

**INTERACTION OF GENTAMICIN WITH PHOSPHATIDYL
SERINE IN HOMOGENOUS AND PHASE SEPARATED
MONO- AND BILAYER FILMS**

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ABSTRACT. Aminoglycoside are antibiotics used to treat serious infections caused by aerobic gram-negative bacilli. These antibiotics and especially Gentamicin possess aggressive nephro- and ototoxicity. Gentamicin accumulates in the renal cortex before renal failure. Furthermore calcium reduces Gentamicin binding to cell membranes and dietary calcium reduces Gentamicin nephrotoxicity. Possible molecular mechanism underlying nephro- and ototoxicity is kidney cell membrane fusion, this event strongly depend on the presence of negatively charged phospholipids i.e. phosphatidyleserine.

The aim of the current work is to study thermodynamically the interactions of Gentamicine and phosphatidyleserin through nanometer scale thin films, Thin Liquid Films – Bilayer Films (TLFs) and Monolayer Films (MFs). Investigation is concentrated on the effect of Gentamicin on the TLFs stability i.e fusion, MFs surface tension and the influence of calcium ions on the discussed thermodynamic parameters. Important part is focused on the significance of lipid phase-separation for the Gentamicin-phosphatidyleserine interactions.

It was found a destabilization effect of Gentamicin on PS TLF and alternatively a stabilization effect of Gentamicin on the phase separated TLFs formed from dipalmitoylphosphatidylcholine/phosphatidyleserine. No drug effect on the neutral phospholipid TLFs and on all other TLFs in presence of calcium was observed, suggesting strictly electrostatic Gentamicin-phosphatidyleserine interactions both in phase separated and homogenous TLFs.

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It was found a perturbation of MFs formed from phosphatidyleserine and beneficial surface behavior of the phase separated MFs due to Gentamicin. No drug effect on all used MFs in presence of calcium was observed.

The authors hope that the present investigation will highlight the molecular mechanism of the Gentamicin nephro-and ototoxicity as well as calcium involvement in this pathology.

KEY WORDS. Thin Liquid Films, Bilayer Films, Monolayer Films, Gentamicin, fusion, calcium

INTRODUCTION

Aminoglycoside are antibiotic drugs, which are used to treat serious infections caused by aerobic gram-negative bacilli (eg, a number of the Enterobacteriaceae, *P. aeruginosa*). These include lower respiratory tract, intra-abdominal, soft tissue, bone or joint, wound, and complicated urinary tract infections; endocarditis; bacteremias; and meningitis (by intrathecal administration).

Gentamicin often is the preferred aminoglycoside for general use in hospitals where the prevalence of bacterial resistance to this agent is low (based on locally generated antimicrobial susceptibility profiles). Its major advantage over tobramycin, amikacin, and netilmicin is lower cost.

Enterococcal endocarditis is a difficult infection to eradicate, with relapse or death occurring in about 20% of cases. In the western world it is a disease of the elderly and accounts for 5–15% of all cases of endocarditis (Olaison et al. 2002). The treatment usually includes β lactam in combination with an aminoglycoside (Wilson et al. 1995 and Working party of the British Society.... 1998). Gentamicin is the most commonly used but recent reports suggest a strong nephrotoxicity (Rougier et al. 2004, Dominguez et al. 1996) and ototoxicity (East et al. 2005; Bates et al. 2002). The strongest ototoxicity effect of Gentamicin was found in-patients with presented chronic renal failure (Crass et al. 1981). Monitoring of plasma concentrations is routine and generally effective in avoiding nephrotoxicity and ototoxicity caused by the drug's narrow treatment window (Gunnar et al. 1984). Nevertheless, toxicity can occur despite apparently satisfactory drug concentrations.

Calcium interacts with aminoglycosides in several ways. It has been reported to diminish aminoglycoside binding or transport by gram-positive and -negative bacteria (Bryan et al. 1977), rabbit vascular smooth muscle (Goodman 1978), renal mitochondria (Kornguth et al. 1980), and human serum proteins (Myers et al. 1978). Furthermore, calcium in nutrient medium decreases in vitro gentamicin antibacterial activity (Dornbusch 1980) and is also reported to reverse or prevent an aminoglycoside - mediated neuromuscular blockade due to calcium reduction of Gentamicin binding to cell membranes (Kornguth et al. 1980). Since gentamicin

accumulates in the renal cortex before renal failure and calcium reduces gentamicin binding to cell membranes, Bennett et al. 1982 examined the effect of dietary calcium loading on gentamicin toxicity in male F344 rats. The results indicate that dietary calcium loading reduces experimental gentamicin nephrotoxicity.

