Eur Biophys J (2006) 35: 281–286
DOI 10.1007/s00249-005-0019-5

BIOPHYSICS LETTER

Victoria Vitkova · Philippe Méléard · Tanja Pott
Isak Bivas

Alamethicin influence on the membrane bending elasticity

Received: 6 June 2005 / Revised: 22 August 2005 / Accepted: 25 August 2005 / Published online: 7 October 2005

Abstract We investigate the bending elasticity of lipid membranes with the increase of the alamethicin concentrations in the membrane via analysis of the thermally induced shape fluctuations of quasi-spherical giant vesicles. Our experimental results prove the strong influence of alamethicin molecules on the bending elasticity of diphytanoyl phosphatidylcholine and dilauroyl phosphatidylcholine membranes even in the range of very low peptide concentrations (less than 10^{-3} mol/mol in the membrane). The results presented in this work, testify to the peripheral orientation of alamethicin molecules at low peptide concentrations in the membrane for both types of lipid bilayers. An upper limit of the concentration of the peptide in the membrane is determined below which the system behaves as an ideal two-dimensional solution and the peptide molecules have a planar orientation in the membrane.

Introduction

The bending elasticity is an important property, governing in a great extent the membrane deformability and stability and thus playing a major role in various cellular processes such as endo- and exocytosis, cellular division, and fusion. The inclusion of peptide molecules in the matrix of a model lipid membrane and the investigation of its elastic properties gives useful information about the peptide role in the membrane stability. Although the first research on this topic was published in 1986 (Evans and Needham 1986), there are few works dedicated to this problem to date (Gerbeaud 1998; Hackl et al. 1997).

The present work focuses on the influence of the amphiphilic peptide, alamethicin, on the lipid bilayer bending elasticity. Alamethicin is a 20-amino acid antibiotic peptide produced by the fungus *Trichoderma viride* and is widely used as a model of a channel-forming peptide in the membrane. It is a peptide with a pronounced antibacterial activity that inhibits the growth of various organisms or causes their cytolysis even at very small alamethicin concentrations in the membrane.

Molecular models of alamethicin derived from X-ray or NMR data, reveal some amphipathicity in its secondary structure (Woolley and Wallace 1992). Folded as helix, alamethicin presents a polar face containing two amino acid (Gln^7 and Glu^{18}) residues, and a corresponding nonpolar part containing four (Val, Aib, Ala and Leu) residues (Sansom 1993). Sedimentation studies have demonstrated that alamethicin is monomeric in most common organic solvents but in aqueous solutions the peptide has a limited solubility and aggregates above concentrations of 10–20 μM (Archer et al. 1991; Rizzo et al. 1987). Taking into account this fact, we have worked with alamethicin concentration in the aqueous phase, not exceeding 7 μM.

During the last two decades the lipid-alamethicin interactions and the mechanism of the alamethicin insertion into lipid bilayers have been the object of detailed research in the literature (Cascio and Wallace 1988; He et al. 1996; Huang and Wu 1991; Olah and Huang 1988; Rizzo et al. 1987; Woolley and Wallace 1993; Wu et al. 1995). The binding of alamethicin to lipid vesicles was found to be lipid and ionic-strength dependent. As a function of the molar concentration, C_p, in the membrane and the type and concentration of the admixtures, dissolved in the water, alamethicin molecules were observed to switch between two states: at

V. Vitkova · I. Bivas (✉)
Liquid Crystal Laboratory, Institute of Solid State Physics,
Bulgarian Academy of Sciences, 72 Tzarigradsko Chaussee Blvd.,
1784 Sofia, Bulgaria
E-mail: bivas@issp.bas.bg

P. Méléard · T. Pott
CNRS UMR 6052"Synthèses et activations des biomolécules",
Equipe "Physico-chimie des systèmes organisés complexes",
Ecole Nationale Supérieure de Chimie de Rennes,
av. du Général Leclerc, 35700 Rennes Beaulieu, France

low C_p, the majority of the peptide molecules bind parallel to the membrane surface (the so-called S-state) (He et al. 1996; Olah and Huang 1988; Woolley and Wallace 1993); and above some critical insertion concentration, most of the peptide molecules insert perpendicularly into the membrane (the so-called I-state) (He et al. 1996; Huang and Wu 1991; Vogel 1987). Circular dichroism spectroscopy and X-ray diffraction have been used as experimental tools. In the present work, we will continue these studies using a completely different method. Via measurements of the membrane bending elasticity we will confirm that at low enough alamethicin concentrations in the bilayer the peptide molecules are in S-state in the membrane. We will determine an upper limit of the concentration of the peptide in the membrane, below which the system behaves as an ideal two-dimensional solution and the peptide molecules have a planar orientation in the membrane.

