) as our model system, in a molar ratio of POPE/POPG = 0.6/0.4 mol/mol. Gel phase–liquid-crystalline phase transition of this mixture is approximately at 20°C (Lopes et al., 2010; Pozo Navas et al., 2005). The temperature during the experiment was well-maintained at 25°C, ensuring our liposomes are in a liquid-crystalline phase.

An extract of polar lipids of *E. coli* (commercially prepared from total lipid extract by precipitation with acetone and extraction with diethyl ether) contains PEs (67%), PGs (23%) and CLs (10%). The POPE/POPG/CL three-component mixture (0.67:0.23:0.1) shows the highest similarity with the *E. coli* extracts based on phase transitions (Lopes et al., 2010). Despite CLs having 4 fatty acids in its molecules (in our case, 4 oleic acids), the dominant types of cardiolipin usually contain only one or two kinds of fatty acids. It was found that the fatty acid composition is relatively resistant to dietary manipulations, suggesting a specific composition is needed for its biological functions (Schlame et al., 2005). Tetraoleoylcardiolipin has a gel to liquid-crystalline phase transition at approximately -15°C (Chen, 2012). Therefore, we assume that our ternary mixture was in liquid-crystalline phase during the experiment as well.

In the present paper, the solubilising effect of a surfactant DDAO on two bacterial model membranes was studied using static light scattering (nephelometry). Because of the amphiphilic nature of the DDAO surfactant and relatively high amount of anionic phospholipids in model membranes,

all experiments were performed in phosphate-buffered saline (PBS, pH 7.5).

MATERIALS AND METHODS

Chemicals

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), 1,3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (sodium salt) (TOCL) purchased from Avanti Polar Lipids (USA), and *N,N*-dimethyl-1-dodecanamine-*N*-oxide (DDAO) obtained from Sigma-Aldrich (Germany) were >99% pure and were used without further purification. Other used chemicals: K_2HPO_4 (CentralChem, Slovakia), KH_2PO_4 (Merck, Germany) and NaCl (Slavus, Slovakia) were of analytical grade.

Liposomes preparation

POPE-POPG ($n_{POPE}:n_{POPG} = 0.6:0.4$ mol/mol) and POPE-POPG-TOCL ($n_{POPE}:n_{POPG}:n_{TOCL} = 0.67:0.23:0.1$ mol/mol/mol) model membranes were prepared. Weighted amounts of dry lipids in glass tubes were co-solubilised by dissolving in chloroform. Chloroform was evaporated under a stream of gaseous nitrogen to dryness, followed by evacuation in a vacuum chamber using rotary oil pump for 8 h. 50 mmol/dm³ PBS buffer with pH 7.5, consisting of 7.6 mmol/dm³ KH_2PO_4 , 42.4 mmol/dm³ K_2HPO_4 and 150 mmol/dm³ NaCl was prepared using redistilled water. Adequate amount of dry lipid film was hydrated with 1.3 ml of PBS buffer to obtain lipid concentration 10 mmol/dm³. Spontaneous formation of multilamellar liposomes (MLLs) was accompanied by occasional vortexing until a homogenous dispersion was obtained. MLLs dispersion was extruded (LiposoFast-Basic Extruder) through a 100 nm polycarbonate filter 51 times (MacDonald et al., 1991; Olson et al., 1979). Unilamellar liposomes (ULLs) dispersion was then used to prepare a set of 25 samples with constant concentration of lipid and increasing concentration of DDAO from 0 to 3.12 mmol/dm³. 5 sets of samples, with lipid concentrations 0.02, 0.04, 0.06, 0.08 and 0.1 mmol/dm³ were prepared for each type of model membrane.

Static light scattering method

Typical three-stage (Lichtenberg et al., 2013a; Lichtenberg et al., 2013b; Želinská et al., 2020) solubilisation experiment is shown in Figure 1. In the first stage, the surfactant partitions into the membrane, until it is saturated (we will refer to this concentration as D_t^{SAT}). Further increase in the DDAO concentration causes a phase transition from lamellar structures (in our case, ULLs) to smaller micelles. This transition continues until all the lamellar structures are completely solubilised (we will call this concentration D_t^{SOL}).

Figure 1. Dependence of normalised intensity of scattered light (I_{NORM}) on the concentration of added DDAO (c_{DDAO}), both types of membrane at a lipid concentration of 0.1 mmol/dm^3 . Inset shows a comparison of the experiment at three different concentrations of POPE-POPG-TOCL (intensity (I) was not normalised).

