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Structural perturbations in phospholipid model membrane systems: The effect of dihalogenated phenol compounds and metal ions

Summary of PhD thesis

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2009

1. Introduction

The study of phospholipid bilayers as representative model systems for biomembranes has lately become an attractive issue in the field of soft condensed matter science. Though, studying the two component lipid/water system instead of the biomembrane itself is a significant abstraction, the interpretation of effects observed in these lyotropic liquid crystals may serve the better understanding of biological membranes. In this thesis the perturbation effects of 2,4-dichloro/dibromo phenol (DCP and DBP) and divalent metal ions (Cu^{2+}, Cd^{2+}) ions on the structure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) vesicles are studied.

The basic interactions in phospholipid/water model membrane systems can be divided into two groups according to the place where the perturbation effect takes place. The effect of dihalogenated phenols on DPPC/water system serves as an example for the perturbation in intrabilayer interactions, while divalent metal ions modify the interbilayer interactions. Dihalogeneted phenols are known to be toxic in small quantities and they evolve specific structural transition in the phospholipid bilayers. The Cu^{2+} and Cd^{2+} ions evaluated in this thesis belong to the trace and toxic metals in living organisms, respectively.

Thus, knowing the changes induced in lipid bilayer structure by the presence of organic molecules enables the design of vesicle systems for applications e.g. as drug carrier systems. The structure and dynamics of sterically stabilized vesicles (or with other word liposomes, SSVs or SSLs) is in the forefront of biophysical studies on this field, since a detailed knowledge about these systems can help in tailoring of the properties of these drug carriers to reach the optimum efficiency.

The localization of divalent metal ions in phospholipid vesicles also connects to the field of application, since the membrane-mimicking systems can be used as "reactors" for nanoparticles. In these applications the fact that these systems are structured in the nanometer scale is exploited.

A. Bóta, Z. Varga, G. Goerigk: Vesicles as reactors of nanoparticles, SAS2006 Kyoto, XII. International Conference on Small-angle Scattering, July 9-13, 2006. Kyoto, Japan

Z. Varga, G. Holló, Á. Orbán, L. Korecz, L. Naszályi Nagy, Gy. Kéri, L. Őrf, Z. Greff, G. Németh, B. Szokol, A. Lőrincz, I. Peták, A. Bóta: Structure and dynamics of sterically stabilized vesicles, Joint Meeting on Medicinal Chemistry, June 24-27, 2009 Budapest, Hungary

Others

A. Wacha, Z. Varga, L. Trif, G. Goerigk, A. Bóta, U. Vainio, ASAXS study of hexagonal W-type barium ferrite nanoparticles, *HASYLAB Annual Report*, 2007, pp. 477-478

A. Wacha, L. Trif, Z. Varga, G. Goerigk, A. Bóta, U. Vainio, SAXS study of hexagonal W-type barium ferrite nanoparticles, *HASYLAB Annual Report*, 2007, pp. 773-774

A. Bóta, Z. Varga, G. Goerigk, Biological systems as nanoreactors: anomalous small-angle scattering study of the CdS nanoparticle formation in multilamellar vesicles, *HASYLAB Annual Report*, 2007, pp. 37-40

Á. Oszlánczi, A. Bóta, Z. Varga, G. Goerigk, Effects of the sulfadiazine on the DPPE/DPPG/water vesicles, *HASYLAB Annual Report*, 2005, 841-842

A. Bóta, Z. Varga, G. Goerigk, Location of copper ions in the DPPC/water vesicles observed by using anomalous small angle X-ray scattering, *HASYLAB Annual Report*, 2005, pp. 909-910

A. Bóta, Z. Varga, G. Goerigk, E. Klumpp, ASAXS study of the localisation of the 2,4-dibromophenol in the DPPC/water multilamellar vesicles, *HASYLAB Annual Report*, 2004

A. Bóta, Z. Varga, G. Goerigk, V. Csokai, S. S. Funari, Effects of the copper ions on the DPPC/water multilamellar vesicles observed by ASAXS technique, *HASYLAB Annual Report*, 2004

2. Literature review

Phospholipids are the main lipid components of biological membranes. Due to the amphiphilic character of these molecules they spontaneously form mesoscale molecular aggregates with different symmetry. Although the self assembly of the phospholipids is diversified, the most common, and the biologically most relevant phase is the lamellar one. The model membrane system studied in this thesis, namely the DPPC/water system, is a lyotropic liquid crystal, with four temperature dependent multilamellar phases[1].