Materials and methods

Two types of membranes were studied, built-up of diphytanoyl phosphatidylcholine (3,7,11,15-tetramethylhexadecanoic, DPhPC, Avanti Polar Lipids Inc., USA) or built-up of dilauroyl phosphatidylcholine (DLPC, Sigma-Aldrich Chimie, France).

Alamethicin (Antibiotic U-22324) was purchased from Sigma Aldrich Chimie (France). Two independent experimental methods were applied in order to quantify the alamethicin content in our samples. The first of them is a spectroscopic one, the Lowry assay (Cascio and Wallace 1988; Lowry et al.1951), permitting the detection of the total quantity of peptide bonds in the solution. When measuring that quantity in our experimental solutions we used a calibration curve, obtained with melittin, whose concentration was determined independently by absorption spectrometry (Ladokhin et al. 2000). In the case of alamethicin we used a modification of the Lowry assay, adapted for more hydrophobic peptides. The modification included the addition of 1 wt% of sodium dodecyl sulfate (SDS, Sigma Aldrich Chimie) to the solution in order to facilitate the peptide dissolution in water. The signal from the alamethicin sample was corrected with a factor of 0.77, equal to the ratio between the numbers of the peptide bonds of alamethicin and melittine molecules.

In our experiments giant unilamellar vesicles, containing alamethicin in their membranes, were prepared by the electroformation method (Angelova and Dimitrov 1988; Gerbeaud 1998) in an appropriate buffer solution with pH 7.4 (Rizzo et al. 1987) (1 mM Tris-(hydroximethyl)-aminomethane, 1 mM Ethylenediaminetetraacetic acid (EDTA), NaOH, Sigma Aldrich Chimie). In all samples the lipid concentration in the experimental cell was in the range between 26.1 and 28.3 μM. In what follows, an average lipid concentration of 27 μM in the experimental cell is used, which leads to an uncertainty of our considerations smaller than 4% (much less than the typical experimental error of our measurements, 10%). In samples with alamethicin the peptide was co-dissolved in the lipid solution and in the buffer solution according to the partition coefficient between the membrane and the aqueous phase (*cf.* Results) (Gerbeaud 1998; Tamm 1994).

Only quasi-spherical fluctuating giant vesicles with no observable defects and diameters between 12 and 30 μm were chosen for the bending elasticity measurements. They were performed on lipid membranes, containing different alamethicin concentrations, via the analysis of the thermal shape fluctuations of giant unilamellar vesicles (Faucon et al. 1989). The studied vesicles were observed in phase contrast under an inverted microscope Axiovert 135 (Zeiss, Germany) with a dry objective (63×). The equatorial cross-sections of each observed vesicle with the focal plane of the objective were recorded via a CCD camera (C3582, Hamamatsu Photonics, Japan) directly connected to a video recorder (U-matic, Sony, Japan). For each studied vesicle a few hundred images (usually 400 and not less than 150), taking one image per second, were digitalized via the NIH image software Scion Image 1.63 (Scion Corp., USA). The vesicle contour was reconstructed for each of the registered images. In the general case it represents a deformed circumference. To analyse it, the deviations of the contour from the mean form, expressed as a function of the polar angle, were developed in a series of Legendre polynomials (Faucon et al 1989; Mitov et al. 1992) (some authors develop the deformations in a series of harmonic functions (Engelhardt et al. 1985; Hackl et al.1997). The time mean squares of the amplitudes for each fluctuation mode were determined from the ensemble of contours. From earlier theoretical treatments it is known that these time mean squares depend on: the radius R_0 of the vesicle; the number q of the mode; the bending elasticity k_c of the membrane; the effective normalised tension

