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EQUINATOXIN II INTERACTIONS WITH ZWITTERIONIC PHOSPHOLIPIDS AT pH 3.0: CALORIMETRIC AND SPECTROSCOPIC STUDY[#]

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Abstract: The nature of the interaction of Equinatoxin II (EqTxII) with small zwitterionic unilamellar phospholipid vesicles at pH 3.0 in 100 mM glycine/100 mM NaCl buffer was investigated by differential scanning calorimetry (DSC), CD-spectropolarimetry and intrinsic fluorescence emission spectroscopy. EqTxII binding to the zwitterionic DPPC vesicles at pH 3.0 does not influence significantly the cooperativity, the enthalpy and the temperature of the gel/liquid-crystaline phase transition of the lipids. Similarly, the zwitterionic DPPC lipids at the same conditions do not influence significantly the EqTxII structural transitions since in the presence or absence of DPPC EqTxII exhibits the same properties. Our results suggest that there is no strong interactions between EqTxII and zwitterionic small unilamellar vesicles in glycine/NaCl buffer at pH 3.0.

INTRODUCTION

Proteins which are capable of changing their water/membrane solubility in response to changes in the environment offer an unique experimental opportunity to

[#] Dedicated to Prof. Dr. Drago Leskovšek on the occasion of his 80th birthday.

study the events associated with the processes of protein-membrane association and insertion. Equinatoxin II (EqTx II) is a member of the family of pore-forming toxins, which exhibits an ability to bind to the natural or model lipid membranes and form pores selective to cations [1]. It consists of 179 amino acid residues (M_w = 19.8 kDa) [2] and in aqueous solutions forms a globular conformation with a defined secondary and tertiary structure. Our previous investigations have shown that EqTxII can undergo structural transitions to other conformational states in response to changes in temperature and/or pH [3, 4]. In particular, fluorescence, CD, UV-spectroscopy and DSC techniques have shown that at low pH EqTxII undergoes a conformational transition into an intermediate state characterized by a non-native secondary structure rich in α-helical content and completely collapsed tertiary structure; a behavior typical of protein existing in so called "molten globule" state [3, 4].

Recently, we have shown that also at very basic pH EqTxII unfolds into a molten globule state characterized by a more expanded α-helical secondary structure and higher stability in the presence of NaCl [5]. This findings encouraged us to start investigating the interactions of EqTxII with model membranes. Our preliminary results [6] have indicated that EqTxII has some preferences for anionic lipids in the liquid-crystalline form since at neutral pH and high lipid to protein ratios it causes a decrease in the enthalpy of phase transitions of anionic L-α-dipalmitoylphosphatidylglycerol (DPPG) lipids and has no effect on the thermotropic behavior of zwitterionic L-α-dipalmitoylphosphatidylcholine (DPPC) lipids [6]. Furthermore, in the presence of anionic lipids EqTxII undergoes two distinguishable transitions with pronounced α-helical secondary structure formation at higher temperatures while the observed conformational transition of EqTxII in the presence of DPPC lipids do not differ much from those observed in triple distilled water. To get some information about the influence of low pH on the interaction of EqTxII with a zwitterionic phospholipid vesicles, we investigated the effect of EqTxII on the phase transitions of DPPC small unilamellar vesicles in 100 mM glycine/100 mM NaCl buffer at pH = 3.0 by differential scanning calorimetry (DSC) and the effect of DPPC lipids

on the conformational transitions of EqTxII at the same conditions by the far-UV CD spectropolarimetry and intrinsic emission fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Preparation of Vesicles. The L-α-dipalmitoylphosphatidylcholine, DPPC, (M_w = 734.0 g/mol) used in this study was purchased from Sigma Chemical Co. and was used without further purification. Suspensions of lipid vesicles for DSC, CD-spectropolarimetry, and intrinsic emission fluorescence spectroscopy were prepared as follows. Pure DPPC and DPPG lipids (5 mg) were dissolved in 10 mL chloroform and chloroform/methanol = 3:1 mixture, respectively. Equimolar mixture of DPPG (M_w = 745.0 g/mol) and DPPC (M_w = 734.0 g/mol) lipids were prepared by mixing solutions of each lipids together at the desired molar ratio of 1 to 1. The lipid solutions were dried by slow evaporation under a constant stream of nitrogen. After removal of the last traces of solvent in vacuum overnight, triple distilled water was added to obtain the desired concentration of lipids (2.5 mg/ml). Multilamellar vesicles (MLV) were prepared by vortexing the lipid suspension for 10 min. MLV were further transformed to small unilamellar vesicles (SUV) by sonication using an ultrasonic disintegrator (150 W) equipped with a microprobe until the milky MLV suspension became clear. The concentration of the lipid solution for DSC and CD measurements was 0.5-1 mg/ml and for fluorescence titration measurements about 1 mg/ml. The concentrations of lipids were determined by dry weight analysis.