Introducing a third component to the lipid/water system is the first step towards real biological membranes. Moreover, the basis of the ecological or physiological role of many small molecules can be predicted based on the physico-chemical description of its interactions with phospholipid model membranes. Halogenated phenols, evaluated in this thesis, come into focus, since these compounds are present in a wide range of different fields of environment. For example bromophenols occur in sponges and algae and also in other marine organisms, as well as in the blood of fish and mammals. Recently, the importance of the elucidation of the brominated phenols' effect has been increased not only by their natural occurrence in the organisms but rather by their industrial application as fungicides and flame retardants (e. g. 2,4,6-tribromophenol, tetrabromobisphenol A). Chlorophenols appear in industry as they exhibit antibacterial effects. On the other hand, these molecules can form in natural waters during photooxidation. The ecological role of these molecules is not clear, but it has been shown, that they have hormone-like effects, and disrupt the cellular Ca^{2+} homeostasis in endocrine cells[2].

It was described previously by Csiszár et al.[3] that 2,4-dichlorophenol induces an interdigitated phase above a critical concentration of 0.5 guest molecule/lipid. This phase is characterised by the reduction of the layer thickness, which is caused by the slipping of carbon chains of the two layers into each other. The periodic distance of the sublattice changes simultaneously. The formation of the interdigitated phase can also be induced by a number of salts and

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[2] Olsen et al. Toxicology Letters, 129 (2002) 55, Hassenklöver et al. Aquatic Toxicology, 76 (2006) 37
[3] Csiszár et al. Chem. Phys. Lipids, 126 (2003) 155

alcohols. However, its structural description and the interpretation of different experimental observations are still unresolved.

Beside the main components of the real cell membranes there are many indispensable chemicals which are present in the millimolar range of concentration, but they account for an important physiological function. Divalent ions fall into this group, so their effect on biomembranes and also on model membranes have attracted increasing attention in recent years[4]. These ions can strongly affect the double layer structure and the conformation of the embedded membrane-proteins. For example, copper(II) ions are known to be released during synaptic transmission which is in connection with the involvement of these ions in the pathology of neurodegenerative diseases such as Alzheimler's, Parkinson's and Creutzfeldt-Jakob diseases[5].

Former and recent studies show that ions strongly influence the local and global properties of the lipid bilayer[6]. Monovalent and divalent ions are known to evolve different perturbation effects due to their different degree of binding to the lipid molecules. It was reported previously in the literature that divalent ions, such as Ca^{2+}, cause the unbinding of the bilayers of a multilamellar vesicle. In other words, the structure of the multilamellar vesicle (MLV) brakes up into an unilamellar system through a second order transition which was described several years ago, but its experimental observation is quite rare, although its biological significance is beyond doubt if one considers e.g. the fission and the fusion of cells[7].

The perturbation effects in liposomal systems are also important for applications purposes. For example, the use of membrane-mimetic systems represents a field of preparation of nanoparticles by wet chemical methods[8], where the starting system contains the electrolyte of the proper metal ion.

The idea of using small unilamellar vesicles for the delivery of different drugs came just a few years after the first observation that

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[5] Everse and Coates, Neurobiology of Aging, 30 (2009) 1011, Strausak et al. Brain Res. Bull. 55 (2001) 175

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[8] Fendler, Membrane-mimetic approach to advanced materials, 1997

[9] Z. Varga, A. Bóta, G. Goerigk, A. Hoell, Distribution of Copper(II) Ions in a Phospholipid Bilayer Studied by Anomalous Small Angle X-ray Scattering and Molecular Dynamics (in preparation)

[10] Z. Varga, A. Bóta, Structure of the interdigitated phase of DPPC bilayers induced by dihalogenated phenols (in preparation)

Presentations-Oral

Z. Varga: Contrast variation X-ray scattering in studying lyotropic liquid crystals, workshop on “Ferroelectric phenomena in liquid crystals”, Liquid Crystal Institute, Kent State University, June 19 - 28, 2007, Kent, U.S.A.