$$\bar{\sigma} = \frac{\sigma R_0^2}{k_c} - 2c_0 R_0,$$

where σ is the tension of the membrane and c_0 is its spontaneous curvature; the time of integration of the video camera (40 ms). The bending elasticity of the membrane was determined via the software specially developed for this aim. Because of the dimension of the vesicles (their radii were of the order of 10 μm) and the characteristics of the set-up, only the modes with numbers $q \leq q_{max} = 15$ were used. The precision of the bending elasticities, calculated in this way, was of the order of 10%. Modifications of the method were proposed, permitting a more precise determination of the contour and increasing the value of q_{max} in the decomposition of the fluctuations of the equatorial cross-section. This is done by increasing the contrast of the contour via dissolution of sugars with the same molar concentrations but different refractive indexes of their aqueous solutions inside and outside of the vesicle (Pécréaux et al. 2004), by better interpolation of the intensities between the points corresponding to the dis-

crete positions of the pixels (Hackl et al.1997), etc. Despite these improvements the overall precision of the determination of the bending elasticity of the vesicle membrane is not yet substantially raised.

The different methods for measurement of the bending elasticity of the membrane—vesicle contour analysis (Mitov et al.1992), aspiration of vesicles in micropipettes (Evans and Rawicz 1990), tether formation (Bo and Waugh 1989), etc.—give not exactly the same values for it (Pécréaux et al. 2004). The explanation of these differences remains an open question, which is out of the scope of the present paper. We would like to note that one of the reasons for these differences could be found in the change of the bending elasticity of the lipid membranes when the aqueous phase contains low molecular weight carbohydrates (Vitkova et al. 2004).

The method, used by us for the preparation of giant vesicles, assures the binding of the alamethicin molecules to both monolayers composing the lipid bilayer. This process is a statistical one and it was possible the surface densities of the inclusion to be not exactly the same for the two monolayers. The differences between these densities are expected to be much less than the densities themselves (higher differences between the densities are statistically improbable). The surface densities of the lipid in the two monolayers could be slightly different, too. These differences create an effective spontaneous curvature of the membrane. As it has been shown (Bivas and Méléard 2003), this spontaneous curvature influences the effective normalised surface tension $\bar{\sigma}$ of the membrane and does not change the bending elasticity k_c.

Results

We obtained the following values for the bending elastic moduli of the pure DPhPC and DLPC membranes in the presence of the chosen buffer (see Materials and Methods):

$$\text{DPhPC bilayers}: k_c = (1.17 \pm 0.10) \cdot 10^{-19}\text{J}$$

$$\text{DLPC bilayers}: k_c = (0.96 \pm 0.08) \cdot 10^{-19}\text{J}$$

Because of the amphiphilic nature of alamethicin, its water solubility cannot be neglected due to the low lipid concentration in the experimental cell (27 μM). Therefore, when forming giant vesicles in the presence of alamethicin the quantity of the peptide, dissolved in the water, must be taken into account.

In the literature the partition constant for the transfer of the peptide from the aqueous solution into the membrane (hydrophobic core or interface) is defined by (Tamm 1994):

$$K_p = \frac{C_p^b}{C_p^f C_l^{tot}} \text{ l/mol} \quad (1)$$

where C_p^b is the bound peptide molar concentration, C_p^f is the free peptide molar concentration and C_l^{tot} is the total lipid molar concentration, all of them referring to the volume of the sample. This relation is relevant for small enough peptide concentrations in the membrane and in the water solution. That is why we apply it only for the analysis of our results at small alamethicin concentrations in the bilayer.

When evaluating the quantities of the peptide, distributed between the membranes, studied by us, and the aqueous phase, the following value of the partition coefficient K_p was used:

$$K_p = 1.3 \times 10^3 \text{ l/mol} \quad (2)$$

It was chosen to be equal to the experimentally determined value of this coefficient for the DOPC-alamethicin-water and egg-yolk phosphatidylcholine-alamethicin-water systems (Archer et al. 1991; Rizzo et al. 1987; Schwartz et al.1986) (the partition coefficients of these two systems are identical).

Taking into account the mean total concentration of the lipid in our experimental cells ($C_l^{tot} = 27$ μM), from Eqs 1 and 2 we obtain:

$$\frac{C_p^b}{C_p^f} \approx 0.035 \quad (3)$$

The total peptide concentration in the sample, C_l^{tot}, is a sum of the bound peptide concentration and the free peptide concentration:

$$C_p^{tot} = C_p^b + C_p^f \quad (4)$$

Therefore, only less than 4% of the peptide in the experimental cell was bound to the membrane and this fact was taken into account in the sample preparation in order to guarantee the necessary peptide concentration in the bilayer.

