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ORIGINAL PAPER

Interaction of gentamicin with phosphatidylserine/phosphatidylcholine mixtures in adsorption monolayers and thin liquid films: morphology and thermodynamic properties

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Abstract Gentamicin possesses strong adverse actions like oto and nephrotoxicity. The latter is a result of strong gentamicin–acid phospholipid interactions, resulting in cell fusion, fission, etc., ions as calcium interact with gentamicin and effectively deter its toxicity. In this work, the interactions of gentamicin and Ca^{2+} with phosphatidylserine/phosphatidylcholine (PS/PC) mixtures of different ratio are experimentally characterized. Special attention is paid to bridge thermodynamic and morphological properties of adsorption monolayers and thin liquid films (TLFs) composed of these lipid mixtures. Our results show that gentamicin decreases the stability of common black TLFs formed of pure PS coupled with suppression of lipid surface adsorption to the monolayers at the air–water interface; also, gentamicin reveals effects of lowering of lipid spreading on the interface and significant loss of material during monolayer cycling, increase of condensed phase, and organization of dense net-like domain monolayer texture. Gentamicin addition results in opposite effects for films formed of DPPC/PS (95:5) mixture. It increases the stability of Newton black TLFs formed by DPPC/PS correlated with faster and stronger surface adsorption and better surface spreading; also, gentamicin lowers the amount of condensed phase and organization of domains of smaller size. We also showed that Ca^{2+} itself decreases the stability of common black TLFs formed of PS accompanied with weaker surface adsorption, formation of higher amounts of condensed phase and organization of domains. In our experiments, Ca^{2+} softens, even deters, the effects of gentamicin on both PS and DPPC/PS films.

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Introduction

Aminoglycosides are antibiotic drugs used in medical practice for treatment of serious infections caused by aerobic Gram-negative bacilli (e.g., Enterobacteriaceae, *P. aeruginosa*). The aminoglycosides participate in a variety of regimens that target treatment of infections in the lower respiratory tract, intra-abdominal, soft tissue, bone or joint, wounds, and urinary tract, endocarditic, bacteremias, and meningitis.

In comparison with other aminoglycosides, gentamicin has lower bacterial resistance (based on locally generated antimicrobial susceptibility profiles) and therefore is used as an antibiotic of general purpose. Another major advantage over other aminoglycosides, e.g., tobramycin, amikacin, and netilmicin, is the lower cost. Recent literature reports suggest strong nephrotoxicity (Antoine et al. 2009; Dominguez et al. 1996; Fujii et al. 2009; Li et al. 2009; Ozbek et al. 2009; Rougier et al. 2004) and ototoxicity (Bates et al. 2002; Behnoud et al. 2009, de Aquino et al. 2008; East et al. 2005), including neonates (Baggio et al. 2009), caused by gentramicin. The plasma concentration monitoring is found to be a routine and in general effective way to avoid the nephrotoxicity and ototoxicity caused by the drug's narrow treatment window (Kahlmeter and Dahlager 1984). Nevertheless, toxicity still occurs despite apparently satisfactory drug concentrations.

Gentamicin belongs to the group of cationic amphiphilic drugs and contains a hydrophobic part (e.g., aromatic ring) and a hydrophilic moiety with nitrogen groups that delivers

a net positive charge at physiological pH (Reasor et al. 2006; Xia et al. 2000). The drug toxicity is related to its ability to (1) interfere with protein synthesis (Selimoglu 2007), and (2) electrostatically bind with negatively charged phospholipid headgroups (Reasor and Kacew 2001).

The positive charge and the amphiphilic properties "lend" high affinity of gentamicin to partition in membranes containing anionic phospholipids (e.g., phosphatidyleserine, PS) due to electrostatic and hydrophobic interactions (Forge et al. 1989; Kubo et al. 1986). In contrast, gentamicin does not bind to membranes composed entirely by neutral lipids (Alexander et al. 1979). In vitro experiments indicate multiple binding sites of aminoglycosides, particularly gentamicin, in the organs of Corti and vestibular maculae, and a lack of binding specificity to the liver, spleen, and heart of guinea pigs (Huy and Deffrennes 1988).

