

**BENDING ELASTICITY OF PHOSPHOLIPID BILAYERS
CONTAINING AN AMPHIPATHIC PEPTIDE WITH LOW
MAMMALIAN CYTOTOXICITY**

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Abstract

Peptide mimetics imitate natural peptides' structure but they could be specifically designed to be more selective concerning their toxicity to mammalian cells. In most cases this specificity is due to their ability to form α -helix in amphipathic environment. In addition, the specific activity depends on the ability of final structure to penetrate cell membrane. Being responsible for the cell integrity and compartmentalization, biomembranes also play a major role in cellular processes, in which the membrane deformations are important. In the present study we probe peptide-membrane interactions for a shortened amino acid sequence KLAKLAK-NH₂ of an antimicrobial peptide with apoptotic effect. The bending rigidity of model lipid bilayers is measured by flicker spectroscopy of quasispherical unilamellar vesicles monitored and analyzed in phase contrast light microscopy. At high peptide concentrations $\sim 30 \mu\text{mol/L}$ and peptide-to-lipid total molar ratios ~ 0.90 bilayer stacking formation is observed. A reduction of the bending constant is reported at peptide-to-lipid total molar ratio ~ 0.80 . The membrane softening indicates peripheral peptide orientation at the lipid bilayer, which is considered a prerequisite for channel formation. Based on KLAKLAK-NH₂ effect on the membrane bending elasticity we provide an evaluation of the peptide partition coefficient characterizing

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its affinity to POPC bilayers. The acquired results might be helpful in efforts to further tailor the pharmacokinetic properties of antimicrobial peptides in combination with strengthened stability towards enzymatic degradation.

Key words: lipid vesicles, GUV, thermal fluctuations, KLAKLAK-NH₂, anticancer peptides, antimicrobial activity

Introduction. The rapid rise in antibiotic resistance defines a vision toward a post-antibiotic era, in which previous effective therapeutic strategies would be no longer relevant establishing the need for new antibacterial drugs. Antimicrobial peptides (AMPs) are small peptides, which constitute an important part of the innate immune system of various organisms. AMPs are produced by granulocytes, macrophages and most epithelial cells in humans and have antimicrobial, antifungal, antiviral and even antiprotozoal activity [1]. In addition, due to similar mechanism of cell penetration many AMPs show anticancer properties [2]. Hundreds of naturally occurring and thousands of synthetically strained AMPs exhibit potential of high effectiveness against various pathogens [3]. AMPs are being intensively studied for their antibacterial activity [4] as well as for the broad-spectrum cytotoxicity to cancer cells [5]. The strong antimicrobial properties combined with low cytotoxicity towards mammalian cells make AMPs suitable candidates for the treatment of bacterial infections, to which traditional antibiotics have no or show weak effect [6].

The primary structure of (KLAKLAK)₂-NH₂ peptide, whose anticancer properties have been reported in relation to mitochondrial membrane-mediated apoptosis [5], represents a 14-residue sequence containing repeated lysine, leucine and alanine residues. (KLAKLAK)_n-NH₂ peptides ($n = 1, 2, 3$) express selective cytotoxicity depending on their length and structure [7]. The introduction of non-proteinogenic amino acids into the peptide structure serves as a successful tool to modify the peptide main activity [8]. As reported previously β -Alanine containing shortened analogues obtained by solid phase synthesis, improve the pharmacodynamic properties and bioavailability of the peptide through increasing its stability against enzymatic degradation [9]. The shortest KLAKLAK-NH₂ analogue has shown the highest selectivity with pronounced lytic effect on gram-negative bacteria combined with low mammalian cytotoxicity tested on mouse fibroblast and human erythrocytes.