Our experimental results for the bending elasticity behaviour for both types of lipid membranes as a function of the ratio C_p^{tot}/C_l^{tot} for a fixed lipid molar concentration in the experimental cell are presented in Fig. 1.

The bending elasticity decrease of DPhPC and DLPC membranes with increasing the alamethicin concentration has a behaviour similar to the results, previously obtained by other authors for the elasticity of a membrane containing amphiphilic additives (Gerbeaud 1998; Hackl et al. 1997).

For low enough concentrations of the additive the system behaves as an ideal two-dimensional solution and the dependence of the bending elasticity on the concentration C_p of the peptide in the membrane, $k_c(C_p)$, can be presented as (Bivas and Méléard 2003; Kralj-Iglic et al. 1996; Leibler 1986; Seifert 1993):

$$k_c(C_p) = A \cdot (1 - B \cdot C_p) \quad (5)$$

where the coefficient A has the physical meaning of the bending elastic modulus of the pure (without any additive) lipid membrane.

The analysis of our experimental data for the bending elasticity behaviour at low peptide concentration in the

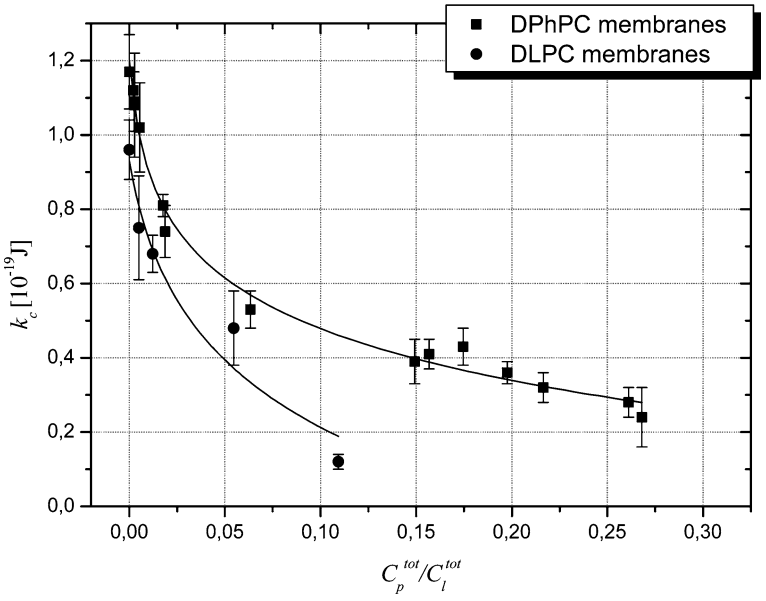

Fig. 1 Evolution of the bending elastic modulus of DPhPC (*filled square*) and DLPC (*filled circle*) membranes as a function of the ratio C_p^{tot}/C_l^{tot} of the total volume concentration C_p^{tot} of the peptide and C_l^{tot} of the lipid in the sample (at $C_l^{tot} = 27$ μM)

bilayer reveals a steep linear decrease (Eq. 5), when increasing the molar alamethicin concentration in the membrane. The best linear fits of the ensemble of the experimental points for each of the lipid bilayers are presented on Fig. 2. The bending elasticity evolution for both types of lipid membranes is similar. The parameters of the linear fits are displayed on the relevant graphics and are as follows:

DPhPC membranes + alamethicin :

$$A \approx 1.14 \pm 0.03; B \approx 467 \pm 70; \quad (6a)$$

DLPC membranes + alamethicin :

$$A \approx 0.90 \pm 0.06; B \approx 571 \pm 143; \quad (6b)$$

In the DPhPC membranes C_p was in the concentration range $(0 \div 6.7 \times 10^{-4})$ mol/mol, while in DLPC membranes this range was $(0 \div 7.6 \times 10^{-4})$ mol/mol.