Z. Varga: A réz élettani szerepe a biomembránok tükrében. Oláh György Doktori Iskola Konferenciája, February 2009, BME, Budapest

Z. Varga: The role of guest molecules in the induced interdigitation of phospholipid bilayers as revealed by anomalous scattering, 1st International ASAXS workshop, Helmholtz-Zentrum Berlin für Materialien und Energie, May 14-15, 2009, Berlin, Germany

Presentations-Poster

A. Bóta, Z. Varga, and G. Goerigk: Effects of toxic molecules/ions on biological model membranes, 19th ECIS Conference, Sept. 18-23. 2005, Geilo, Norway

A. Bóta, Z. Varga, G. Goerigk: Displacement of metal ions in vesicles, Annual Users Meeting, DESY HASYLAB, Jan. 27. 2006. Hamburg, Germany

Z. Varga, A. Bóta, G. Goerigk: Location of dibromophenol in vesicle system, Annual Users Meeting , DESY HASYLAB, Jan. 27. 2006. Hamburg, Germany

Z. Varga, A. Bóta, G. Goerigk: Location of dihalogenated phenols in vesicle system as determined by anomalous small angle X-ray scattering, XII. International Conference on Small-angle Scattering, July 9-13, 2006. Kyoto, Japan

6. Publications

Papers

[1] Z. Varga, A. Bóta, G. Goerigk, Localization of Dibromophenol in DPPC/Water Liposomes Studied by Anomalous Small-Angle X-ray Scattering, *J. Phys. Chem. B.* (2006) **110(23)**, 11029-11032., IF: 4.115, C(IC): 3(1)

[2] A. Bóta, Z. Varga, G. Goerigk, Biological Systems as Nanoreactors: Anomalous Small-Angle Scattering Study of the CdS Nanoparticle Formation in Multilamellar Vesicles, *J. Phys. Chem. B.* (2007) **111(8)**, 1911-1915. IF: 4.086,C(IC): 3(2)

[3] Z. Varga, A. Bóta and G. Goerigk, Localization of dihalogenated phenols in vesicle systems determined by contrast variation X-ray scattering, *J. Appl. Cryst.* (2007). **40**, s205-s208, IF: 3.629, C(IC): 0(0)

[4] A. Bóta, Z. Varga, G. Goerigk, Vesicles as reactors of nanoparticles: An anomalous small-angle X-ray scattering study of the domains rich in copper ions, *J. Appl. Cryst.* (2007). **40**, s259-s263, IF: 3.629, C(IC): 0(0)

[5] A.Bóta, Z. Varga, G. Goerigk, Structural Description of the Nickel Part of a Raney-Type Catalyst by Using Anomalous Small-Angle X-ray Scattering, *J. Phys. Chem. C* (2008). **112(12)**; 4427-4429., IF: 3.396, C(IC): 2(0)

[6] Z. Varga, A. Bóta and G. Goerigk, Unbinding Transition in Lipid Multibilayers induced by Copper(II) Ions, *J. Phys. Chem. B* (2008) **112(29)**, 8430-8433., IF: 4.189, C/IC: 1(0)

[7] G. G. Nair, C. A. Bailey, S. Taushanoff, K. Fodor-Csorba, A. Vajda, Z. Varga, A. Bóta, A. Jákli, Electrically Tunable Color by Using Mixtures of Bent-Core and Rod-Shaped Molecules, *Advanced Materials* (2008), **20**, 3138-3142., IF: 8.191, C(IC): 2(1)