The physical state of membranes (composition, structure, elasticity, surface charge, etc.) determines the deformability of the lipid bilayer, which is substantial in the cases, when the activity of the guest molecule is governed by its conformational changes. The ability of lipid bilayers to deform easily in hydrodynamic flows is a prerequisite for the successful use of functionalized liposomes in pharmacology for transporting medicinal substances into the bloodstream of the human organism. Hence, the investigation of all factors possibly altering the membrane deformability underlies liposome-based developments in pharmacology and

biomedicine. Giant unilamellar vesicles (GUVs) with diameters at the micrometer scale similar to the typical cell sizes (5–100 μm), are being successfully exploited to model the lipid matrix of biological membranes [10]. GUVs allow controlling numerous parameters as membrane composition, viscosity and concentrations of solutes in the aqueous environment, and thus providing the opportunity for direct visualization of particular membrane-related phenomena. In what follows we study phosphatidylcholine GUVs to probe the morphology, stability and bending rigidity of lipid membranes containing KLAKLAK-NH₂ peptide, which is known to exhibit antimicrobial properties and low cytotoxicity towards human erythrocytes [7]. Our previous study on KLAKLAK-NH₂ molecule revealed very low cytotoxicity of this peptide against model BALB/3T3 cells but moderate antibacterial activity against *Escherichia coli* K12 407 strain at 20 μM [9]. Taking into account these findings, herein, we investigate the peptide interactions with model lipid bilayers in order to probe the AMP's physicochemical effect on membrane morphology and mechanics.

Materials and methods. All chemicals used for vesicle preparation were analytical grade and used as purchased without further purification. The phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, C₄₂H₈₂NO₈P, molar mass 760.08 g/mol), purchased from Avanti Polar Lipids (Alabama, USA), was used to produce GUVs. The organic solvents methanol and chloroform were supplied by Sigma-Aldrich (Germany). For the preparation of all aqueous solutions bidistilled water from a quartz distiller, filtered through 0.2 μm sterile syringe filters (Corning Incorporated, Germany) was used. KLAKLAK-NH₂ (C₃₆H₇₁N₁₁O₇, molar mass 770.02 g/mol), whose primary structure was obtained by solid phase synthesis (SPPS) is described in detail in [9].

GUVs preparation. POPC and POPC/KLAKLAK-NH₂ GUVs were produced in bidistilled water using the electroformation method [11]. Vesicle suspensions were prepared on two indium tin oxide (ITO)-coated glass plates, separated by a polydimethylsiloxane (PDMS, Dow Corning, Germany) spacer with thickness of 5 mm. The first step consisted in spreading out on the ITO-coated side of each electrode 50 μg of POPC or POPC/KLAKLAK-NH₂ solution with total concentration of 1 g/L in chloroform-methanol solvent (9:1 volume parts). The complete evaporation of the organic solvents was ensured under vacuum. Prior to electroformation the electroformation chamber was completely filled with bidistilled water. Quasispherical unilamellar vesicles were obtained after the application of AC electric field (0.4 V/mm peak-to-peak amplitude, 10 Hz) to the electroformation cell for a couple of hours, according to previously established protocols [12].

Observations of the morphology of POPC/KLAKLAK-NH₂ GUVs. The morphology of POPC GUVs in the presence of KLAKLAK-NH₂ at concentrations up to 28.7 $\mu\text{mol/L}$ in the electroformation cell was monitored by a Zeiss Axiovert 100 inverted microscope (Zeiss, Germany) operating in phase contrast and equipped by a long working distance dry objective ($\times 63$). The recording

was carried out by a CCD camera (C3582, Hamamatsu Photonics, Japan) connected to a frame grabber (DT3155, Data Translation, USA, 768×576 8-bit pixels, $0.172 \mu\text{m}/\text{pixel}$) as a part of a Linux-operating station for digitization and analysis.

Bending elasticity measurements. In its liquid state the lipid bilayer is characterized by two mechanical parameters, related to its resistance to stretching and bending. The membrane tension $\sigma = k_s \Delta A/A_0$ depends on the relative area change $\Delta A/A_0$ of the bilayer with the coefficient k_s standing for the membrane stretching modulus. In the experiments conducted here on symmetrical membranes facing identical aqueous medium on both sides of the bilayer, the bending modulus k_c is defined via the density of the bending energy F_c and the curvature of the lipid membrane resulting from its bending [13], namely $F_c = k_c(c_1 + c_2)^2/2 + k_G c_1 c_2$. In the latter k_G denotes the saddle splay (Gaussian) modulus; and $(c_1 + c_2)$ and $c_1 c_2$ stand for the total curvature and the Gaussian curvature, respectively.