Preparation of the Protein Solution. EqTxII was isolated from the sea anemone *Actinia Equina L.* [2], stored as a powder at -10 °C and used without any further purification. EqTxII solutions for the CD-measurements in the far- and near-UV CD range and for the DSC measurements were prepared directly by dissolving the protein in 100 mM glycine/100 mM NaCl buffer, pH=3.0, to obtained the concentration ~ 1 mg/ml and diluting it by liposomes to obtain the appropriate lipid to protein ratio. The protein concentration was determined spectrophotometricaly at 20 °C by using the extinction coefficient ε_{280nm} = 1870 cm^{-1} $(g/ml)^{-1}$ before DPPC lipids

were added. For fluorescence measurements EqTxII solutions (c = 0.01 mg/ml) were prepared daily from defrozen aqueous stock solution by diluting it with buffer solution and titrated with DPPC.

Differential Scanning Calorimetry. DSC experiments were performed in a micro-DSC Calorimeter from Setaram (Caluire, France) described elsewhere [7]. The sample and the reference cells of optimal operational volume of 0.8 ml were used. Calibration was performed with special cells using the Joule effect. Thermograms of DPPC in 100 mM Gly/100 mM NaCl buffer pH=3.0 in the presence of EqTxII at different lipid (L) to protein (P) molar ratios (L/P = 100:1, 50:1 and 20:1) were recorded over the temperature range from 20 to 60 °C at the heating rate of 0.5 °C/min. The base lines obtained with vessels filled with equal quantities of solvent were subtracted from the lipids/protein thermograms. The first DSC scan was used to obtain values for temperature, T_m, and the enthalpy, ΔH, of the phase transition as described previously [7]. The second scan was used to assess the reversibility of the phase transition.

Circular Dichroism (CD) Spectropolarimetry. CD experiments were performed on an AVIV Model 62A DS Spectropolarimeter (Aviv Associates, Lakewood, NJ, USA) equipped with a thermoelectrically controlled cell holder. CD spectra of EqTxII at different molar ratios of added lipids (L/P = 100:1 to 10:1) were measured in the far-UV range (200 - 250 nm) in 0.25 mm pathlength quartz cuvettes. The concentration of lipids were kept constant at all L/P ratios to have the same contribution from light-scattering effect. Spectra were measured at 5 ^oC intervals in the temperature range from 5 to 95 ^oC with an averaging time of 3 s, an equilibration time of 2 min and the band width of 1 nm. Spectra were base-line-corrected, smoothed by using a polynomial fitting function (Aviv Associate, Inc.) and converted to mean residue ellipticity, $[\Theta]_\lambda$, by using the relation

$$[\Theta]_\lambda = \frac{M_o \cdot \Theta_\lambda}{100 \cdot c \cdot l} \qquad (1)$$

in which M_o is the protein mean residue molar weight, Θ_λ is the measured ellipticity in degrees, c is the concentration in g/ml and l is the path length in dm. The value of M_o = 110.6 g/mol was obtained from M_w of EqTxII (19.8 kDa) by dividing it with the

number of aminoacid residues (179) constituting the EqTxII molecule.

Intrinsic Emission Fluorescence Spectroscopy. All emission fluorescence measurements were performed in a Perkin-Elmer Model LS-50 Luminescence spectrometer with a water thermostated cell holder using a 1 cm path length quartz cuvette. Slit widths with a nominal band pass of 5 nm were used for both excitation and emission beams. An excitation wavelength of 280 nm was used and the emission spectra were recorded in the range from 300 to 500 nm. Isothermal titration of aqueous solution of EqTxII with DPPC liposomes were performed at 25 and 48 °C by incrementally adding 2.5 to 50 μl aliquots of lipid solution into 2 ml solution of 0.01 mg/ml ($5.0 \cdot 10^{-7}$ M) of EqTxII. After each addition, the emission spectrum was recorded. The same titration procedure was repeated without EqTxII to obtain the fluorescence emission spectra of pure solvent (background intensity), which were subtracted from the corresponding emission spectra of EqTxII. Then the emission spectra of EqTxII were multiplied by the dilution factor and corrected for PM-tube response using fluorescence spectrum of Quinine sulphate ($c = 2.5 \cdot 10^{-7}$M) in 0.1 M solution of perchloric acid as a standard. The temperature in the cell was measured with a Fe/Fe-constantan thermocouple.