In the last stage, only lipid–surfactant mixed micelles and residual surfactant monomers are present. Typical feature of this transition is the decrease of the size of the particles, which can be observed using static light scattering (nephelometry). We have also evaluated a concentration of DDAO that causes the scattered light intensity to decrease by 50% compared to the value in the first phase. We will refer to this parameter as D_t^{MID} . We will use the term “critical concentration” for all three D_t parameters.

Static light scattering experiments were carried out using Fluoromax 4 spectrofluorometer (Horiba Jobin Yvon, USA) with a controlled temperature of 25°C . Scattering of the light with 600 nm wavelength at a 90° angle was measured for 6 seconds with a step 0.1 second. Average intensity of the scattered light was calculated. Samples were measured 2 h after the DDAO was added to the ULLs dispersion.

Data evaluation and partition coefficient calculation

The method used to evaluate the data and perform the calculations was previously described in Želinská et al. (2020). To determine the D_t^{SAT} and D_t^{SOL} parameters, the nephelometry data were fitted using two bilinear functions (each parameter was fitted individually):

$$y = (kx + q)0.5(1 - (x - D_t)/|x - D_t|) + (mx + D_t(k - m) + q)0.5(1 + (x - D_t)/|x - D_t|) \quad (1)$$

where $y = kx + q$ represents the equation of the straight line before the critical concentration (D_t), and $y = mx + r$ is

the equation of the straight line after the critical DDAO concentration was passed. Using the bilinear function, the searched for parameter, D_t , is found as an intersection point of these two straight lines. An example of the fitting is shown in the Figure 2 (full line). We can see that both fits (one for D_t^{SAT} and the other for D_t^{SOL}) overlap perfectly creating a continuous line, because the equation for the straight line in the second stage is identical for both fits.

The numerical value of D_t^{MID} was determined by fitting the dependence of scattered light intensity (I) on the DDAO concentration (c_{DDAO}) with reverse sigmoid function (Figure 2, dashed line):

$$I = I_{\text{min}} + (I_{\text{max}} - I_{\text{min}})/(1 + \exp(-(c_{\text{DDAO}} - D_t^{\text{MID}})/dc_{\text{DDAO}})) \quad (2)$$

where I_{max} is the maximum intensity, I_{min} is the minimum intensity and dc_{DDAO} represents the width of the second stage. Molar partition coefficient (K_p) is defined as the ratio of molar concentration of amphiphile in the lipid phase to its molar concentration in the aqueous phase in the equilibrium state. To determine K_p of DDAO between the POPE-POPG or POPE-POPG-TOCL bilayers and our water-based buffer, we used a method previously described in more detail in Želinská et al. (2020). The experimentally acquired dependence of the critical DDAO concentrations (D_t) on the concentration of lipid (c_L) was fitted with the function:

$$D_t = R_e \cdot \frac{\rho_B}{M_L} \cdot \frac{1}{K_p} + R_e \cdot c_L \quad (3)$$

Figure 2. Dependence of scattered light intensity (I) on the concentration of added DDAO, $c_{\text{POPE+POPG+TOCL}} = 0.06 \text{ mmol/dm}^3$. Arrows indicate the critical concentrations D_t^{SAT} , D_t^{SOL} acquired using bilinear functions (full line) and D_t^{MID} using reverse sigmoid function (dashed line).

where the slope of this linear dependence is the effective molar ratio (R_e) of the amount of DDAO integrated into the bilayer to the amount of lipid. The density of bilayer (ρ_b) was measured using vibrational densitometer DMA 4500M (Anton Paar, Austria), following the same procedure as in our previous study (Gallová et al., 2017). Molar mass (M_b) of the bilayer was calculated using mole fractions of lipids (X_i) constituting the bilayer. For example, $M_{\text{POPE+POPG}} = X_{\text{POPE}}M_{\text{POPE}} + X_{\text{POPG}}M_{\text{POPG}}$. The constant ratio of the density of bilayer (ρ_b) and its molar mass (M_b) was denoted as a fixed parameter ($1,350 \text{ mmol/dm}^3$ for POPE-POPG and $1,234 \text{ mmol/dm}^3$ for POPE-POPG-TOCL) during the fitting. All three (D_t^{SAT} , D_t^{MID} , D_t^{SOL}) dependencies were fitted (Figure 3) simultaneously with Function 3 using Multi-Data Fit Mode in Origin software (Version 2019b, OriginLab Corporation, Northampton, MA, USA). The global fit fitted the individual linear dependencies and their slopes (R_e) and calculated one shared partition coefficient (K_p) for all three dependencies.