The membrane bending modulus was measured by thermal shape fluctuation analysis (TSFA) performed on quasispherical vesicles [14,15]. The method is based on Legendre analysis of the time-averaged angular autocorrelation function of the vesicle radius, $\xi(\gamma)$ as discussed in detail in [14]. It has been shown that the amplitudes $B_n(k_c, \bar{\sigma})$ of the n -th mode in the decomposition of $\xi(\gamma)$ with respect to Legendre polynomials, depend on the bending constant k_c and the reduced membrane tension $\bar{\sigma} = \sigma R_0^2/k_c$, where R_0 stands for the vesicle radius [14].

The stroboscopic illumination of the observed vesicles, synchronized with the camera, permitted fast modes of vesicle fluctuations to be recorded and processed [15]. All measurements of the membrane bending rigidity were performed at $37 \pm 0.1^\circ\text{C}$. The temperature control was effectuated by a two-channel system operating both with the microscope plate and with the oil-immersed objective ($100\times$, numerical aperture 1.25; $0.106 \mu\text{m}/\text{pixel}$). TSFA was performed on visibly fluctuating quasispherical vesicles with diameters larger than $10 \mu\text{m}$. For every vesicle studied several hundred images were captured once per second for calculation of the membrane bending constant and tension as described in detail in [15]. The reported values of the membrane bending modulus were obtained over an ensemble of not less than six GUVs collected from at least two different preparations.

Results and discussion. According to the GUVs' preparation protocol applied here the total lipid concentrations C_L^{tot} in the electroformation cell ranged from 33 up to $37 \mu\text{M}$. For each membrane composition studied, the vesicle morphology was monitored by means of phase contrast microscopy. Electroformations produce a high yield of quasispherical GUVs with single-component POPC bilayers as shown in Fig. 1. In the control samples a large number of POPC vesicles met all qualification criteria for TSFA [15], thus providing the accumulation of bending elasticity data (Table 1). Upon addition of KLAKLAK-NH₂ at total

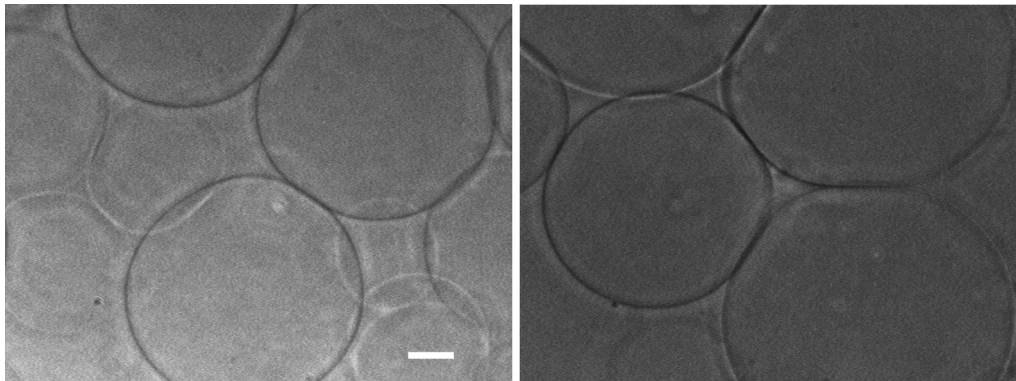


Fig. 1. Phase-contrast snapshots of two different POPC vesicle preparations in the electroformation cell; the bar corresponds to 10 μm

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Bending elasticity modulus of POPC and POPC/KLAKLAK-NH₂ membranes; measurements performed in bidistilled water, pH 5.6, at 37 °C; C_p^{tot} and C_L^{tot} stand for the total molar concentrations of the peptide and the lipid, respectively; GF corresponds to the goodness of fit (χ^2 test); N gives the number of GUVs in the batch

Sample	C_p^{tot} , μM	C_L^{tot} , μM	$C_p^{\text{tot}}/C_L^{\text{tot}}$	k_c , 10^{-19} J	GF	N
POPC (control)	0	37	0	1.29 ± 0.03	0.59	17
POPC/KLAKLAK-NH ₂	20	36.1	0.554	1.35 ± 0.08	0.50	12
POPC/KLAKLAK-NH ₂	26.7	33.6	0.795	1.12 ± 0.11	0.77	6