RESULTS AND DISCUSSION

Figure 1 shows the DSC thermograms of DPPC vesicles in 100 mM Gly/100 mM NaCl buffer at pH=3.0 at various DPPC/EqTxII molar ratios. In the absence of protein DPPC exhibits endothermic transition at T_m = 44 ± 1 °C (panel A), which corresponds to the highly cooperative gel/liquid-crystalline (chain melting) phase transition of the lipid. The accompanying enthalpy of this chain melting of DPPC in glycine buffer at pH 3.0, ΔH, is equal to 36 ± 4 kJ/mol and lower than the corresponding enthalpy of DPPC lipids in triple distilled water reported to be ΔH = 39 ± 4 kJ/mol at 43 °C [6]. The observed small change in ΔH and T_m of the phase transition of DPPC at pH = 3.0 in comparison with neutral pH is consistent with the

literature data [8]. In the presence of low to moderate levels of EqTxII the observed chain melting transition of DPPC is not influenced significantly by EqTxII as indicated by the ΔH values of the gel/liquid-crystalline phase transition observed at 44 °C at DPPC/EqTxII ratios of 100:1 and 50:1 to be 36 ± 4 kJ/mol and 32 ± 4 kJ/mol, respectively (Fig. 1 B, C).

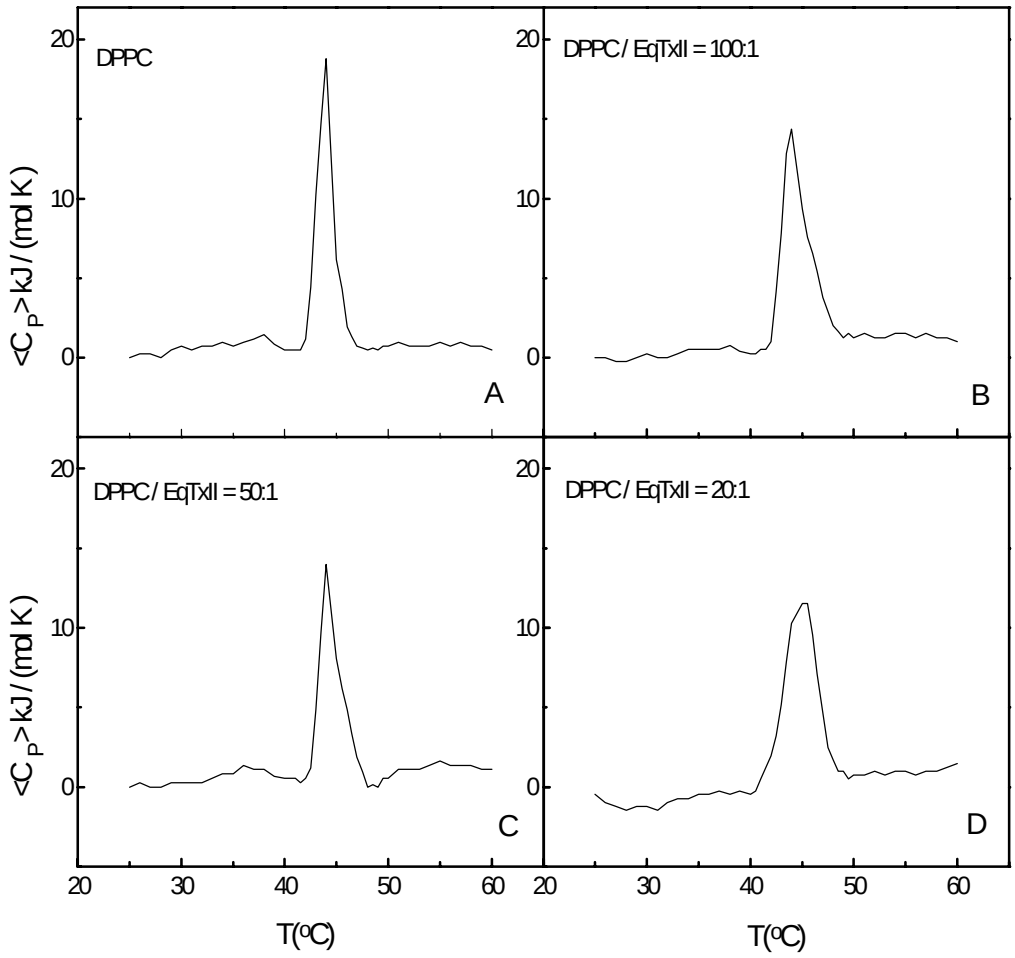

Figure 1: *The apparent excess heat capacity curves of DPPC vesicles in 100mM Gly/100 mM NaCl pH=3.0 in the presence of EqTxII. (A) DPPC vesicles alone, c = 1 mg/ml; (B) DPPC/EqTxII = 100:1, c_{DPPC} = 1 mg/ml and c_{EqTxII} = 0.27 mg/ml; (C) DPPC/EqTxII = 50:1, c_{DPPC} = 1 mg/ml and c_{EqTxII} = 0.54 mg/ml; (D) DPPC/EqTxII = 20:1, c_{DPPC}= 0.5 mg/ml and c_{EqTxII} = 0.67 mg/ml.*

At DPPC/EqTxII ratio of 20:1 the ΔH of chain melting obtained from the corresponding DSC thermograms (Fig. 1 D) is at 45 °C equal to 25 ± 4 kJ/mol, a

value that still adds up to 65 % of the ΔH value of pure DPPC vesicles. This result differs significantly from the one observed at neutral pH where at DPPC/EqTxII ratio of 20:1 the phase transition of DPPC vesicles almost disappears [6]. Apparently, at pH = 3.0 the electrostatic interactions between the surface of DPPC and the EqTxII molecules are shielded by small ions which are present at high concentrations in the buffer solution (100 mM glycine/100 mM NaCl). Furthermore, at pH = 3.0 the surface net charge on the DPPC lipids might become positive (the pK_a of negatively charged phosphate of the DPPC lipids is $pK_a \sim 2 - 3$ [8]) and thus repulsive to the EqTxII molecules whose net