RESULTS AND DISCUSSION

Solubilisation of POPE-POPG and POPE-POPG-TOCL ULLs induced by non-ionic surfactant DDAO was studied at five different lipid concentrations. Decrease of the lipid particle sizes, which is typical for the solubilisation process, was examined using nephelometry. We can see examples of the experiments in Figure 1. The inset of Figure 1 shows

raw solubilisation data for POPE-POPG-TOCL membranes at three lipid concentrations. We can see that the method is sensitive not only for the changes in the size of the scattering particles, but for their concentration as well. The main part of Figure 1 shows a solubilisation experiment comparison of the two types of membrane we investigated, at the same lipid concentration 0.1 mmol/dm^3 . Intensity of the scattered light was normalised (to the highest detected intensity) for better comparison. All dependencies follow the three-stage process described earlier. All dependencies were fitted with bilinear functions (Equation 1) and reverse sigmoid function (Equation 2) to obtain critical concentrations D_t^{SAT} , D_t^{MID} , D_t^{SOL} . An example of the fitting functions can be seen in Figure 2 (critical concentrations are indicated by arrows).

All acquired D_t^{SAT} , D_t^{MID} , D_t^{SOL} values are plotted as a function of concentration of the lipid in Figure 3. We can see their values increase linearly with increasing concentration of lipid for both types of membrane. The only exception is the saturation concentration D_t^{SAT} for POPE-POPG membranes. The reason is that for POPE-POPG membranes, the intensity of scattered light in the first phase of solubilisation was increasing, as opposed to staying approximately the same, as was the case for POPE-POPG-TOCL membranes (an example can be seen in Figure 1). Surfactant-induced increase in the particle size has been reported in literature (Kragh-Hansen et al., 1998). The mentioned study shows that DDAO induced a fusion of small ULLs to larger vesicles before the transition into micelles. The

Figure 3. Linear dependencies of critical DDAO concentrations on the concentration of POPE-POPG (left) and POPE-POPG-TOCL (right). The figure shows the global fit used to calculate the effective molar ratios (R_e) and the partition coefficients (K_p). D_w^{SAT} represents the concentration of DDAO surfactant in the water phase at the stage of complete saturation of the lipid bilayers by DDAO.

ULLs interacting with DDAO were composed of sarcoplasmic reticulum lipid extract (lipid composition was not specified), and the buffer in use contained 0.1 mmol/dm³ CaCl₂. Binding of Ca²⁺ ions to the membrane are known as an important factor involved in the membrane fusion (Martens & McMahon, 2008). Our buffer did not contain such ions, and the ULLs lipid composition was different as well. The reason for such increase in intensity needs to be studied further. The increase in the intensity made the fitting of D_t^{SAT} less reliable, as we can see the error bars (Figure 3) are greater than for any other parameter. Nevertheless, this parameter was not excluded from further analysis, because doing so have not provided significantly different results. All three critical concentrations were fitted with the global function (Equation 3, Figure 3) as described earlier. Instrumental weighing was applied during the fitting, because it uses the square of the reciprocal of the error values, so points with smaller error values have more weight. Results of the fitting are shown in Table 1.

D_w represents the concentration of surfactant in the water phase at particular stage of the solubilisation process. D_w values were obtained by extrapolation of the global function to zero lipid concentration (see Figure 3, where D_w^{SAT} is depicted as an example). D_w values (D_w^{SAT} , D_w^{MID} , D_w^{SOL}) were obtained as a y-coordinates of the intersection points of the global function with the y-axis. The critical micellar concentration of DDAO at 27°C in its non-ionic form (at pH > 7) is 2.1 mmol/dm³ (Herrmann, 1962). All our calculated values of D_w are smaller than this value, which means that DDAO molecules did not form micelles during the solubilisation experiment. Therefore, we propose that the solubilisation process in this study took place by the transbilayer mechanism (Kragh-Hansen et al., 1998).

The effective molar ratio (R_e) of the amount of DDAO integrated into the bilayer to the amount of lipid is a constant, independent of the concentration of lipid and specific for surfactant–lipid mixture. The R_e (we will be using values for D_t^{MID} concentration for comparisons) values were 4.6 ± 0.3 for POPE-POPG and 6.3 ± 0.3 for POPE-POPG-TOCL. We have determined the partition coefficient of DDAO in bilayers consisting of POPE-POPG $K_p = 5,300 \pm 400$ and for the POPE-POPG-TOCL $K_p = 6,500 \pm 500$.

