CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 6, (2001) pp 283 – 290

Received 13 May 2001
Accepted 20 July 2001

X-RAY DIFFRACTION AND NEUTRON SCATTERING STUDIES OF AMPHIPHILE - LIPID BILAYER ORGANIZATION

PAVOL BALGAVÝ[1], DANIELA UHRÍKOVÁ[1], JANKA KARLOVSKÁ[1], MARTINA DUBNIČKOVÁ[1], NORBERT KUČERKA[1], FERDINAND DEVÍNSKY[1], IVAN LACKO[1], JOZEF ČIŽMÁRIK[1], KARL LOHNER[2], GÁBOR DEGOVICS[2], GERT RAPP[3], SERGEJ YARADAIKIN[4], MICHAIL KISELEV[4], ACHMED ISLAMOV[4] and VALENTIN GORDELIY[4]

[1]Faculty of Pharmacy, Comenius University, SK-832 32 Bratislava, Slovakia, [2]Institute of Biophysics and X-Ray Structure Research, Austrian Academy of Sciences, A-8042 Graz, Austria, [3]Max Planck Institute for Colloid and Interface Science Golm/Potsdam, c/o HASYLAB, DESY, D-22603 Hamburg, Germany, [4]Frank's Laboratory of Neutron Physics, Joint Institute for Nuclear Research, 141980 Dubna, Russia

Abstract: The lipid bilayer thickness d_L, the transbilayer distance of lipid phosphate groups d_{PP} and the lipid surface area A_L of fluid hydrated bilayers of lamellar phases of egg phosphatidylcholine or dipalmitoylphosphatidylcholine containing N-alkyl-N,N-dimethylamine N-oxides (CnNO), 1,4-butanedi-ammonium-N,N'-dialkyl-N,N,N',N'-tetramethyl dibromides (GSn) or mono-hydrochlorides of [2-(alkyloxy)phenyl]-2-(1-piperidinyl)ethylesters of carbamic acid (CnA) were obtained by X-ray diffraction, and the bilayer thickness in extruded unilamellar dioleoylphosphatidylcholine vesicles containing C12NO was obtained by the neutron scattering. The values of d_L, d_{PP} and A_L change linearly up to the 1:1 amphiphile:lipid molar ratio. The slopes of these dependencies increase for d_L and d_{PP} and decrease for A_L with an increasing number of carbons n in the amphiphile long hydrocarbon substituent ($18 \geq n \geq 8$ for CnNO, $16 \geq n \geq 9$ for GSn, $12 \geq n \geq 5$ for CnA), while the opposite trends are observed for the short substituent ($8 \geq n \geq 6$ for CnNO, $9 \geq n \geq 7$ for GSn, $5 \geq n \geq 3$ for CnA). In case of long substituents, the effects on d_L, d_{pp} and A_L are caused by the decrease in the difference between the lipid and amphiphile hydrocarbon chain lengths and by the increase in their van der Waals attraction. The short substituent amphiphiles are mobile and exchange between multiple binding sites in the bilayer, minimizing the bilayer surface area.

Key Words: Bilayer Thickness, Lipid Surface Area, Phosphatidylcholines, Liposomes, Surfactants, X-Ray Diffraction, Neutron Scattering, NMR.