Aramaki et al. 1989 postulated a possible mechanism of Gentamicin nephrotoxicity. They studied interactions of Gentamicin with a phospholipid model membrane (liposomes) and found an increased turbidity due to the drug presence. The effect was dependent on the concentration of acidic phospholipids in the liposome membrane. It was found from electron microscopic observations that the increased turbidity of liposome suspensions due to Gentamicin presence was caused by liposome fusion. Furthermore Alexander et al. observed microelectrophoretically (Alexander et al. 1979) that Gentamicin binds avidly to acid phospholipids and mixtures between acid and neutral phospholipids and no binding of Gentamicin was found for neutral phospholipids. Other investigators demonstrated that Gentamicin binds avidly the acid phospholipid – phosphatidylserine (Kubo et al. 1986; Chung et al. 1985; Forge et al. 1989).

The aim of the current work is to study thermodynamically the interactions of Gentamicine and phosphatidyle serin, an acid phospholipid, through a nanometer scale thin films formed at the air/liquid interface. The investigation is also concentrated on the effect of calcium ions on the drug-lipid interactions on the one hand and on the influence of lipid phase separation (denoted to be important in the drug-membrane interactions in Jutila and Kinnunen 2001) in the thin film on the other. The lipids chosen for the purpose of the work are brain phosphatidylserine (PS), dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC). They were used to obtain homogenous – DMPC (zwitterionic), PS (negatively charged) and phase separated - DPPC/PS thin films.

The thin films are regarded in the current work as model membrane systems. Two thin films, Thin Liquid Films – bilayer films (TLFs) and Monolayer Films (MFs), were carefully selected for the purpose of the present work. The former, being of several types, are composed of two mutually adsorbed lipid monolayers, oriented “head-to-head”, (Fig. 1A-C), thus representing the contact area occurring between the cis-monolayers of two fusion bilayer membranes (Kuhl et al. 1996). Further the lipid TLFs has been successfully used as a model system to study membrane-membrane fusion, adhesion, interaction, biosurfactant behavior at interfaces, lung surfactant, etc. (Lalchev et al. 1995; Lalchev Z, 1997; Exerova 1988). The lipid MF (Fig. 1D) can be regarded as one half of TLFs and was used for a long time as a model system for studying the interaction of membrane components with different molecules and ions injected into the liquid subphase (Lalchev et al. 1996; Lalchev and Mackie 1999; Panaiotov 2000)

MATERIAL AND METHODS

Material

Brain phosphatidylserine (PS), dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC). “Avantyl Polar Lipids”. Gentamicine was purchased from “Sigma”. NaCl was purchased from “Merck”. Solutions were made with bidistilled water with conductivity less than 1 μ S.

Methods

Thin Liquid Films (TLFs)

TLFs were formed by the method of Scheludko and Exerowa (Exerowa 1998) using the modified measuring cell as previously described by Lalchev et al 1999. A biconcave drop (50 μ l volume) of the phospholipid dispersion (pH=6.8-7.0; C_{el} =0,5 M NaCl) was incubated into the cylinder of the measuring cell at $T=37^{\circ}\text{C}$ for 30 minutes. After sucking the solution from the drop thick TLF is formed (Fig. 1). Further the film spontaneously gets thinner and after some characteristic film thinning time, t_{0-1} (sec), critical film thickness (300 \AA) is reached. Then a Black Spot (BS), local thinning in the film, appears (as schematically shown in Fig.1 A), expands with characteristics rate to fill up the whole area of the film. The kinetic of this process was measured by BS expansion time t_{1-2} (sec) detecting the time from the formation of the first black spot to the moment of its expansion to the whole film area, i.e. to black TLF formation. At different experimental conditions two types of black films is possible to be formed- common black films, CBFs, (Fig. 1B) and Newton black films, NBFs, (Fig. 1C).