charge at pH = 3.0 is estimated from amino acid composition to be 25+ [9]. The fact, that EqTxII has only a moderate effect on the enthalpy of the overall chain-melting phase transition of the first scanning experiment even at L/P ratio 20:1 suggests that EqTxII interactions with zwitterionic DPPC vesicles do not significantly alter the energy of hydrocharbon chain packing in both the gel and liquid-crystalline states. The shape of the thermograms obtained from the second scanning experiments as well as the corresponding cooperativities and temperatures of the phase transitions were changed, suggesting that the phase transition of DPPC in the presence of EqTxII at low pH is not a reversible process as it is in the triple distilled water [6].

In the Figure 2 (A, B & C) are presented the CD spectra of EqTxII in the far-UV CD range at temperatures between 5 and 95 °C and L/P ratios of 100:1, 50:1 and 20:1, respectively. The CD spectra of EqTxII at all L/P ratios are identical to the one observed for EqTxII in 100 mM glycine/100 mM NaCl buffer at pH 3.0 in the absence of DPPC [4]. At all L/P ratios the secondary structure of EqTxII remains stable with increasing the temperature up to 45 °C. Above this temperature a pronounced transition is observed. At L/P = 100:1 and 50:1 the temperature of the transition, T_m, determined from the temperature dependent changes in the molar ellipticity, $[\Theta]_\lambda$, followed at 208 and 235 nm is 50 ± 1 °C while the conformational transition followed at 217 nm is not completed up to 95 °C. Similarly, at higher EqTxII concentrations e.g. lower L/P ratio (20:1) the observed transition at $T_m = 52 \pm 1$ °C (Fig. 2 C, D) can also be obtained only from CD melting curves measured at 208 and 235 nm. At all L/P ratios, the EqTxII shows similar conformational changes as were observed for EqTxII

transition in the same buffer solution in the absence of DPPC lipids and which were characterized as the protein transitions from its native to molten globule state [4]. For such partial unfolding of EqTxII into molten globule state at pH 3.0 it has been shown that it is accompanied by a total collapse of the EqTxII tertiary structure and switching of its secondary structure into a form rich in α-helical content [4]. The results we are presenting here show similar behavior of EqTxII also in the presence of DPPC lipids.