INTRODUCTION

Amphiphilic substances with long linear hydrocarbon substituents (*n* being the number of carbon atoms in the substituent) are widely used in agriculture, the food industry, pharmacy and medicine. It is believed that these substances act at the biological membrane level [1]. It has been suggested that the lateral expansion of the phospholipid bilayer of biological membranes caused by the intercalation of amphiphilic substances between phospholipid molecules and the mismatch between their hydrocarbon chain lengths results in the creation of voids ("free volume V_{free}") in the bilayer hydrophobic region (see [2] for references). At high amphiphile concentrations, V_{free} can be eliminated via the formation of various non-bilayer phospholipid-amphiphile aggregates resulting in membrane solubilization. At low amphiphile concentrations, the elimination of V_{free} via hydrocarbon chain ***trans-gauche*** isomerisation and/or interdigitation should result in a bilayer thickness d_L change. At a constant amphiphile concentration ***in the bilayer***, one would expect that the surface area A_L per phospholipid on the bilayer-aqueous phase interface should not depend on *n* (the amphiphile polar group is the same in the homologous series) and the d_L change should be smaller when the lengths of amphiphile and lipid hydrocarbon chains are comparable. In this paper, we summarize the results of our small-angle X-ray diffraction (SAXD), small-angle neutron scattering (SANS) and ^{31}P-NMR experiments testing these assumptions on model phospholipid membranes. We studied three homologous series of amphiphiles: N-alkyl-N,N-dimethylamine-N-oxides (CnNO), gemini surfactants 1,4-butanediammonium-N,N'-dialkyl-N,N,N',N'-tetramethyl dibromides (GSn), and monohydrochlorides of [2-(alkyloxy)phenyl]-2-(1-piperidinyl)ethyl esters of carbamic acid (CnA). As model phospholipid membranes, we used fluid lamellar phases composed of hydrated egg yolk phosphatidylcholine (EYPC) or dipalmitoylphosphatidylcholine (DPPC), and large unilamellar vesicles of dioleoylphosphatidylcholine (DOPC) prepared by extrusion.

MATERIALS AND METHODS

CnNOs, GSns, CnAs and EYPC were prepared and purified in our laboratories; DPPC and DOPC were purchased from Avanti Polar Lipids, ^2H_2O from Izotop and the other chemicals from Lachema. All the chemicals used were of analytical grade; the solvents (except ^2H_2O) were redistilled before use. Amphiphile and lipid were mixed in organic solvents; the solvents and traces of water were then removed by evaporation under gaseous N_2 followed by an oil pump evacuation and prolonged drying over P_2O_5. Water was added to this dry

mixture in a glass tube. The tube was flame-sealed and its content mechanically homogenized and equilibrated at a temperature higher than the gel-fluid phase transition temperature of the pure phospholipid. Finally, the hydrated mixture was equilibrated at room temperature in darkness. For SAXD, the waxy samples were pressed between two Mylar foils or mica windows in a sample holder, and the liquid dispersions were filled in glass capillaries (ø≤1 mm). Scrupulous care was taken to prevent water evaporation or adsorption during sample preparation and measurement. For ^{31}P-NMR spectroscopy, the dispersions were transferred to precision Wilmad NMR tubes (ø≤1 mm). For SANS, unilamellar liposomes (~1 wt. % of lipid in $^{2}H_2O$) were prepared by 25 or more extrusions through two stacked polycarbonate filters with 50 nm diameter pores using the LiposoFast Basic extruder (Avestin). The samples were put into 1 or 2 mm Hellma quartz cells.

The diffractograms of samples containing CnNO were obtained predominantly on a diffractometer equipped with a conventional X-ray source, Kratky compact camera (A. Paar) and linear position sensitive detector (M. Braun). The samples containing CnA or GSn and EYPC were measured on a DRON-4-07 diffractometer (LNPO Burevestnik) (see [3] for references). The diffraction data for DPPC+CnA samples were obtained using an X13 double focusing monochromator-mirror camera in the EMBL Outstation at the Deutsches Elektronen Synchrotron (DESY) in Hamburg on a DORIS storage ring (see [4] and references therein). The SANS measurements were performed on a small-angle time-of-flight axially symmetric neutron scattering spectrometer YuMO in an IBR-2 fast pulsed reactor in JINR, Dubna (see [3, 5] for references). ^{31}P-NMR spectra were recorded on Bruker AM 300 or Varian VXR 300 NMR spectrometers at 121.4 MHz using a deuterium lock, HF pulse width 45-55º and an interpulse relaxation delay of 0.7-1.0 sec. The spectra were recorded using strong proton inverse gated proton decoupling. Exponential multiplication of free induction decays corresponding to 50 Hz line broadening was applied prior to their Fourier transformation.