The probability (W) for formation of stable black TLFs depends strongly on the phospholipid concentration (Exerowa 1998) and can be calculated by the equation $W=\Delta N / N$, where N is the total number of trials (at least 50 for each concentration) and ΔN is the number of trials in which stable black films are formed. Thus, W varies between 0 and 1 indicating that the films always rupture ($W=0$) and that the films always are formed stable ($W=1$). The dependence $W(C)$ is extremely steep which allowed to define a threshold concentration (C_t) as the minimum phospholipid concentration at which $W=1$ and stable films are always formed (Lalchev 1984). It is proven that $W(C)$ dependence is sensitive to the composition, molecular shape and phase state of the film forming phospholipids (Lalchev 1997 and Exerowa 1998).

Monolayers

The adsorbed monolayers of DMPC, PS and DPPC/PS were formed in the Langmuir trough and the surface tension (mN/m) was measured by the method of Wilhelmy with accuracy ± 0.5 mN/m as previously described (Christova et al. 1998). . The surface tension, maximum and minimum, were measured after observation of equilibrium surface tension due to 50/100 % compression/decompression of the film surface. PS and DPPC/PS were used for compression/decompression experiments at

the corresponding CMC concentrations. Experiments were conducted at T=37°C, pH 6.8-7.0 and electrolyte concentration $C_{el}=0,5$ M NaCl in presence and absence of 10-3 M calcium.

Lipid phase state

Lipid phase heterogeneity was obtained by strictly following the protocol suggested by Jutila et al. 2001. The lipids were mixed in proportion 95/5 (mol/mol) DPPC/brain PS. The lipid phase state for the homogenous films were determined according to the Caffrey et al. 1993, liquid-crystalline for PS and DMPC at the used in the study experimental conditions.

RESULTS AND DISCUSSION

Thin Liquid Films

Probability of CBFs formation as dependence of brain PS is presented on Fig 2. The threshold concentration - Ct for PS was 200 µg /ml. At Ct the Gentamicin was added in to the film meniscus at 5×10^{-6} M. This Gentamicin concentration was found to be the minimal with destabilization effect on the PS CBFs. The destabilization is presented at Fig 2. with a shift in PS Ct to higher PS concentrations, which in presence of 5×10^{-6} M Gentamicin was 210 µg /ml. The Ct for PS in presence of calcium was 360 µg/ml. In presence of calcium the PS TLFs thin to NBFs in comparison to the calcium absence where the films were CBFs. The change in the type of PS TLFs from CBFs to thinner NBFs is due to the neutralization of the PS head negative charge in presence of calcium, resulting in strong decrease of the repulsive forces among the bilayer film surfaces. No effect of on the stability of PS NBFs in presence of calcium Gentamicin was observed, suggesting electrostatic Gentamicin - PS interactions.

Fig. 2

The probability of NBFs formation as dependence of DPPC/PS bulk film concentration is presented on Fig 3. The threshold concentration for DPPC/PS was 200 µg /ml. The Gentamicin stabilization effect is presented as a lowering of DPPC/PS Ct to $\frac{1}{2}$ DPPC/PS, Ct = 100 µg /ml, and the further stabilization of DPPC/PS NBFs were due to Gentamicin presence. The minimal Gentamicin concentration at which stabile NBFs were observed was 10^{-2} M Gentamicin. In presence of calcium no effect of Gentamicin on the NBFs stability was observed (data not shown), suggesting predominantly electrostatic Gentamicin-PS interactions.

Fig. 3

It was found no destabilization effect of Gentamicin on TLFs from DMPC. No effect was observed in presence of calcium.

It was found no effect of Gentamicin on the hydrodynamic behavior of all used in the study TLFs.

Gentamicin is hydrophilic and $\sim+3$ charged antibiotic drug, and its binding to liposomes requires the presence of acidic phospholipids (Brasseur et al., 1984; Chung et al., 1985; Kubo et al., 1986). The electrostatic association of Gentamicin to liposomes results in charge neutralization and tightening of lipid packing (Gurnani et al., 1995). Due to its net positive charge ($\sim+3$) Gentamicin molecules should be able to complex with three negatively charged phospholipids.

We attempt to explain the DPPC/PS increased stability in presence of Gentamicin with faster surface rearrangement and better surface spreading of DPPC/PS colloidal particles on both of the film surfaces, resulting in faster formation of densely packed lipid monolayers on the film interfaces. Alternatively PS colloidal particles were more rigid due to Gentamicin-PS interactions and further formation of complex of 3 molecules PS and one Gentamicin. The colloidal particles possessed slow surface rearrangement and spreading. The conclusions were further confirmed by the results from the MFs.