Discussion

Bruckner et al. (2000) have studied the influence of toluene inclusion on the bending elasticity of lipid membranes. They considered the observed decrease of this quantity as a manifestation of the decrease of the acyl–acyl interactions, caused by the toluene molecules, and as one of the consequences of the lateral diffusion of these molecules and their exchange between the membrane and the surrounding water. These authors assumed that the presence of inclusion in the bilayer influences the bending elasticity if the lateral diffusion of these guest molecules is fast enough. It has been shown (Bivas and Méléard 2003) that the change of the bending constant k_c is really related to the lateral redistribution of the molecules, incorporated in the lipid matrix, but it depends mainly on their "geometric" characteristics—their ability to induce spontaneous curvature in the bilayer and their mean area per molecule. The modification of k_c does not depend on the value of the coefficient of the lateral diffusion of the inclusion in the membrane.

As it can be seen from our experimental results (Fig. 1), the bending elasticity sharply decreases in the region of low peptide concentrations, while for higher peptide concentrations the rate of k_c decrease is much smaller. The alamethicin molecule can be considered approximately as a slightly bent cylinder (Fox and Richards 1982. At low enough concentration the alameticin molecules are in a monomeric form in the membrane and form an ideal two-dimensional solution. In (Bivas and Méléard 2003) it has been shown that for the case of additives with low enough concentration, having symmetrical molecules, symmetrically inserted in lipid bilayer, the coefficient B has a modulus |B| of the order of 1. Consequently, if the peptide were symmetrically inserted in the membrane, piercing it, the coefficient B should have a modulus of the order of 1, while our experimental results show that it is of the order of 500 for both lipids used by us. Therefore, at low concentrations C_p the alameticin molecules in these bilayers have peripheral orientations, in excellent agreement with the results of Wu et al. (1995) for DPhPC bilayers.

On the basis of our experimental results we were able to estimate the upper limit of the alamethicin concentration in the membrane below which the peptide molecules form an ideal two-dimensional solution in the bilayer and the dependence of k_c on C_p is given by a function of the type in Eq. 5. This estimation is $1/B$, which for the case of DPhPC membranes is $\sim 2 \times 10^{-3}$ mol/mol, and for DLPC membranes is $\sim 1.8 \times 10^{-3}$ mol/mol. Above these concentrations the peptide molecules no longer behave as an ideal two-dimensional solution in the membrane, otherwise the