RESULTS AND DISCUSSION

SAXD patterns of samples containing intermediate amounts of water ($5 \le n_W = H_2O:PC \le 25$ mol/mol) and less than ~1 mol amphiphile per mol of PC consisted of Bragg diffraction peaks characteristic of one-dimensional lamellar phases wherein lipid bilayers are separated by water layers. Using the Bragg equation, the repeat period d of the lamellar phase was determined from the reciprocal spacings of the maxima of SAXD peaks. Supposing that the molecular volumes of amphiphile, water and lipid located in the bilayer are additive, the values of d_L and A_L were obtained from d according to [6]. The experimental points of d_L (A_L) obtained at $n_W \le 25$ were fitted by the function $f=f_\infty+(f_0-f_\infty)exp(-n_W/b)$ ($f=d_L$ or A_L) where f_0 and f_∞ are the values of f at zero and

full hydration, respectively, and b is a constant [3]. The values of d_L and A_L presented below were calculated from these fits for the n_W given except for the CnA+DPPC mixtures. The absolute values of structure factors were obtained as roots of integral intensities of diffraction peaks. The integral intensities were corrected using the Lorentz factor, the polarization factor, and the factor corresponding to the geometry of the experiment. From the set of structure factors obtained at different hydrations and using the Shannon's sampling theorem, the signs of structure factors were determined. The bilayer electron density profile was then calculated by the Fourier series and structure factors corresponding to the first four experimental diffraction orders as described in [3]. The bilayer thickness was then characterized by the distance d_{PP} between the electron density peaks corresponding to the transbilayer positions of phosphatidylcholine phosphate groups. The value of d_{PP} decreased with increasing hydration till some critical value of n_W and then remained approximately constant up to n_W~25. The mean of d_{PP} from this region is presented below.

The SANS spectra were evaluated as described in detail in [5, 7, 8]: From the Kratky-Porod plot $\ln[I(Q)Q^2]$ vs. Q^2 of SANS intensity $I(Q)$ in the range of scattering vector values Q corresponding to the interval $0.001\ \text{Å}^{-2} \le Q^2 \le 0.006\ \text{Å}^{-2}$, the liposome bilayer radius of gyration R_g and the bilayer thickness parameter $d_g = 12^{0.5} R_g$ were obtained. The value of d_g is equal to the steric thickness of the PC bilayer with no water molecules penetrating its polar region [5, 7, 8].

The proton-decoupled ^{31}P-NMR spectra of CnA+EYPC mixtures displayed a broad axially symmetric lineshape characteristic of the phospholipid bilayers in large unilamellar liposomes (>150 nm), multilamellar liposomes and lamellar liquid crystalline phase L_α. The effective ^{31}P-NMR chemical shift anisotropy $\Delta\sigma_{eff}$ was evaluated as the distance between the extrema of the first derivative of the spectrum and corrected for the Lorentzian linewidth broadening using simulated spectra. The spectra were computer simulated as described earlier [9-11]. The absolute value of $\Delta\sigma_{eff}$ increased with the increase in the CnA:EYPC molar ratio. This effect has been ascribed to the change in EYPC headgroup conformation caused by the electrostatic repulsion of the positively charged CnA amine and EYPC choline groups [11].

Selected experimental results are presented in Figs. 1-3. The values of d_L, d_{PP}, d_g and A_L are linearly dependent on the CnNO:PC (Figs. 1-2), GSn:PC and CnA:PC (not shown) molar ratios. The slopes Δd_L, Δd_{PP}, Δd_g and ΔA_L of these linear dependencies give the change of bilayer parameters at 1:1 molar ratio. For C12NO, $\Delta d_L = 0.28 \pm 0.03$ nm (EYPC, SAXD) and $\Delta d_g = 0.29 \pm 0.06$ nm (DOPC, SANS) were obtained from the data in Fig.1, i.e. at different bilayer hydrations and in different model membrane systems.