Monolayer films

The equilibrium surface tension (mN/m), γ_{eq} , vs. lipid concentration (M) in presence and absence of Gentamicin are shown in Fig. 4. It can be seen that PS dispersion reach a plateau values of $\gamma_{eq} = 69.1$ mN/m at 10^{-6} M brain PS. In comparison in presence of Gentamicin a plateau values of $\gamma_{eq} = 71.1$ mN/m at the same concentration of PS. CMC can be determined: 1×10^{-6} for PS in presence and absence of Gentamin.

Alternatively DPPC/PS dispersion reached a plateau values of γ_{eq} equal to 70.2 mN/m at lipid mixture concentrations of 10^{-6} M. Gentamicin resulted to decrease of γ_{eq} plateau values of 67.0 mN/m at lipid mixture concentrations of 10^{-9} M DPPC/PS. CMC can be determined: 1×10^{-6} for DPPC/PS and 1×10^{-9} in presence of Gentamin.

Fig. 4

The results observed, suggest perturbation of the adsorbed monolayer formed from PS in presence of Gentamicin in comparison to the Gentamicin absence. Alternatively the presence of Gentamicin for DPPC/PS resulted to better surface lipid colloidal particle reorganization and spreading, and more densely packed MFs presenting better surface properties in comparison to DPPC/PS in absence of Gentamicin. Furthermore a shift in CMC to lower DPPC/PS concentrations was observed

The equilibrium surface tension (mN/m), γ_{eq} , vs. lipid concentration (M) in presence and absence of Gentamicin and calcium are shown in Fig. 5. The γ_{eq} plateau values for brain PS dispersion in presence of 10^{-3} M calcium were 70 mN/m at 10^{-5} M brain PS. The same γ_{eq} values and the same brain PS concentration were observed in presence of Gentamicin. The results clearly suggested no effect of Gentamicin on the

brain PS lipid colloidal suspension in presence of calcium. CMC were determined: 1×10^{-5} M brain PS in presence of calcium and in presence of calcium and Gentamicin.

For DPPC/PS (Fig. 5) the γ_{eq} plateau values of 69.25 mN/m were observed at 10^{-5} M DPPC/PS. In presence of Gentamicin the same γ_{eq} plateau values equal to 69.25 mN/m were observed but also a shift in the minimal DPPC/PS concentration to 10^{-7} M DPPC/PS was found.

Fig. 5

The results observed suggest no effect of Gentamicin due to calcium presence for brain PS lipid colloidal suspension. For the lipid mixture DPPC/PS in presence of calcium Gentamicin resulted only in shift of CMC to lower lipid concentration, from 10^{-4} M DPPC/PS to 10^{-7} M DPPC/PS in presence of Gentamicin.

On Fig. 6 the maximal and minimal surfaces tension, γ_{max} and γ_{min} , are plotted vs. compression decompression cycle. It is shown almost linear decrease (of 10mN/m) of γ_{max} for PS in presence of Gentamicin and practically no decrease in γ_{max} for PS in the Gentamicin absence, suggesting a decreased spreading and new PS colloidal particle surface reorganization to the “cleared” by the compression air/water interface in presence of Gentamicin. Better surface properties in the Gentamicin absence were determined. Furthermore lower γ_{max} values = 62.5 mN/m, for the third cycle, were observed, for brain PS adsorbed MFs in comparison to PS in presence of Gentamicin where $\gamma_{max} = 66.0$ mN/m, for the third cycle.

It was found (Fig. 6) strong and linear increase (of about 20 mN/m for the third cycle) in γ_{min} for PS in presence of Gentamicin. Alternatively brain PS presents practically no change in γ_{min} for the first and the second cycle and an increase of 10 mN/m for the third cycle. Since the electrostatic association of Gentamicin to liposomes results in charge neutralization and tightening of lipid packing (Gurnani et al., 1995) we postulate aggregation PS molecules by Gentamicin and as a result solid like brain PS MFs behavior.

Fig. 6

On Fig. 7 are presented maximal and minimal surface tension, γ_{max} and γ_{min} , plotted vs. compression decompression cycle for the DPPC/PS adsorbed MFs. It is shown practically no change in γ_{max} for DPPC/PS MFs and a decrease of DPPC/PS γ_{max} with 3 mN/m between the first and the third cycle in presence of Gentamicin. Furthermore lower γ_{max} values = 69.0 mN/m, for the first cycle, for DPPC/PS adsorbed MFs in presence of Gentamicin, were observed in comparison to $\gamma_{max} = 72$ mN/m for the pure DPPC/PS adsorbed MFs.