[8] Z. Varga, Sz. Berényi, B. Szokol, L. Őrfi, Gy. Kéri, I. Peták, A. Hoell, A. Bóta, A Closer Look at the Structure of Sterically Stabilized Liposomes: A Small-Angle X-ray Scattering Study, *J. Phys. Chem. B* (submitted)

exposure of phospholipids to excess water gives rise to lamellar structures (later become known as vesicles or liposomes). Until 1990s, the use of liposomes as therapeutic vectors was hampered by their toxicity, the lack of knowledge about their biochemical behaviour and the fact that pure phospholipid/water vesicles are rapidly removed from the blood circulation by the reticuloendothelial system. After the pioneering work of Papahadjopoulos and co-workers[9] the use of sterically stabilized (polyethylene-glycol-lipid conjugate (PEGylated lipid) containing) phospholipid vesicles (or liposomes) as drug delivery systems become widespread[10], since they have shown that the incorporation of PEG-conjugated phospholipids results in improved stability of the phosphatidylcholine vesicles as well as a longer circulation time in blood vessels.

Since the properties of liposomes depend strongly on the lipid composition, size, surface charge and the method of preparation, different drugs with different chemical characters require different formulations, which need a detailed knowledge about the structural and dynamical perturbation effects of each component.

The biophysical characterization has started with the discovery of these systems[11]. Studies applying scattering methods, optical and different electron spin resonance spectroscopic techniques as well as calorimetric investigations revealed many properties of these systems, on the other hand there are still ambiguous points on this field.

[9] Papahadjopoulos et al. PNAS 88 (1991) 11460
[10] Gregordias, Liposome Technology, Vol. 2, 2006
[11] Bouwstra et al. Chem. Phys. Lipids, 64 (1993) 83, Belsito et al. Biophys. Chem. 75 (1998) 33, Liu et al. Coll. Surf. A, 212 (2003) 227

3. Experiments and simulations

For the characterization of the changes in the layer structure of DPPC/water multilamellar vesicles, **small-angle and anomalous small-angle X-ray scattering (SAXS, ASAXS)** were used. In the case of a vesicular system, the periodically packed phospholipid bilayers cause Bragg-reflections in the scattering pattern in the position of $q_n=2\pi n/d$[12]. The shape of the curves carries information about the fluctuations of the individual layers which can be traced on the multilamellarity of the system, since multilamellarity reduces with the strengthening of the undulations. With model fitting procedures, both the bilayer electron density profile, and also the structure factor of the layer arrangement can be deduced from the scattering curve.

I used contrast variation by means of anomalous scattering for the determination of the distribution of the bromophenols and divalent metal ions along the normal of the bilayers. The basis of the method is the energy dependency of the scattering factors of the atoms, which are complex quantities and show a strong variation with the X-ray energy in the vicinity of the absorption edge of the atom or ion under consideration.

For the thermotropic characterization of phase transitions in the studied systems I used **differential scanning calorimetry (DSC)**. Since both the pre- and main transitions have a first order character, they manifest in endothermic peaks on the DSC curves. The morphology of the studied systems was visualized by **freeze fracture combined electron microscopy**.

I studied the interdigitated phase induced by the halogenated phenol compounds by means of **molecular dynamics (MD) simulations** at atomic details. I also used MD for the interpretation of the changes in the ASAXS curves, hence the localization of the metal ions, in the case of the DPPC/$CuCl_2$ system.

[12] The scattering variable (q) is defined by $q=(4\pi/\lambda)\sin(\theta)$, where λ is the wavelength of the X-ray beam, while 2θ is the scattering angle.

5. Novel scientific results

1.) I introduced the anomalous scattering formalism for the structural characterization of guest molecules and ions in macroscopically not-oriented lamellar systems [1]. In multilamellar vesicles of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocoline (DPPC) the localisation of Cu^{2+} ions and 2,4-dibromophenol molecules is described by the application of Stuhrmann-equations for the separation of the scattering contribution of the resonant atoms and ions. I estimated the effective ratio of DBP molecules inside the bilayers.

2.) Based on the small- and wide angle X-ray scattering results together with the simulations, I gave the atomic scale structural description of the interdigitated gel phase of DPPC bilayers induced by dihalogenated phenols [3, 9]. Using the quantitative analysis of the ASAXS curve I described the bounding ratio of the guest molecules to the phospholipids (based on the procedure described in [5]).