Regarding the difference between POPE-POPG and the cardiolipin-containing model membrane, the latter was more stable against the DDAO-induced solubilisation. As we can see in Table 1, the R_e values were higher at all three evaluated critical concentrations. The calculated partition coefficient was higher as well. Assuming it is caused purely by the presence of cardiolipin would be premature. Domain formation in bacteria membranes can also play an important role. These domains, reported to be enriched in cardiolipin, have been suggested to play an important role in regulatory functions of the cell (Epand & Epand, 2009 and references therein). Many studies with bacterial model membranes tend to neglect the importance of the presence of cardiolipin in the model membranes. Further investigation is needed, because it appears cardiolipin is an important part of bacterial membrane, even though its function is not yet completely known. We would like to continue the research of cardiolipin-containing model membranes in the future, for example using fluorescence microscopy with the help of cardiolipin specific dye 10-N-nonyl acridine orange.

The partition coefficients and R_e values calculated in the present study are greater than have been reported for the interaction of DDAO with mammalian model

Table 1. Global fit (Equation 3) results of POPE-POPG and POPE-POPG-TOCL static light scattering data.

Critical DDAO concentration	POPE-POPG		POPE-POPG-TOCL	
	R_e	D_w [mmol/dm ³]	R_e	D_w [mmol/dm ³]
D_t^{SAT}	3.7 ± 0.2	0.9 ± 0.1	5.0 ± 0.3	1.0 ± 0.1
D_t^{MID}	4.6 ± 0.3	1.2 ± 0.2	6.3 ± 0.3	1.2 ± 0.2
D_t^{SOL}	5.1 ± 0.3	1.3 ± 0.2	7.5 ± 0.4	1.4 ± 0.2
K_p	$5,300 \pm 400$		$6,500 \pm 500$	

R_e is the effective ratio of the amount of DDAO integrated into the lipid bilayer to the amount of lipid. D_w is the concentration of surfactant in the water phase. K_p is molar partition coefficient of DDAO between the bilayer and water phase.

membranes. For example, in our recent article (Želinská et al., 2020), we have researched the interaction of DDAO with dioleoylphosphatidylcholine (DOPC) and cholesterol (CHOL)-enriched DOPC liposomes (33 mol% of CHOL). The R_e values for the D_t^{MID} concentration were 2.2 ± 0.3 in the case of DOPC ULLs and 1.8 ± 0.3 for DOPC-CHOL type of membrane. Also, the calculated partition coefficients were reported smaller: $2,300 \pm 400$ (for DOPC) and $2,100 \pm 600$ (for DOPC-CHOL). Other studies reported smaller molar partition coefficients for mammalian model membranes as well. For example, Hrubšová et al. (2003) calculated $K_p = 500 \pm 200$ in a system egg yolk phosphatidylcholine (EYPC) ULLs/water. R_e for the D_t^{MID} concentration was 0.6 ± 0.2 . MLLs of EYPC were reported to have a molar partition coefficient of DDAO equal to $1,200 \pm 400$ (Karlovská et al., 2004a). In these two studies, only a dependence of D_t^{MID} on the lipid concentration was used for the calculations.

Our data and calculations show us that the partitioning of DDAO between the aqueous phase and the bacterial model membranes is higher than it is in the case of mammalian model membranes, and yet, the bacterial ULLs needed more DDAO to become solubilised. Because the hydrophobic part of the studied phospholipids is very similar, we suggest that the reason for such big differences might be caused by the

polar parts of the phospholipids. In comparison with PCs, the polar part of PEs has smaller diameter (the choline group in PCs is bigger than the ammonium group) and binds smaller amount of water molecules. The ammonium group is able to form hydrogen bonds with phosphate and oxygen atoms of adjacent molecules. A molecular dynamics simulation (Murzyn et al., 2005) of POPE-POPG (in the proportion 3:1) with Na⁺ ions (to neutralise the negative charge of POPG) has shown that POPE molecules interact readily with POPG and other POPE molecules with hydrogen bonds and water bridges. These bonds strengthen interlipid contacts in the bilayer. These physico-chemical properties of PCs and PEs-PGs-containing membranes might be the reason for our findings, but further analysis is needed. Therefore, in the future, we would like to widen this research to monitoring the effect of DDAO on fluidity of the membrane and the process of pore formation using fluorescent probes.

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