About 4 mN/m lowering of γ_{max} during cycles for DPPC/PS in presence of Gentamicin was observed, suggesting fast re-spreading of the MFs during decompression and little free air/water interface for lipid adsorption. 6mN/m higher

γ_{\max} was found for DPPC/PS in comparison to presence of Gentamicin, which can be explained with better colloidal particle surface reorganization and more lipids on the interface due to Gentamicin presence.

For γ_{\min} (Fig. 7) a difference of 6mN/m for the final cycle and lower γ_{\min} (for all the cycles) for DPPC/PS in comparison to DPPC/PS in presence of Gentamicin was observed. The data suggests better surface behavior for the DPPC/PS in presence of Gentamicin in comparison to the Gentamicin absence.

Fig. 7

CONCLUSIONS

Aminoglycoside are antibiotic drugs, which are used to treat serious infections caused by aerobic gram-negative bacilli (eg, a number of the Enterobacteriaceae, *P. aeruginosa*). These include lower respiratory tract, intra-abdominal, soft tissue, bone or joint, wound, and complicated urinary tract infections; endocarditis; bacteremias; and meningitis (by intrathecal administration). Unfortunately these antibiotics and especially Gentamicin were found to possess aggressive nephrotoxicity (Rougier et al. 2004, Dominguez et al. 1996) and ototoxicity (East et al. 2005; Bates et al. 2002). It was found that Gentamicin accumulates in the renal cortex before renal failure and that calcium reduces gentamicin binding to cell membranes. Bennett et al. in 1982 examined the effect of dietary calcium loading on gentamicin toxicity in male F344 rats. The results indicate that dietary calcium loading reduces experimental Gentamicin nephrotoxicity.

In 1989 Aramaki et al. postulated a possible mechanism of Gentamicin nephrotoxicity. They studied interactions of Gentamicin with a phospholipid model membrane (liposomes) and found an increased turbidity due to the drug presence. The effect was dependent on the concentration of acidic phospholipids (phosphatidyleserine) in the liposome membrane. It was found from electron microscopic observations that the increased turbidity of liposome suspensions due to Gentamicin presence was caused by liposome fusion.

The aim of the current work is to study thermodynamically the interactions of Gentamicine and phosphatidyleserin through a nanometer scale thin films formed at the air/liquid interface. The investigation is primary concentrated on the effect Gentamicin on the TLFs stability (closely connected with fusion) and MFs surface behavior (surface tension and surface dynamic parameters) on the one hand and the influence of calcium ions on the discussed thermodynamic parameters on the other. Important part of the study is also focused on the influence of the lipid phase separation (denoted to be important in the drug-membrane interactions in Jutila and Kinnunen 2001) in the thin films.

It was found strong destabilization effect of Gentamicin on CBF formed from PS. The calcium presence, in 10^{-3} M, resulted in no effect of Gentamicin on the stability of NBFs formed from PS.

Alternatively it was found a strong stabilization effect of Gentamicin on NBFs formed from the lipid mixture DPPC/PS. The calcium presence, in 10^{-3} M, resulted in no effect of Gentamicin on the stability of NBFs formed from the lipid mixture DPPC/PS.

It was found no effect of Gentamicin on the stability of TLFs formed from DMPC. The calcium presence, in 10^{-3} M, resulted also in no effect of Gentamicin on the properties of DMPC TLFs.

It was found no effect of Gentamicin on the TLFs hydrodynamic behavior.

The effect of Gentamicin on the stability of TLFs formed by PS suggests strong film monolayers perturbation followed by film monolayer fusion. The results were confirmed by other investigators (Aramaki et al. 1989) which postulate that the possible cause for the Gentamicin nephrotoxicity is kidney cell membrane fusion and depends on the presence of negatively charged phospholipids. We found an opposite effect of Gentamicin, an effect of stabilization the PS TLFs, when tested on the phase separated TLFs formed by DPPC/PS. These conclusions were further confirmed by the results obtained from the experiments with MFs.

The results observed from the MFs, suggest perturbation of the adsorbed monolayer formed from PS in presence of Gentamicin in comparison to the Gentamicin absence. Alternatively the presence of Gentamicin for DPPC/PS resulted to better surface lipid colloidal particle reorganization and spreading, and more densely packed MFs presenting better surface properties in comparison to DPPC/PS in absence of Gentamicin. Furthermore a shift in CMC to lower DPPC/PS concentrations was observed.