The values of d_L, d_{PP}, A_L, Δd_L, Δd_{PP}, ΔA_L and $\Delta\sigma_{eff}$ display biphasic dependencies on n (Figs. 2-3). The decrease in ΔA_L and A_L with *increasing* n for long chain amphiphile homologs ($n \ge 8$ for CnNO, $n \ge 9$ for GSn, $n \ge 5$ for CnA) is

most probably caused by the van der Waals attraction between lipid and amphiphile hydrocarbon chains which increases with *n*. The cause of the decrease in ΔA_L and A_L with ***decreasing n*** for short chain amphiphile homologs might be a changed location of the short chain homologs in the bilayer in comparison with the long chain homologs. While the long chain homologs are anchored in the bilayer core due to their strong attraction to acyl chains of PC, the short chain homologs are most probably more mobile and can exchange between multiple binding sites in the bilayer, thus minimizing the free energy also by the minimalization of bilayer surface area.

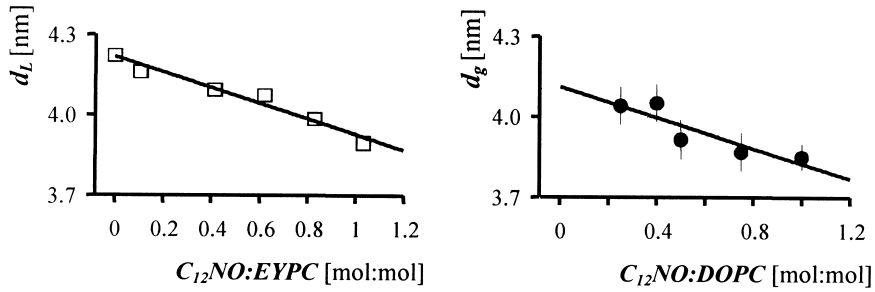

Fig. 1. EYPC and DOPC bilayers in the presence of C12NO at 20°C: □ - EYPC, n_W=12, SAXD, ● - DOPC, $n_W \rightarrow \infty$, SANS.

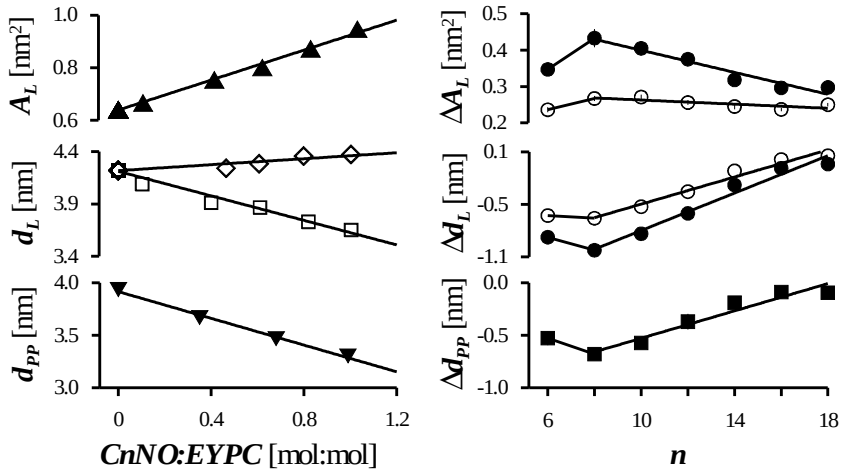

Fig. 2. EYPC bilayers in the presence of CnNO at 20°C: □ -C8NO, ▼ - C10NO, ▲- C12NO, ◊ - C16NO. Hydration: □▼◊○ - n_W=12, ● - $n_W \rightarrow \infty$.