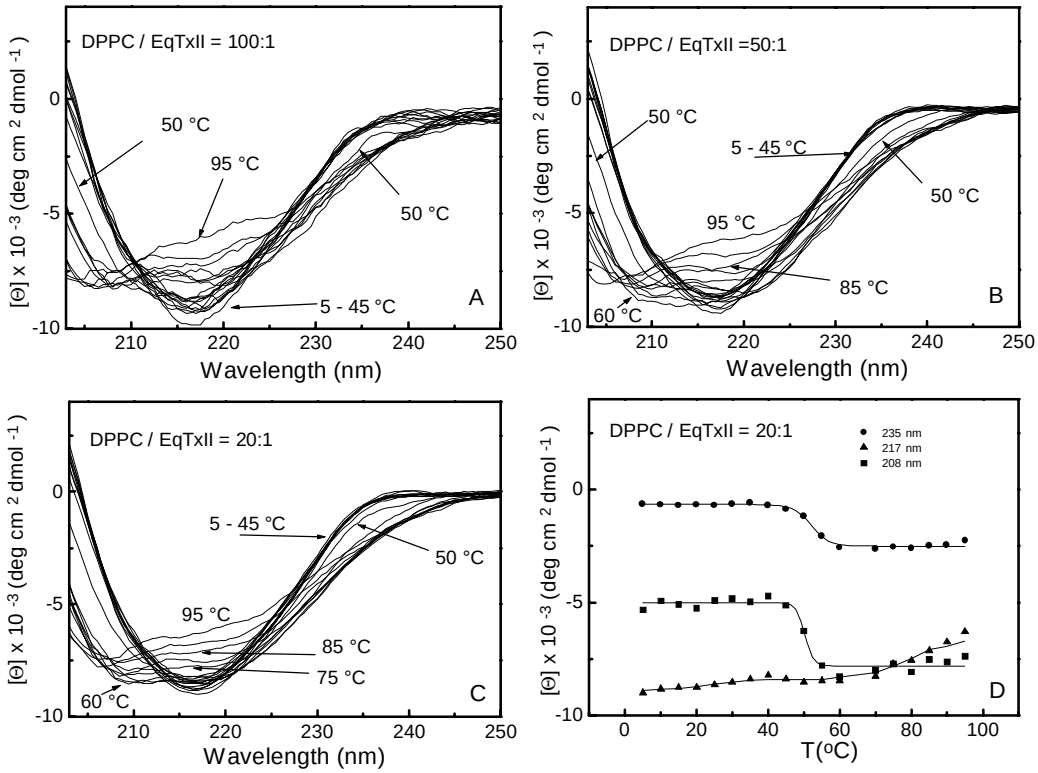

Figure 2: *Temperature-dependence of the far-UV CD-spectra of EqTxII in the presence of the zwitterionic DPPC vesicles at pH 3.0. (A) DPPC/EqTxII = 100:1, c_{DPPC} = 1 mg/ml and c_{EqTxII} = 0.27 mg/ml; (B) DPPC /EqTxII = 50:1, c_{DPPC} = 1 mg/ml and c_{EqTxII} = 0.54 mg/ml; (C) DPPC/EqTxII = 20:1, c_{DPPC} = 0.5 mg/ml and c_{EqTxII} = 0.67 mg/ml; (D) CD-melting profile of EqTxII in the presence of DPPC at DPPC/EqTxII = 20:1 followed at 235, 217 and 208 nm.*

The results of fluorescence titration of EqTxII performed at two different pH, in 100 mM Gly/100 mM NaCl buffer and triple distilled water, and at two different temperatures, bellow the gel/liquid-christalline phase transition of DPPC at 25 °C and

above it at 48 °C, are presented in Figure 3. No significant difference in the quenching of the protein intrinsic fluorescence emission intensity has been observed when in triple distilled water EqTxII was titrated by DPPC either at 25 °C or 48 °C [6].