The interaction of the drug with anionic phospholipids is known to result in liposome aggregation and fusion (Aramaki and Tsuchiya 1989; Bambeke et al. 1995) and in kidney cell membrane fusion (Aramaki and Tsuchiya 1989). A major feature associated with the toxicity of gentamicin and numerous other cationic antibiotics is phospholipidosis, the excessive storage of "indigestible" lipid structures in the lysosomes (Vitovic et al. 2008). The phenomenon is most probably caused by the attractive electrostatic interactions of the drugs with the anionic lipid's headgroups that reduce the repulsion between the lipid bilayers and result in their adhesion or fusion in large multilamellar myeloid bodies. The resultant neutralization of the membrane's negative surface charge and decrease of total surface area makes the lipid aggregates insusceptible to the action of phospholipases and to further catabolism.

Recent studies show that some chemical agents, e.g., a 20-residue basic peptide N-WASP181-200 and its PEGylated version, inhibit renal accumulation of the aminoglycoside antibiotics such as gentamicin and arbekacin (Fujii et al. 2009). Other works demonstrate that peptide fragments that bind to the aminoglycoside binding receptor, such as megalin and/or peptides that interact with acidic phospholipids in a similar to the aminoglycosides fashion, might be useful to prevent aminoglycoside-induced nephrotoxicity (Kim et al. 1991; Watanabe et al. 2004). In this study, we focus on calcium; it is known that this ion interacts with aminoglycosides (e.g., gentamicin) in several ways including deterring the toxicity of gentamicin. Experimental data show that Ca^{2+} diminishes aminoglycoside binding or transport by Gram-positive and -negative bacteria (Bryan and Elzen 1977), rabbit vascular smooth muscle (Goodman 1978), renal mitochondria (Kornguth et al. 1980), and human serum proteins (Myers et al. 1978). Ca^{2+} in nutrient medium decreases in vitro the gentamicin antibacterial activity (Dornbusch 1980) and reverses, as well as prevents, the aminoglycoside-mediated neuromuscular blockade due to reduced gentamicin binding to cell membranes (Kornguth et al. 1980). In addition, Bennett et al. observed lower gentamicin nephrotoxicity in dietary calcium-loaded F344 rats (Bennett et al. 1982). Thus, it is worth quantitatively studingy, on a model level, the interactions between gentamicin, anionic phospholipids, and Ca^{2+}.

The adhesion/fusion of the lipid bilayers is due to changes of the normal interactions between the oriented head-to-head outer membrane monolayers. Thus, it makes sense to examine the interaction of gentamicin (in the absence or presence of Ca^{2+}) with phospholipids in a model membrane system specially designed to probe the head-to-head interactions between the two approached monolayers. Such systems are the thin liquid films (TLFs) also known as foam films. TLFs, being of several types, are composed of two mutually adsorbed, oriented "head-to-head", phospholipid monolayers (Fig. 1a–c), thus representing the contact area occurring between the *cis*-monolayers of two adhering or fusing membranes (Georgiev and Lalchev 2004; Georgiev et al. 2007; Naydenova et al. 1990). They are already successfully used as a model system to study membrane–membrane interactions, like adhesion and fusion (Georgiev and Lalchev 2004; Georgiev et al. 2007; Naydenova et al. 1990), and find numerous applications varying from examination of the anti-adhesive properties of polymer-grafted surfaces (Georgiev and Lalchev 2004) to the prenatal diagnostics of the stability of the pulmonary surface film (Eksperova et al. 1984; Lalchev 1997, 2007). As a complementary model system, adsorption monolayers are used, which can be considered as one half of a foam film. Therefore, by combining the models of thin liquid films and monolayers, we intend to develop an adequate methodology to quantitatively study the interactions that take place between gentamicin (representative phospholipidosis-inducing drug), anionic phospholipids (e.g., PS), and Ca^{2+} ions.

We have investigated the equilibrium adsorption, dynamic monolayer compression/decompression behavior, and kinetics of lipid adsorption, and compared the thermodynamic data with corresponding Brewster angle microscopy (BAM) micrographs. Some BAM studies recently provide evidence for the formation of condensed phase domains, and thus revealed a first-order phase transition in adsorption layers and demonstrated for aqueous DHBAA (*N*-dodecyl-hydroxybutiric acid amide) solutions (Hénon and Meunier 1993). Following this idea, we also aim to link the thermodynamic properties of adsorption monolayers and TLFs with the adsorption layer morphology (surface texture and domain size and shape) since it would provide various, potentially complementary, qualitative and quantitative experimental characteristics of the studied interactions.