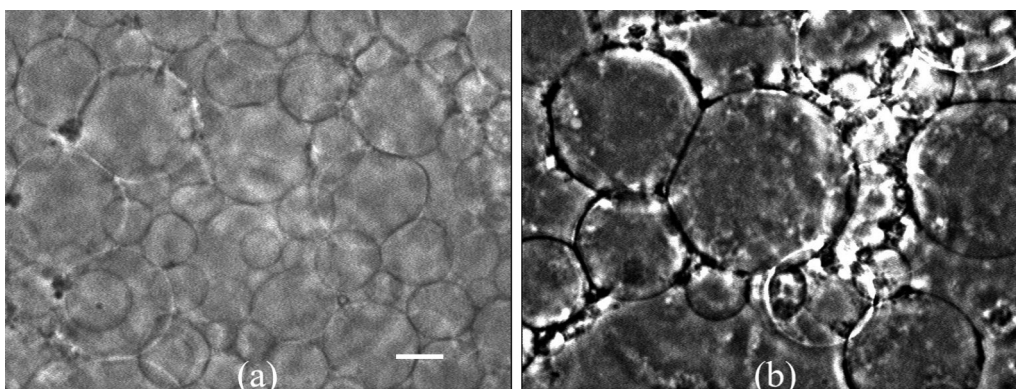


Fig. 2. Phase-contrast micrographs of (a) GUV electroformation at 26.7 μM KLAKLAK-NH₂ and 33.6 μM POPC; and (b) at 28.7 μM KLAKLAK-NH₂ and 33 μM POPC in the electroformation cell; the bar corresponds to 10 μm

peptide concentration $C_p^{\text{tot}} > 20 \mu\text{M}$, GUV suspensions were characterized by smaller vesicles, some of them exhibiting membrane defects, which hindered the contour determination (Fig. 2a).

Peptide-lipid aggregates and foam-like membrane structures were formed at high KLAKLAK-NH₂ content in the sample. The experiments with peptide concentrations higher than $27 \mu\text{M}$, corresponding to $C_p^{\text{tot}}/C_L^{\text{tot}} \sim 0.90$, did not produce quasispherical GUVs appropriate for TSFA (Fig. 2b). The resulting vesicles are non-fluctuating and therefore, not suitable for determination of the membrane bending modulus by TSFA. Furthermore, the vesicle morphology provides evidence that at $C_p^{\text{tot}}/C_L^{\text{tot}} \sim 0.90$ the peptide strongly interacts with the lipid bilayer by promoting the membrane stacking (Fig. 2b). Phosphatidylcholine membranes are characterized by apparent negative charge in water at neutral pH [16] resulting from the arrangement of water dipoles in the lipid head neighbourhood. Taking into account the presence of two positively charged lysine residues under physiological conditions, the origins of the aggregate formation, shown in Fig. 2b, are probably related to the electrostatic interactions between KLAKLAK-NH₂ molecules and POPC bilayers.

The bending rigidity of lipid bilayers was probed at two KLAKLAK-NH₂ concentrations near the lowest concentration at which the peptide expresses cytotoxic and antiproliferative activity in vitro as reported previously [9]. The membrane bending modulus k_c was measured by thermal shape fluctuation analysis of quasispherical GUVs. At nearly 0.80 peptide-to-lipid molar ratio in the bulk sample we measure membrane softening, characterized by a $\sim 15\%$ -reduction of the bending modulus compared to its value of the control POPC bilayer (Fig. 3).