3.) The critical unbinding of the layers is described in DPPC/water MLVs upon the addition of $CuCl_2$ to the system in the millimolar range of concentration in the pretransition range [6]. I explain the observed effects with the enhanced electrostatic repulsion via the formation of the rippled gel phase.

4.) The utility of the lipid/divalent metal ion systems as reactors for nanoparticles is discussed by the determination of the distribution of the ions along the bilayer normal [2, 4] based on ASAXS measurements. On the example of Cu(II) ions, I have shown, that the metal ions localize in the headgroup region of the bilayers, given by a Gaussian distribution with a ~5 Å standard deviation.

5.) It was shown by means of SAXS, that the distribution of the hydrophilic chains of the polyethylene glycol (PEG) in sterically stabilized vesicles is asymmetric. Based on model fitting procedure the ratio of the PEG chains in the inner/outer leaflet is found to be 0.24:0.76.

6.) I have developed a computer program for desmearing of the small-angle scattering curves obtained from a laboratory X-ray apparatus. The program uses the direct and indirect methods described in the literature simultaneously, so the optimal method for the concrete curve can be selected.

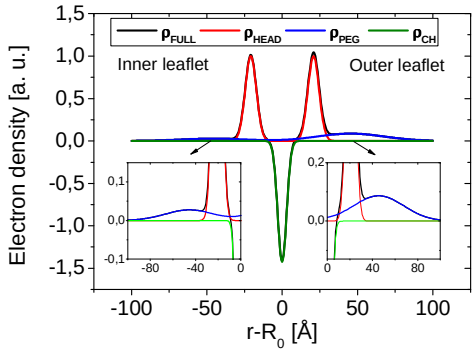

Fig. 4: The radial electron density profile of the studied sterically stabilized vesicles according to the model fits to the SAXS curves.

4.5. SAXS data evaluation

I have developed a computer program for the desmearing of small-angle scattering curves obtained from laboratory X-ray apparatus. It is based on the direct and indirect methods described in the literature. The novelty of this program is that it enables the desmearing with both processes at the same time. Since the choice of the method depends on the character of the scattering curve, the comparison can be made directly. (This program was used for the correction of the laboratory SAXS curves presented in this thesis, and those published in [7].)

4. Results and discussion

4.1. Anomalous scattering formalism of multilamellar vesicle

I introduced the anomalous scattering formalism for the structural characterization of guest molecules and ions in macroscopically not-oriented lamellar systems [1]. In general, the scattering amplitude of the system under consideration can be divided into two terms: A(q)=U(q)+[f'(E)+if"(E)]V(q) where V(q) (pure-resonant term) is the Fourier transform of the partial electron density of the resonant atoms or ions[13] which is bromine in the case of DPPC/DBP system and Cu^{2+} in the case of the DPPC/$CuCl_2$ system. By measuring the scattering curves at three different energies near to the X-ray absorption edge of the element under consideration, V(q) can be directly calculated. I used a Gaussian model for the description of the partial electron density of the resonant atoms. With this procedure I could separately analyze the scattering contribution of the bromine atoms in the case of the DPPC/DBP system as shown in Fig. 1. In the case of the DPPC/Cu^{2+} system simultaneous fitting of the ASAXS curves was performed, because the low amount of Cu^{2+} ions did not enable the separation of V(q).

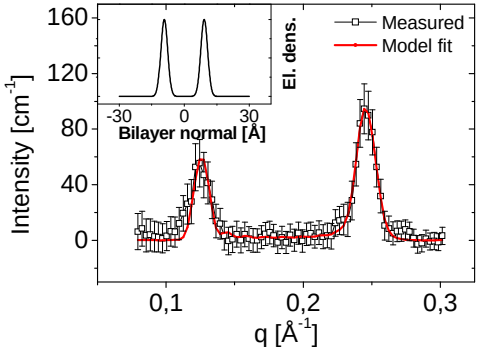

Fig. 1: The pure resonant curve of the DPPC/DBP system. The inset shows the distribution of the bromine atoms along the normal of the bilayer according to the model fitting.