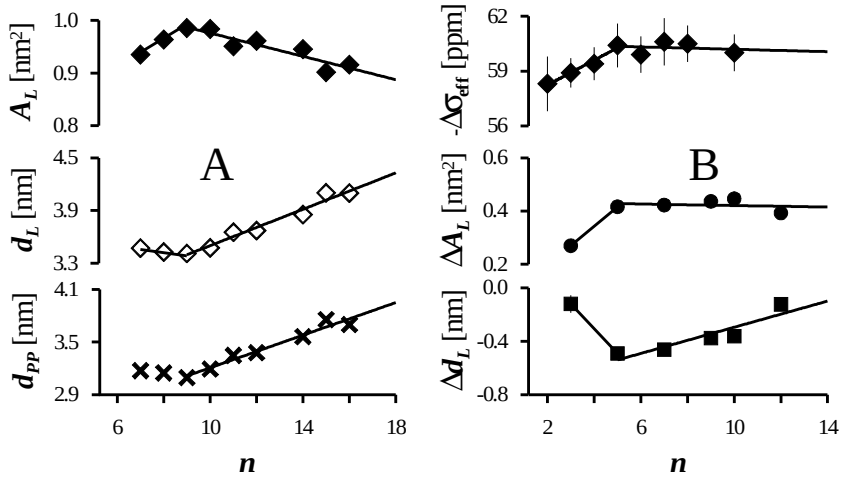

Fig. 3. EYPC bilayers in the presence of GSn (A) at n_W=18 and GSn:EYPC=0.4 mol/mol; EYPC and DPPC bilayers in the presence of CnA (B) at n_W=20 (●■) or n_W=40 (¿), CnA:PC=1 mol/mol. The samples with EYPC were measured at 20°C (¿Ð¯) and the samples with DPPC at the reduced absolute temperature $T_r=(T-T_c)/T_c$=0.035 (●■) where T_c is the CnA:DPPC gel-fluid transition temperature.

Two different binding sites for tertiary amine amphiphiles were found earlier in neutron diffraction and NMR experiments. It was shown that the deuterated C1A methyloxy substituent is located at two different positions – 1.28 nm and 2.18 nm from the bilayer center in oriented solid-like L_β phase C1A:DPPC=1:1 bilayers at 97% humidity and 20°C [12]. Two different tertiary amine binding sites in fluid phosphatidylcholine bilayers of the L_α phase at high hydration were observed by deuterium NMR spectroscopy of specifically deuterated tetracaine (4 carbon atoms in the linear hydrocarbon substituent on the aromate) and procaine (no hydrocarbon substituent on the aromate) [13]. It is possible that the second binding site located closer to the bilayer – water interface is populated more frequently by short chain CnA (or other short chain amphiphiles) and that the surface area is significantly smaller in this case. The changing population of CnA binding sites with the length of the hydrocarbon substituent can explain also the biphasic dependence of $\Delta\sigma_{eff}$ on n (Fig.3). Since the value of $\Delta\sigma_{eff}$ depends on the electrostatic interaction of the CnA charged group with the phospholipid N^+-P^- dipole, the changes in $\Delta\sigma_{eff}$ at a constant CnA:EYPC molar ratio indicate changes in the time averaged distance of the charged amphiphile and lipid groups. It is worth noting that these are observed predominantly for $n\leq5$, while $\Delta\sigma_{eff}$ remains constant (within experimental error) for long chain CnA homologs. Thus, it can be concluded that 1) the short chain homologs are mobile within the bilayer, 2) this mobility decreases with n, and 3) the long chain homologs are anchored in the bilayer as expected.

The primary aim of our experiments was to study the effect of amphiphile linear hydrocarbon chain length on lipid bilayer thickness and lipid surface area. The number of hydrocarbon chains, the polarity of the amphiphiles, the amphiphile head group dimension and conformation, the halide counterions, the bilayer surface charge density, the bilayer phase state (fluid or solid-like) etc. influence the incorporation of amphiphiles into lipid bilayers (see [1, 14] and references therein) and all these factors also influence bilayer thickness and surface area. However, when comparing the data within each of the homologous series studied, the observed dependencies are similar irrespective of the number of chains, polarity of the compounds or type of counterions: the surface area decreases for long chain compounds presumably because of van der Waals attraction of lipid and amphiphile chains and the surface area deviates from this tendency for short chain compounds because they are not anchored into the lipid bilayer like the long chain compounds; the bilayer thickness reflects these changes. We are convinced that the other factors influence the observed tendencies quantitatively but not principally.