The results presented unravel the effect of KLAKLAK-NH₂ peptide on the lipid bilayer's deformability, which is closely related to membrane-mediated processes in the biological cell. The primary structure of the studied peptide represents a doubled sequence of three amino acids. The presence of the charged lysine and the non-polar leucine and alanine determines the amphiphilic nature of the peptide, thus suggesting partitioning between the membrane and the aqueous surrounding [17]. For small enough (bound) peptide concentrations in the membrane, C_p^b , the partition coefficient for the transfer of an amphiphilic peptide from the aqueous solution to the hydrophobic core or interface of the lipid bilayer is given by $K_p = C_p^b/(C_p^f C_L^{\text{tot}})$, where $C_p^f = C_p^{\text{tot}} - C_p^b$ stands for the (free, unbound) peptide concentration in the bulk phase [18]. To the best of our knowledge, literature data about KLAKLAK-NH₂ coefficient of partitioning between the bilayer and the bulk phase still lack. Therefore, all results are referred to the peptide-to-lipid molar ratio $C_p^{\text{tot}}/C_L^{\text{tot}}$ between the bulk concentrations of two substances. The reduction of the bending modulus at $C_p^{\text{tot}}/C_L^{\text{tot}} \sim 0.80$ implies surface orientation of the peptide at the membrane as previously shown [19,20]. In our previous study [20] we have demonstrated that less than $C_p^b/C_L^{\text{tot}} = 10^{-3} \text{ mol/mol}$ of the AMP alamethicin in the membrane strongly influences the bilayer bending elasticity. Based on

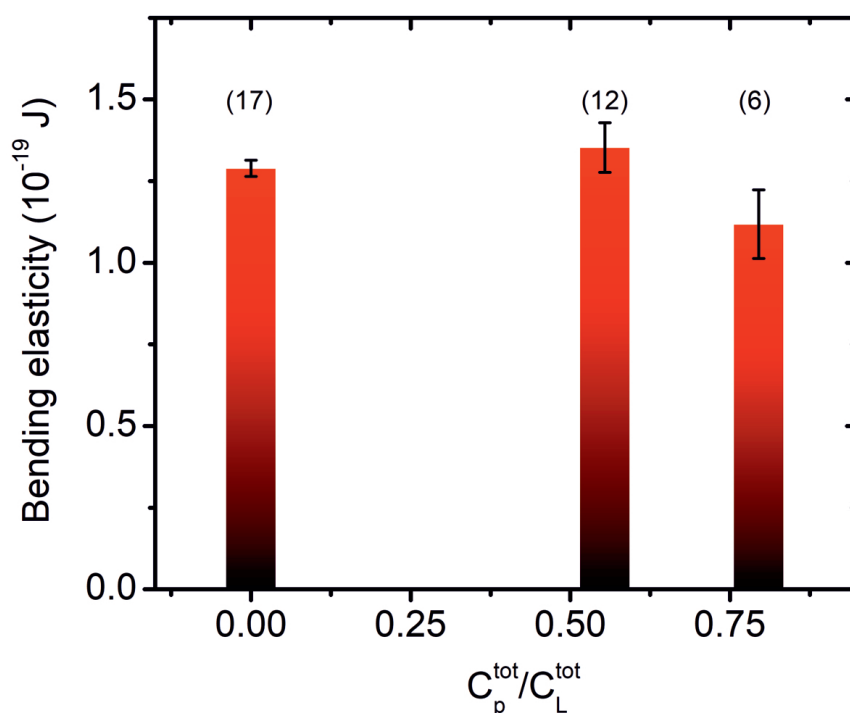


Fig. 3. TSFA results about the bending modulus of POPC membranes upon addition of KLAKLAK-NH₂; C_p^{tot} and C_L^{tot} denote the total molar concentrations of the peptide and the lipid, respectively

these findings and the results reported above, and taking into account the differences in molecular structure and properties of alamethicin and KLAKLAK-NH₂, we present a rough estimation of KLAKLAK-NH₂ partition coefficient K_p . Upon the assumption that the sensibility of the TSFA method to detect the presence of AMPs peripherally oriented at the membrane surface corresponds to the above C_p^b/C_L^{tot} threshold, for KLAKLAK-NH₂ we determine $K_p = 0.07 \times 10^3$ L/mol. This value is ~ 20 times lower than K_p for alamethicin [18], which is much more hydrophobic than KLAKLAK-NH₂ and therefore, readily transferred from the bulk phase into the membrane [17].

The results reported above are expected to valorise the interest to the in-depth analysis of the membrane bending elasticity as a biomarker of peptides' physicochemical effect on lipid membranes. The investigation of the lipid bilayers' structural and mechanical properties provides important information for the determination of various structural and functional parameters of membranes. The knowledge acquired on the interactions of the antimicrobial peptide KLAKLAK-NH₂ with phosphatidylcholine bilayers represents also an important step in the development of various applications of lipid membrane systems such as in pharmacology and biomedicine.

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