It was found no effect of Gentamicin due to calcium presence for brain PS lipid colloidal suspension. For the lipid mixture DPPC/PS in presence of calcium Gentamicin resulted only in shift of CMC to lower lipid concentration, from 10^{-4} M DPPC/PS to 10^{-7} M DPPC/PS.

The results from the dynamic MFs parameters γ_{\min} and γ_{\max} suggest better surface properties for DPPC/PS, in presence of Gentamicin, in comparison to the Gentamicin absence. Alternatively the surface dynamic parameters suggest bad surface packing and solid like behavior for MFs formed from PS due to Gentamicin presence in comparison to pure PS monolayers.

The authors hope that the present investigation will highlight the molecular mechanism of the Gentamicin nephro- and ototoxicity as well as calcium involvement in this pathology.

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FIGURE

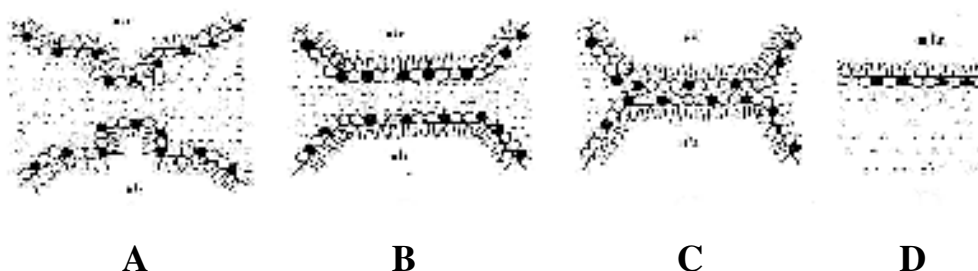


Fig. 1. Schematic representation of TLFs and monolayers at the air/water interface used in our study. Two types of TLFs are shown: Common black Film (B) and Newton Black Film (C). Thick TLF with black spot (A) is also presented. On D is shown phospholipid monolayer, which can be regarded as a half of bilayer film.

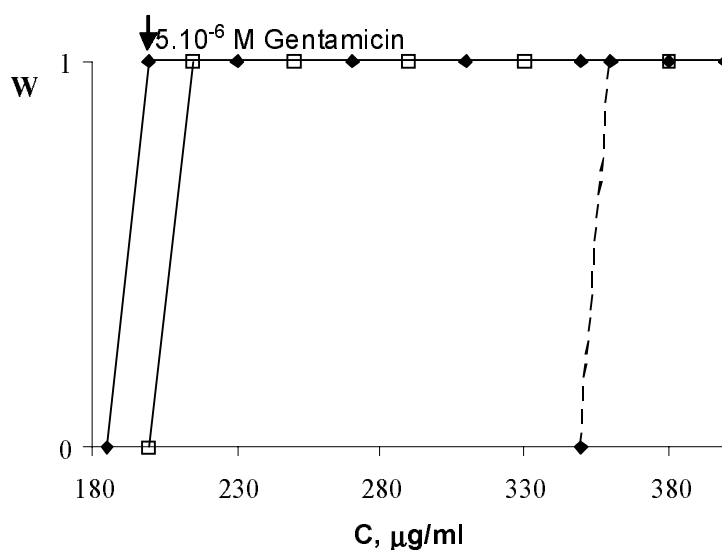


Fig. 2. Probability of CBF formation as dependence of brain PS (full line diamonds) bulk film concentration and in presence of Gentamicin (full line open squares). The probability of NBF formation as PS bulk film concentration in presence of calcium (dashed line full squares) is also presented.

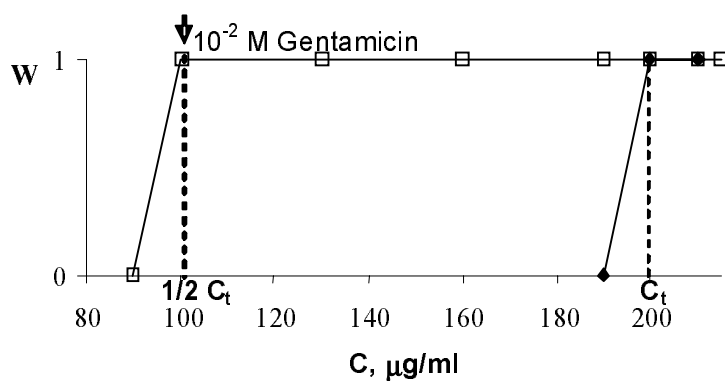


Fig. 3. Probability of NBFs formation as dependence of DPPC/PS (full line diamonds) bulk film concentration. The Gentamicin stabilization effect is presented as a decrease the DPPC/PS C_t to $1/2$ DPPC/PS C_t and stabilization of the NBFs due to Gentamicin presence (full line open squares).