Acknowledgements. This study was supported by the Slovak Ministry of Education VEGA grants 1/7704/2000, 1/7277/2000 and 1/5008/1998, and by the Comenius University grants UK/104100 and UK/6100. Synchrotron beam time was provided under the European Community – Access to Research Infrastructures action of the Improving Human Potential Programme. SAXD and SANS experiments were supported by the Action Austria-Slovakia, by the Austrian Academy of Sciences and by JINR project 07-4-1031-99/03. P.B. thanks Prof. S. Przestalski and the Polish Academy of Sciences for the invitation to Wrocław and for the travel grant.

REFERENCES

1. Przestalski, S., Sarapuk, J., Kleszczynska, H., Gabrielska, J., Hladyszowski, J., Trela, Z. and Kuczera, J. Influence of amphiphilic compounds on membranes. **Acta Biochim. Pol.** 47 (2000) 627-638.
2. Balgavý, P. and Devínsky, F. Cut-off effects in biological activities of surfactants. **Adv. Colloid Interface Sci.** 66 (1996) 23-63.
3. Balgavý, P., Dubničková, M., Uhríková, D., Yaradaikin, S., Kiselev, M. and Gordeliy, V. Bilayer thickness in unilamellar extruded egg yolk phosphatidylcholine liposomes: A small_angle neutron scattering study. **Acta Phys. Slov.** 48 (1998) 509-533.
4. Holopainen, J.M., Lemmich, J., Richter, F., Mouritsen, O.G., Rapp, G. and Kinnunen, P.K.J. Dimyristoylphosphatidylcholine/C16:0-ceramide binary liposomes studied by differential scanning calorimetry and wide- and small-angle X-ray scattering. **Biophys. J.** 78 (2000) 2459-2469.

5. Uhríková, D., Balgavý, P., Kučerka, N., Islamov, A., Gordeliy, V. and Kuklin, A. Small-angle neutron scattering study of the n-decane effect on the bilayer thickness in extruded unilamellar dioleoylphosphatidylcholine liposomes. **Biophys. Chem.** 88 (2000) 165-170.
6. Luzzati, V. X-ray diffraction studies of lipid-water systems. In: **Biological Membranes** (Ed. D.Chapman) pp. 71-124, Academic Press, London, New York, 1968.
7. Balgavý, P., Dubničková, M., Kučerka, N. and Kiselev, M.A. Bilayer thickness and lipid interface area in unilamellar extruded 1,2-diacylphosphatidylcholine liposomes: A small-angle neutron scattering study. **Biochim. Biophys. Acta** 1512 (2001) 40-52.
8. Balgavý, P., Kučerka, N., Gordeliy, V.I. and Cherezov, V.G. Evaluation of small-angle neutron scattering curves of unilamellar phosphatidylcholine liposomes using a multishell model of bilayer neutron scattering length density. **Acta Phys. Slov.** 51 (2001) 53-68.
9. Balgavý, P., Gawrisch, K. and Frischleder, H. Effect of N-alkyl-N,N,N-trimethylammonium ions on phosphatidylcholine model membrane structure as studied by ^{31}P NMR. **Biochim. Biophys. Acta** 772 (1984) 58-64.
10. Uhríková, D. Structural changes of lipid bilayers - effects of hydration, temperature and amphiphilic impurities. **PhD. Thesis**, Faculty of Mathematics and Physics, J. A. Comenius University, Bratislava, 1993.
11. Balgavý, P., Uhríková, D. and Ondriaš, K. Effect of local anesthetic and β-blocker carbisocaine on phospholipid polymorphism. **Biológia (Bratislava)** 49 (1994) 863-869.
12. Balgavý, P., Gordeliy, V.I. and Syrykh, A. G. Influence of local anesthetic heptacaine hydrochloride on the structure of lipid membranes from dipalmitoyllecithin. **JINR Communication** 14-91-387 (1991) 1-14.
13. de Paula, E. and Schreier, S. Molecular and physicochemical aspects of local anesthetic-membrane interaction. **Braz. J. Med. Biol. Res.** 29 (1996) 877-894.
14. Kleszczynska, H., Sarapuk, J. and Rozycka-Roszak, B. Modification of mechanical properties of model membranes by some bifunctional surfactants. **Cell. Mol. Biol. Lett.** 5 (2000) 67-73.