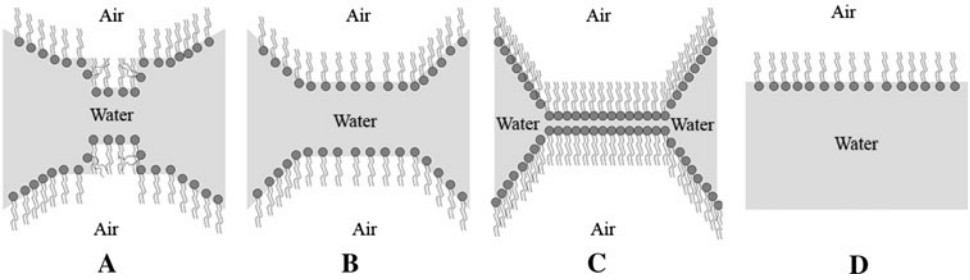

Fig. 1 **a**–**c** Schematic representation of thin liquid films (TLFs) and monolayer at the air–water interface. **a** TLF with black spot. **b** Common black film. **c** Newton black film. **d** Phospholipid monolayer, which can be regarded as one half of the TLF

Materials and methods

Materials

Brain phosphatidylserine (PS) with average Mw 812.041 (stearoyl and oleoyl as the predominant fatty acids), dipalmitoylphosphatidylcholine (DPPC), and dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids. Gentamicin was purchased from Sigma. NaCl and $CaCl_2$ were purchased from Merck.

Lipid dispersions were made with bidistilled water with conductivity less than 1 μS and further used to form adsorption monolayers and TLFs. Film forming dispersions used (stock solutions of PS and DPPC/PS (95/5, mol/mol mixture) had total concentration = 1,000 μg/ml) and constant (evaluated with conductivity measurements) ionic strength $I = 0.25$ M. For the experiments in the absence of Ca^{2+}, the desired ionic strength was reached by using NaCl solution only, while for the experiments in the presence of Ca^{2+}, (1 mM), NaCl and $CaCl_2$ solutions were mixed.

Gentamicin was dissolved in the corresponding electrolyte solution and sonicated for up to 1 min before usage. The gentamicin solution was involved in the monolayer studies by injection of 20 μl into the monolayer subphase and of 5 μl into the menisci of TLF-forming biconcave drop, as described elsewhere (Georgiev and Lalchev 2004; Naydenova et al. 1990). Experiments were conducted at 37°C and pH 6.8–7.0.

Methods

Thin liquid films

Thin liquid films (TLFs) were formed by the method of Scheludko and Exerowa (Exerowa and Krugliakov 1998) using the modified measuring cell as previously described by Lalchev (1997). A biconcave drop (50 μl volume) of the phospholipid dispersion was incubated in the cylinder of the measuring cell at 37°C for 30 min. After aspiration of solution from the drop, thick TLF is formed (Fig. 1). Next, the film spontaneously gets thinner and, after some characteristic film thinning time, t_{0-1} (s), critical film thickness (ca. 30 nm) is reached. Then a black spot (BS) local thinning in the film appears (as schematically shown in Fig. 1a), and expands with atcharacteristic rate to fill the whole area of the film. The kinetic of this process was measured by BS expansion time t_{1-2} (s), 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 film formation. Under different experimental conditions, two types of black films (BF) can 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 very strongly on the phospholipid concentration (Exerowa and Krugliakov 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 whether the films always rupture ($W = 0$) or whether the films are always stable ($W = 1$). The dependence $W(C)$ is extremely steep which permits definition of a threshold concentration (C_t) as the minimum phospholipid concentration at which $W = 1$ and stable films are always formed (Lalchev 1984, 2007). It has been proved that $W(C)$ dependence, in addition to the concentration, is very sensitive to the composition, molecular shape, and phase state of the film forming phospholipids, etc. (Exerowa and Krugliakov 1998; Lalchev 1997). The equilibrium water thickness of the thin liquid films was measured by the microinterferometric technique (Exerowa and Krugliakov 1998) with resolution of 0.5 nm.