[13] Here the atomic scattering factor of the resonant element is $f = f_0 + f'(E) + if"(E)$, where f_0 is equal to the atomic number, while f' and f" are the energy dependent anomalous scattering factors of the element.

4.2. Perturbation in the intrabilayer interactions: The structure of the interdigitated phase induced by dihalogenated phenols

Based on the small- and wide angle X-ray scattering results together with MD simulations, I gave the atomic scale structural description of the interdigitated gel phase of DPPC bilayers induced by DCP and DBP [3, 9]. The electron density profiles along the normal of the bilayers obtained from the SAXS measurements and from the simulations show good agreement as seen in Fig. 2.

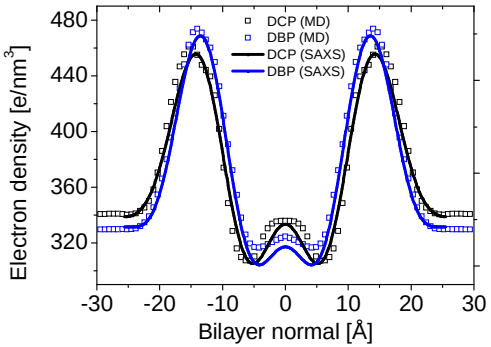

Fig. 2: Electron density profiles of the halogenated phenol/DPPC systems from MD (symbols) and from SAXS measurements (solid lines)

4.3. Perturbation in the interbilayer interactions: The effect of divalent metal ions

I have shown that critical unbinding of the layers takes place in DPPC/water MLVs upon the addition of $CuCl_2$ to the system in the millimolar range of concentration in the pretransition range [6]. SAXS measurements have shown that the loss of correlation between the lamellae can be observed in a MLV system upon the addition of the $CuCl_2$. The unbinding appears at lower concentration when the samples are heated above the pretransition temperature. A schematic phase diagram of the system is shown in Fig. 3. The proposed reason for the latter is the increased repulsive electrostatic interaction due to the appearance of the surface modulation in the ripple gel phase. The observed effects reveal a new aspect of the unbinding phenomenon since only the transition induced by the steric repulsion due to the layer fluctuations has been considered so far. It is shown in this thesis that the

unbinding can also be triggered by the change in the electrostatic interactions.

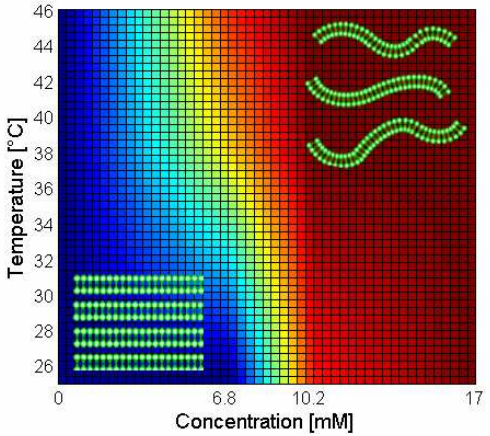

Fig. 3: The phase diagram constructed from the SAXS results of the DPPC/$CuCl_2$ system. The colorbar represents the ratio of the well ordered (blue) and unbound (red) bilayers.

I have discussed the utility of the lipid/divalent metal ion systems as reactors for the preparation of nanoparticles by the determination of the distribution of the ions along the bilayer normal [2, 4]. I have shown on the example of Cu^{2+} ions, that the metal ions localize in the headgroup region of the bilayers, given by a Gaussian distribution with a ~5 Å standard deviation.

4.4. The structural characterization of the sterically stabilized vesicles

It is shown by means of SAXS, that the distribution of the hydrophilic polyethylene glycol chains (PEG) in sterically stabilized vesicles is asymmetric, as shown in Fig. 4. The results indicate a more asymmetric localization than expected from theoretical predictions. However, those model calculations neglect the specific chemical character of the lipids. Based on my model fitting procedure the ratio of the PEG chains in the inner/outer leaflet is found to be 0.24:0.76.