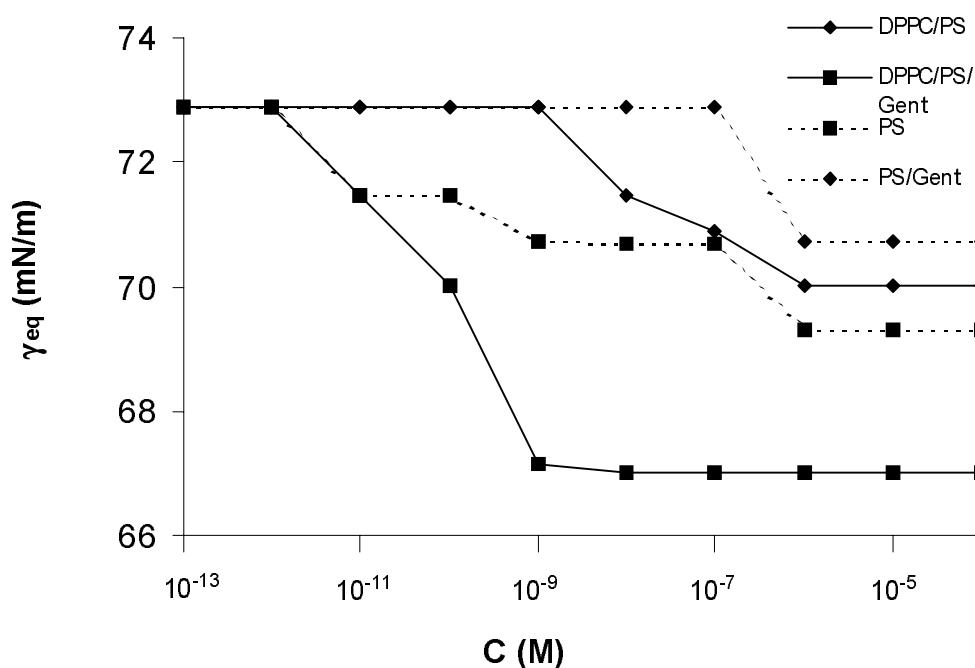


Fig. 4. Equilibrium surface tension (mN/m), γ_{eq} , vs. lipid concentration (M), and in presence of Gentamicin.

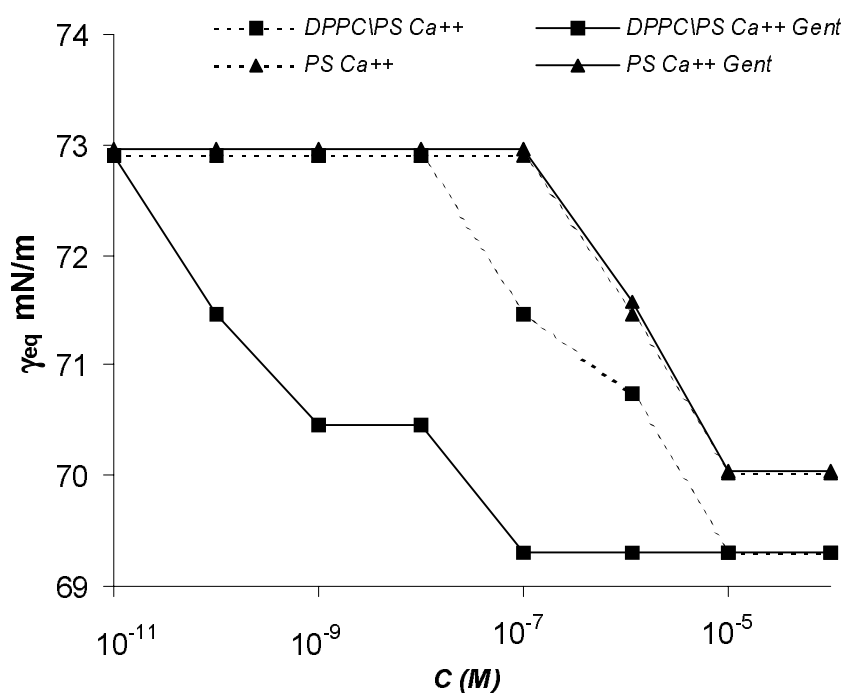


Fig. 5. Equilibrium surface tension (mN/m), γ_{eq} , vs. lipid concentration (M) at 10^{-3} M Calcium, and in presence and absence of Gentamicin.