Monolayers (MFs)

The adsorbed monolayers were composed of a PS and DPPC/PS (95/5, mol/mol) mixture in the presence of Ca^{2+}, and gentamicin was formed in a Langmuir trough and the surface tension γ (mN/m) was measured by the method of Wilhelmy with an accuracy ±0.5 mN/m as previously described (Christova et al. 1999; Georgiev and Lalchev 2004). The equilibrium γ (γ_{eq}) was measured after 30-min delay for adsorption of the lipid and lipid/gentamicin mixtures. During compression/decompression of the monolayers, maximum γ and minimum γ were measured at 100% of the area at the end of decompression and 50% of the initial area at the end of compression, respectively. PS and DPPC/PS

were used for compression/decompression experiments at the corresponding equilibrium γ/concentration plateau values (details provided in Results").

Adsorption kinetics and BAM

Adsorption monolayers composed of PS and DPPC/PS (95/5, mol/mol) in the presence of Ca^{2+} and gentamicin were formed in a Langmuir trough and the surface pressure π (mN/m) during time measured with an accuracy ± 0.01 mN/m in Teflon coated Kibron Microtrough-X (Kibron, Helsinki, Finland) with an area of 118 cm^2. The subphase volume was 20 ml. The instrument uses the Wilhelmy method with a platinum wire probe attached to the microbalance sensor head. The stock lipid dispersions were applied with a Hamilton microsyringe trough injection of 20 µl of stock solution (concentration = 1,000 µg/ml) into the subphase to reach the desired concentration. The trough temperature (37°C) was controlled within ± 0.5°C. Each measurement was repeated at least three times with each separate sample.

Brewster Angle Microscopy (BAM) micrographs were taken simultaneously during the measurements of adsorption kinetics with micro BAM2 (Nima Technology) mounted on the trough corpus.

Results

Thin liquid films

The probability for black film formation on PS concentration dependence (Fig. 2) reveals the formation of stable common black films[1] by phosphatidyleserine (PS) at threshold concentration $C_t = 200$ µg/ml ($W = 1$, black squares). An amount of 5×10^{-6} M gentamicin in the film forming solution destabilizes and ruptures the film at 200 µg/ml ($W = 0$, open squares, Fig. 2), shown as a shift of C_t to higher values (210 µg PS/ml) for $W = 1$. In the presence of Ca^{2+}, the PS forms Newton black films[2] at $C_t = 360$ µg/ml. The addition of gentamicin to the PS/Ca^{2+} has no influence on the film stability or film type (Fig. 2).

The W/C dependence of mixed DPPC/PS (95/5 mol/mol) films in the presence of gentamicin is shown on Fig. 3. Stable Newton black films are formed from DPPC/PS only above $C_t = 200$ µg/ml. The addition of 1×10^{-2} M gentamicin provokes the formation of stable black films ($W = 1$) even at half (100 µg/ml) of the sample concentration (200 µg/ml). The stabilization is concentration dependent—with the addition of only 1×10^{-6} M gentamicin, stable NBFs are formed at 185 µg/ml. No changes of the black film stability or type for the samples DPPC/PS and DPPC/PS/gentamicin occurred in the presence of Ca^{2+} ions up to 1 mM.