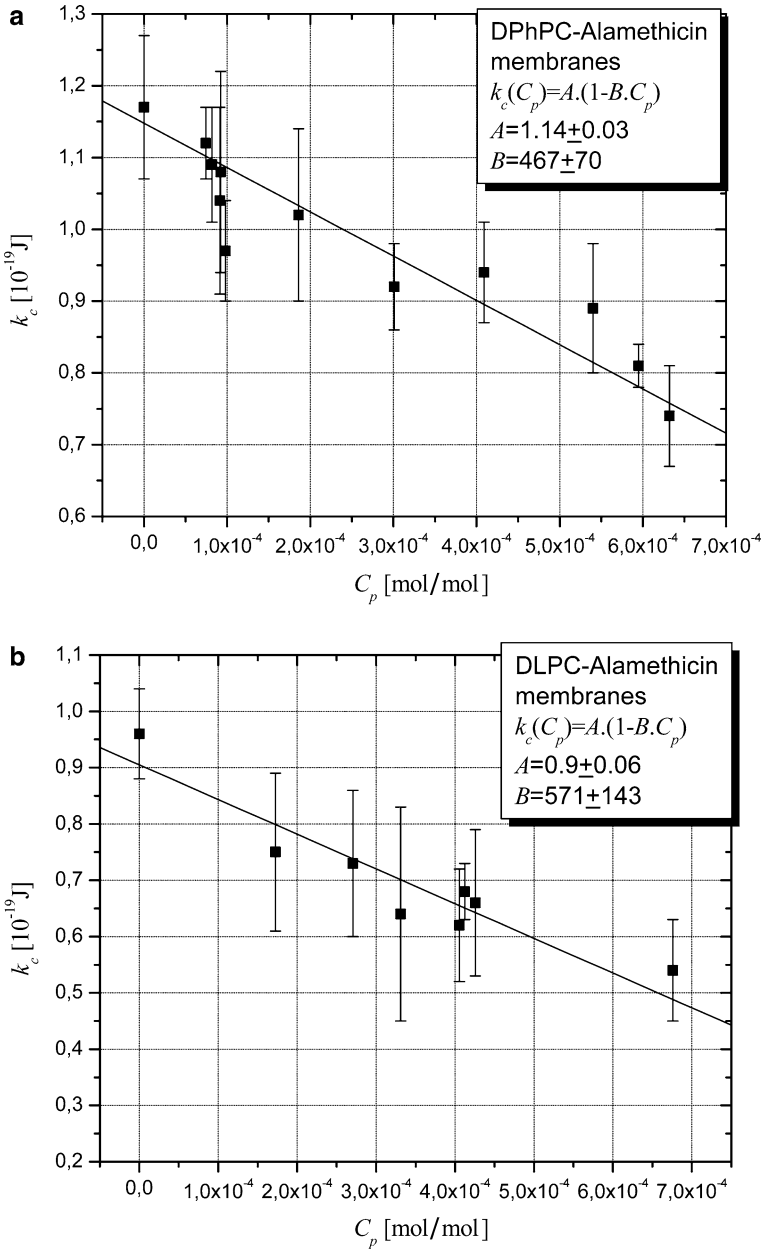

Fig. 2 Bending elasticity, k_c, measured by us at different relative peptide concentrations, C_p, in the membrane and calculated values of the parameters of the best linear fit $K_c\ (C_p) = A\ (1-B{\cdot}C_p)$; **a** DPhPC + alamethicin; **b** DLPC + alamethicin

bending elasticity of the membrane should become negative and the membrane should be destabilised, while the experimental data show that the membranes are stable even when they contain alamethicin with concentration much higher than this limit.

He et al. (1996) showed that for DPhPC bilayers the critical concentration of insertion is about 2.5×10^{-2} mol/mol and that for concentrations higher than 6.7×10^{-2} mol/mol alamethicin molecules completely insert into the membrane and form well-defined pores. Woolley and Wallace (1993), studying DOPC-alamethicin membranes, have detected three spectroscopically distinct states, one corresponding to peptide in the aqueous phase and the other two corresponding to two differently bound to the membrane peptides. For the DPhPC-alamethicin system, Huang and Wu (1991) discovered a phase-transitionlike behaviour between a state in which the majority of alamethicin molecules are in S-state, and a state in which the majority of alamethicin molecules are in I-state at $C_p > 7\times10^{-3}$ mol/mol. According to Olah and Huang (1988) alamethicin molecules with $C_p = 12.5\times10^{-3}$ mol/mol in DLPC multibilayers are preferably perpendicular to the bilayer membrane.

The comparison with the results, presented in this paper, shows that one more concentration interval exists, approximately between 2×10^{-3} and 7×10^{-3} mol/mol, in which the alamethicin molecules no longer behave as an ideal two-dimensional solution but preserve their S-state in the membrane.

Conclusions

The experimental results presented in this work prove the strong influence of alamethicin molecules on the bending elasticity of different lipid membranes even in the range of very low peptide concentrations (less than 10^{-3} mol/mol in the membrane). Our experimental results show that at low alamethicin concentrations the peptide molecules are oriented peripherally on the membrane. A value of the peptide concentration in the membrane was estimated below which the peptide molecules behave as an ideal two-dimensional solution and possess peripheral orientation on the bilayer.

Acknowledgements The authors wish to thank Drs. Jean-Claude Talbot, Thierry Biben, and Marin D. Mitov for the fruitful discussions and collaboration. The contribution of the Bulgarian National Science Foundation (contract MUF-1203/02) is acknowledged.

References

Angelova M, Dimitrov DS (1988) A mechanism of liposome formation. Progr Colloid Polym Sci 76:59–67

Archer SJ, Ellena JF, Cafiso DS (1991) Dynamics and aggregation of the peptide ion channel alamethicin. Biophys J 60:389–398

Bivas I, Méléard P (2003) Bending elasticity of a lipid bilayer containing an additive. Phys Rev E 67:012901

Bo L, Waugh RE (1989) Determination of bilayer membrane bending stiffness by tether formation from giant, thin-walled vesicles. Biophys J 55:509–517

Cascio M, Wallace BA (1988) Conformation of alamethicin in phospholipid vesicles: implications for insertion models. Proteins Struct Funct Genet 4:89–98