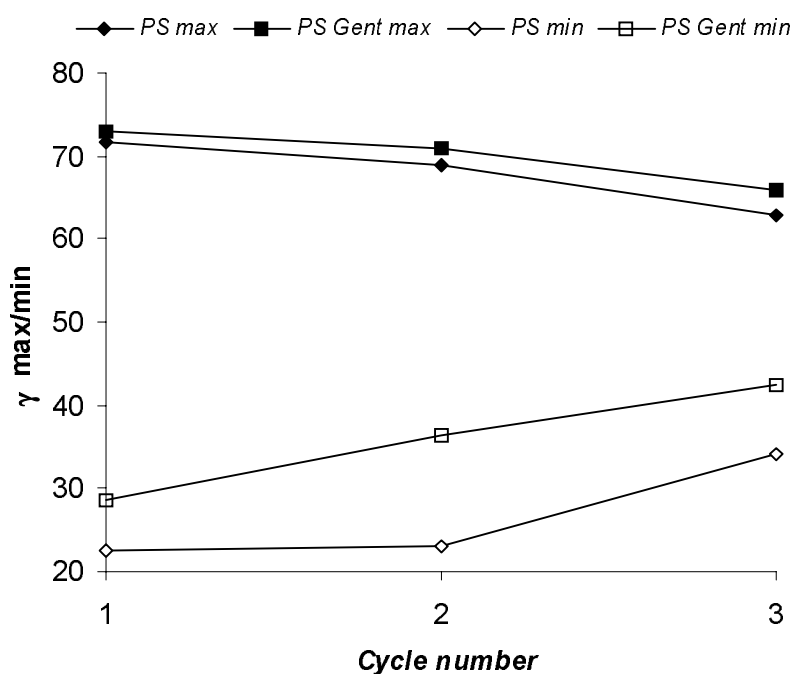


Fig. 6. Maximal and minimal surface tension, γ_{max} and γ_{min} , vs. compression-decompression cycle.

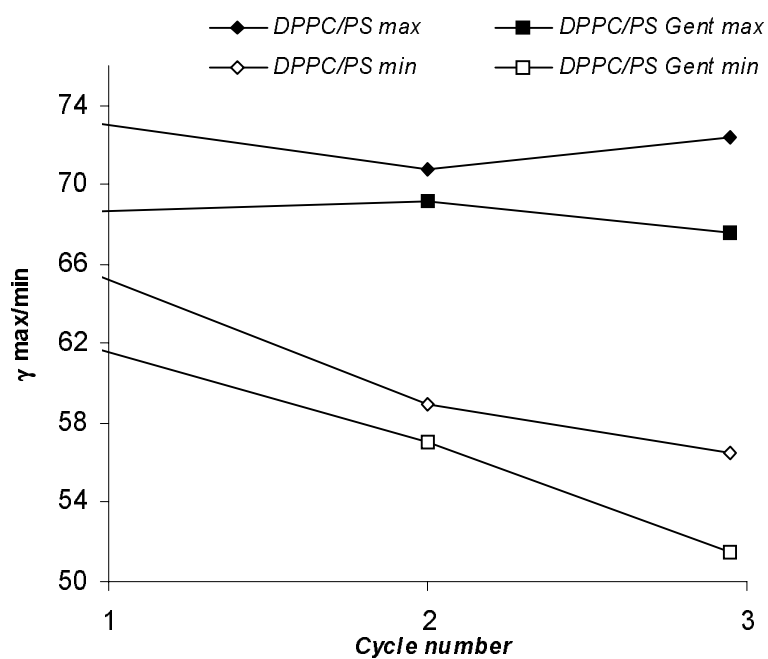


Fig. 7. Maximal and minimal surface tension, γ_{max} and γ_{min} , vs. compression decompression cycle.