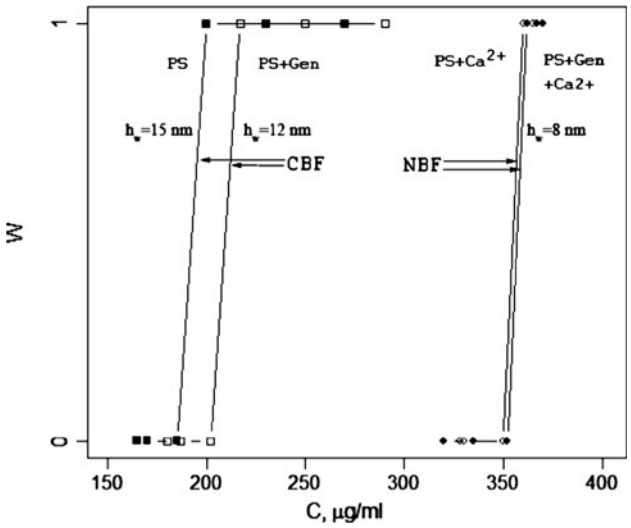

Fig. 2 Dependence of the probability for black film formation (W) on PS concentration (C) for film forming solutions of pure PS (*black squares*), PS plus 10^{-6} M gentamicin (*open squares*), PS in 1 mM Ca^{2+} (*black diamonds*), and PS in 1 mM Ca^{2+} plus 1×10^{-6} M gentamicin (*open diamonds*). Films that are formed in the absence of Ca^{2+} are of CBFs type, while films formed in the presence of Ca^{2+} are of NBFs type. The equilibrium water thickness (h_w) of the thin liquid films is measured by the microinterferometric technique with resolution of 0.5 nm. Experiments were conducted at 37°C, $C_{el} = 0.25$ M, and pH 6.8–7.0

To determine the effects of gentamicin and Ca^{2+} on the stability and type of films composed entirely by zwitterionic lipids, we studied TLFs formed by DMPC. DMPC forms stable CBFs and the addition of gentamicin and Ca^{2+} has no detectable effects on film stability or film type (data not shown).

In summary,our results indicate experimentally detectable and opposite effects of gentamicin on the stability of thin films: a destabilization (increase in the C_t values) of TLFs made by pure PS and stabilization (decrease in the C_t values) of films formed by the mixture DPPC/PS. Finally, the addition of Ca^{2+} to PS films abolished the effect of gentamicin, e.g., the drug does not rupture Newton films formed by PS/Ca^{2+}.

The addition of 10^{-6} M gentamicin was the minimal concentration at which reproducible effects on the stability of foam films are observed, and it was selected for rest of the experiments. The drug concentrations used in assessments of the antibiotic toxicity in vivo vary significantly (Antoine et al. 2009; Bryan and Elzen 1977; Fujii et al. 2009; Hénon and Meunier 1993; Huy and Deffrennes 1988, 1990; Li et al. 2009; Ozbek et al. 2009). Experimental

[1] Bilayers containing a water core between the film monolayers.

[2] Bilayers without water core between the film monolayers.

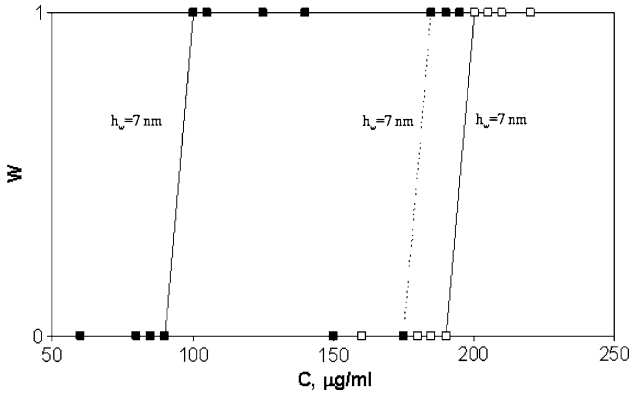

Fig. 3 Dependence of the probability for Newton black films formation (*W*) on the phospholipid concentration (*C*) for film forming solutions containing the mixture DPPC/PS (95/5, mol/mol). Stable DPPC/PS films are formed above $C_t = 200$ µg/ml (*full line*, *open squares*). Addition of 1×10^{-6} M (*dashed line*, *full squares*) and 1×10^{-2} M (*full line*, *full squares*) gentamicin to DPPC/PS films at 185 and 100 µg/ml provokes stable film formation instead of rupture of DPPC/PS control films (without gentamicin) at this concentration. The equilibrium water thickness (h_w) of the thin liquid films is measured by the microinterferometric technique with resolution of 0.5 nm. Experiments were conducted at 37°C, $C_{el} = 0.25$ M NaCl, and pH 6.8–7.0