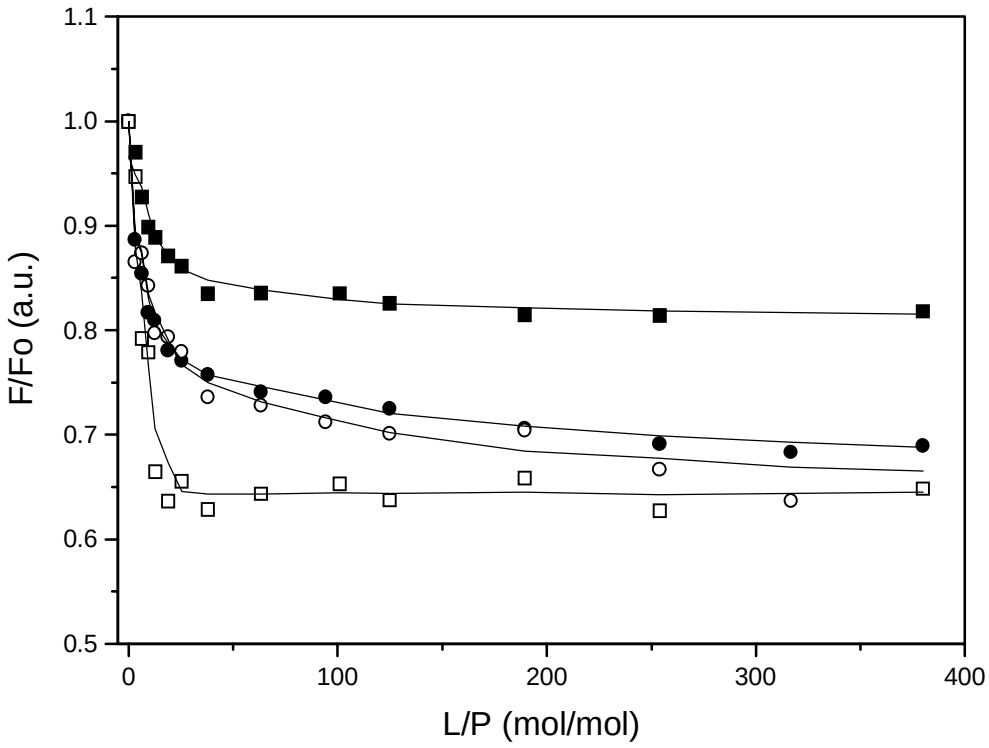

Figure 3: *The relative intrinsic emission fluorescence intensity, F/Fo, at 333 nm of EqTxII (c_{EqTxII} = 0.01 mg/ml) titrated by DPPC vesicles prepared in triple distilled water (●, ○) and by DPPC vesicles prepared in 100 mM Gly/100 mM NaCl buffer pH 3.0 (■, □) at 25 °C (solid symbols) and 48 °C (open symbols), λ_{exc} = 280 nm, c_{DPPC} = 1 mg/ml. Fo stands for the fluorescence intensity of EqTxII in triple distilled water or buffer at pH 3.0 without DPPC.*

Our results further show that at pH 3.0 and 25 °C the observed quenching of EqTxII intrinsic fluorescence is less expressed than in pure water. These results suggest that at given conditions the protein fluorophores are in close proximity of the surface of DPPC vesicles. The non observed shifts in the position of the EqTxII fluorescence emission maxima, λ_{max} = 332 nm ± 1 nm, after titrating it with DPPC indicate that upon EqTxII binding to the vesicles the overall polarity of the environment surrounding the protein fluorophores remains unchanged. This also indicates that

EqTxII molecules when interacting with DPPC vesicles do not undergo any conformational changes and do not insert into the membranes with parts that contain fluorophores. Taken together the observed differences in the behavior of EqTxII in the presence of zwitterionic DPPC lipids at two different pH indicate that the protein-vesicle electrostatic interactions seem to play an important role in protein-membrane recognition.

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POVZETEK

Naravo interakcij med ekvinatoksinom II (EqTxII) in majhnimi enoplastnimi zwiterionskimi fosfolipidnimi liposomi v 100 mM glicinu/100 mM NaCl pri pH = 3.0 smo raziskovali z diferenčno dinamično kalorimetrijo (DSC), CD-spektropolarimetrijo in lastno fluorescenčno emisijsko spektroskopijo. Vezava EqTxII na zwitterionske DPPC liposome ne vpliva znatno na kooperativnost, entalpijo in temperaturo faznega prehoda lipidov med plastno gelsko in tekoči podobno fazo. Podobno, tudi zwitterionski lipidi pri istih pogojih ne vplivajo znatno na konformacijske prehode EqTxII, kajti EqTxII kaže podobne lastnosti, kot brez prisotnih lipidov. Naši rezultati kažejo na to, da ni izrazitih interakcij med EqTxII in majhnimi enoplastnimi zwitterionskimi liposomi v 100 mM glicinskem/100 mM NaCl pufru pri pH 3.0.