Engelhardt H, Duwe H-P, Sackmann E (1985) Bilayer bending elasticity measured by Fourier analysis of thermally excited surface undulations of flaccid vesicles. J Physique Lett 46:L395–L400

Evans E, Needham D (1986) Giant vesicle bilayers composed of mixtures of lipids, cholesterol and polypeptides. Faraday Discuss Chem Soc USA 81:267–280

Evans E, Rawicz W (1990) Entropy-driven tension and bending elasticity in condensed-fluid membranes. Phys Rev Lett 64:2094–2097

Faucon JF, Mitov MD, Méléard P, Bivas I, Bothorel P (1989) Bending elasticity and thermal fluctuations of lipid membranes. Theoretical and experimental requirements. J Physique 50:2389–2414

Fox RO Jr, Richards FM (1982) A voltage-gated ion channel inferred from the crystal structure of alamethicin at 1.5 A resolution. Nature 300:325–330

Gerbeaud C (1998) Effet de l'insertion de protéines et de peptides membranaires sur les propriétés mécaniques et les changements morphologiques de vésicules géantes. PhD Thesis, University of Bordeaux I

Hackl W, Seifert U, Sackmann E (1997) Effects of fully and partially solubilized amphiphiles on bilayer bending stiffness and temperature dependence of the effective tension of giant vesicles. J Phys France II 7:1141–1157

He K, Ludtke SJ, Heller WT, Huang HW (1996) Mechanism of Alamethicin Insertion into Lipid Bilayers. Biophys J 71:2669–2679

Huang HW, Wu Y (1991) Lipid-alamethicin interactions influence alamethicin orientation. Biophys J 60:1079–1087

Kralj-Iglic V, Svetina S, Zeks B (1996) Shapes of bilayer vesicles with membrane embedded molecules. Eur Biophys J 24:311–321

Ladokhin AS, Jayasinghe S., White SH (2000) How to measure and analyse tryptophan fluorescence in membrane properly and why bother. Anal Biochem 285:235–245

Leibler S (1986) Curvature instability in membrane. J Physique 47:507–516

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Mitov MD, Faucon JF, Méléard P, Bothorel P (1992) Thermal fluctuations of membranes. In: Gokel GW (ed) Advances in supramolecular chemistry, vol 2. JAI Press Inc, pp 93–139

Olah GA, Huang HW (1988) Circular dichroism of oriented alpha-helices. I. Proof of the exciton theory. J Chem Phys 89:2531–2538

Pécréaux J, Döbereiner H-G, Prost J, Joanny J-F, Bassereau P (2004) Refined contour analysis of giant unilamellar vesicles. Eur Phys J E 13:277–290

Rizzo V, Stankowski S, Schwartz G (1987) Alamethicin incorporation in lipid bilayers: a thermodynamic study. Biochemistry 26:2751–2759

Sansom MS (1993) Alamethicin and related peptaibols—model ion channels. Eur Biophys J 22:105–124

Schwartz G, Stankowski S, Rizzo V (1986) Thermodynamic analysis of incorporation and aggregation in a membrane: application to the pore-forming peptide alamethicin. Biochim Biophys Acta 861:141–151

Seifert U (1993) Curvature-induced lateral phase segregation in two-component vesicles. Phys Rev Lett 70:1335–1338

Tamm LK (1994) Physical studies of peptide-bilayer interactions. In: White SH (ed) Membrane protein structure: experimental approaches. Oxford University Press, New York

Vitkova V, Genova J, Mitov MD, Bivas I (2004) Mechanical properties of lipid mono- and bilayers in the presence of small carbohydrates in the aqueous phase. C R Acad Bulg Sci 57(6):55–60

Vogel H (1987) Comparison of the conformation and orientation of alamethicin and melittin in lipid membranes. Biochemistry 26:4562–4572

Woolley GA, Wallace BA (1992) Model ion channels: gramicidin and alamethicin. J Membrane Biol 129:109–136

Woolley GA, Wallace BA (1993) Temperature dependence of the interaction of alamethicin helices in membranes. Biochemstry 32:9819–9825

Wu Y, He K, Ludtke SJ, Huang HW (1995) X-Ray diffraction study of lipid bilayer membranes interacting with amphiphilic helical peptides: diphytanoyl phosphatidylcholine with alamethicin at low concentrations. Biophys J 68:2361–2369