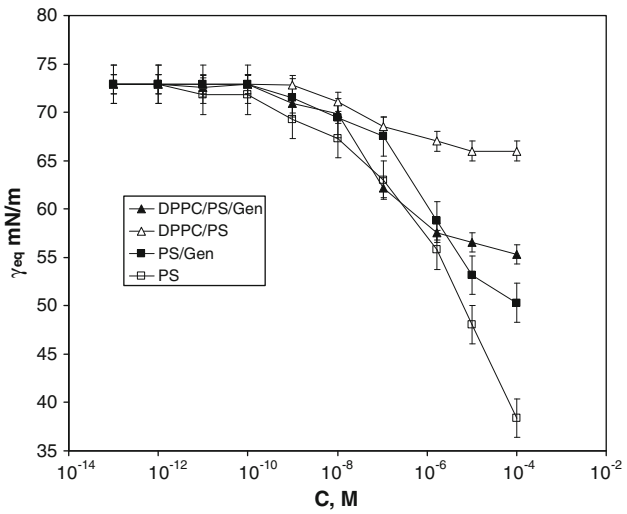

Fig. 4 Dependence of equilibrium surface tension (γ_{eq}) on the total lipid concentration (*C*) of adsorption monolayers made from PS (*open squares*), PS in the presence of 10^{-6} M gentamicin (*black squares*), DPPC/PS (*open triangles*), and DPPC/PS in the presence of 10^{-6} M gentamicin (*black triangles*). Experiments were conducted at 37°C, pH 6.8–7.0, and electrolyte concentration $C_{el} = 0.25$ M NaCl

screening for binding sites of gentamicin in homogenates containing cochlear and vestibular structures use a concentration range of gentamicin, 10^{-9} to 10^{-3} M gentamicin, and observe dissociation constants for gentamicin, respectively 1.2×10^{-6} and 3×10^{-7} M (Huy and Deffrennes 1988). A model membrane study on the detachment of cytochrome c by cationic drugs (including gentamicin) from monolayers of acidic phospholipids utilized drug concentrations of 2×10^{-6} to 6.3×10^{-7} M (Jutila 2001). Thus, the concentration of 10^{-6} M gentamicin used in our experiments correlates well with the amounts used by other researchers in model membrane studies of gentamicin–lipid interactions.

Monolayer films

Data on equilibrium surface tensions

Figure 4 shows the equilibrium surface tensions (γ_{eq}) concentration dependence of monolayers formed by adsorption of PS or the mixture DPPC/PS (95/5, mol/mol) from saline solution subphase and 10^{-6} M gentamicin. The graph reveals that the curve showing the dispersion of PS reaches plateau values at $\gamma_{eq} = 38$ mN/m (at $\sim 10^{-4}$ M PS) while the curve of PS/gentamicin reaches a plateau at higher values, $\gamma_{eq} = 50$ mN/m. The curve representing the dispersion of DPPC/PS reaches a detectable plateau at $\gamma_{eq} = 67$ mN/m (at 10^{-5} M total concentration of the mixture), and the addition of gentamicin results in decrease of the surface tension values to 57 mN/m (at 10^{-6} M of the mixture). Therefore, the concentration at which a plateau in the curve for DPPC/PS is observed is an order of magnitude higher in comparison to the curve for DPPC/PS with additional gentamicin. We can conclude that gentamicin significantly alters the behavior of the PS dispersions, and especially the solubility and surface activity dispersions containing the DPPC/PS.

Although all the samples in the previous set of experiments are subjected to sonication at the moment of preparation and just before injection of the solution in the subphase (at experiment time), the PS data show variability of ±2 mN/m (represented by error bars in Fig. 4). Similar data behavior is observed for the samples containing DPPC/PS but to a lesser extent—approximately ±1 mN/m. After analysis of the data from multiple experiments with PS and PS/gentamicin, the curves show clear differences above concentrations of 10^{-6} M PS. The data curves for DPPC/PS and DPPC/PS/gentamicin show clear differences above concentrations of 10^{-5} M DPPC/PS.

The dependences of equilibrium surface tension of adsorption monolayers made from PS and DPPC/PS dispersions on the lipid bulk concentration with gentamicin (10^{-6} M) and Ca^{2+} (1 mM) in the subphase are shown in Fig. 5. The γ_{eq} plateau value at PS dispersion in the presence of calcium is 42 mN/m at 10^{-5} M PS. A slightly different γ_{eq}/C curve is observed for the sample in the presence of gentamicin. The curve shows near 5 mN/m higher plateau values and a different curve slope. However, both curves reach a plateau value at the same concentration (10^{-5} M PS). Similarly to the data shown in Fig. 4, we observed variability (±2 mN/m) of the γ_{eq}/C data. The two

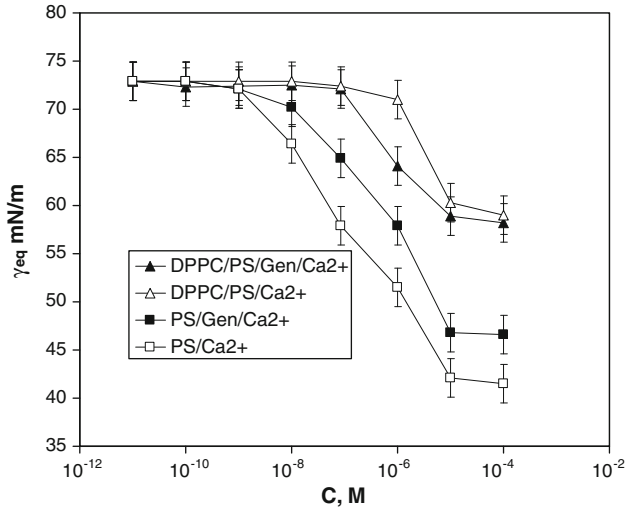

Fig. 5 Dependence of equilibrium surface tension (γ_{eq}) on the total lipid concentration (C) of adsorption monolayers made from PS in the presence of Ca^{2+} (*open squares*), PS in the presence of Ca^{2+} and 10^{-6} M gentamicin (*black squares*), DPPC/PS in the presence of Ca^{2+} (*open triangles*), and DPPC/PS in the presence of Ca^{2+} and 10^{-6} M gentamicin (*black triangles*). Experiments were conducted at 37°C and pH 6.8–7.0

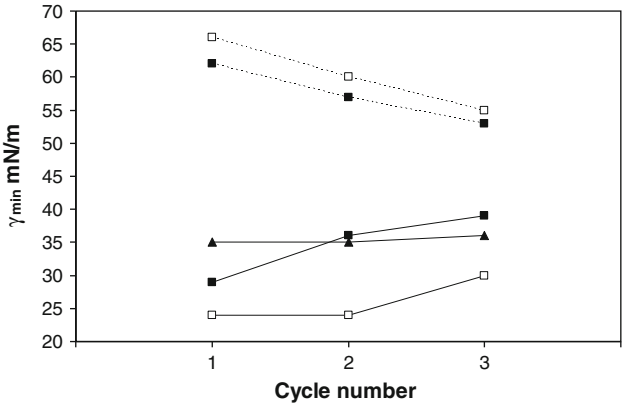

Fig. 6 Dynamic surface tension (γ_{min}) values of PS (*solid line*) and DPPC/PS (*dotted line*) adsorbed monolayers on the number of compression/decompression cycles. *Open squares* denote pure DPPC/PS (200 μg/ml) and PS (concentration = 200 μg/ml) monolayer samples; *black squares* indicate gentamicin presence (PS and DPPC/PS with concentration 210 and 100 μg/ml, respectively). *Black triangles* indicate presence of Ca^{2+} instead of gentamicin in the PS samples (concentration = 360 μg/ml). The presence of Ca^{2+} and gentamicin produces similar results to the *black triangles* curve, e.g., gentamicin does not change the dynamic surface properties of the PS/Ca^{2+} samples. Ca^{2+} does not have measurable effects on the samples DPPC/PS (not shown). PS and DPPC/PS concentrations for all samples represent the threshold concentration at which stable black films are formed as shown in Figs. 2 and 3. Experiments were conducted at 37°C, pH 6.8–7.0, and electrolyte concentration C_{el} = 0.25 M NaCl in the presence and absence of 10^{-6} M gentamicin

curves show detectable differences above concentrations equal to 10^{-8} M PS.

Figure 5 demonstrates a γ_{eq} plateau value equal to 59 mN/m for the sample DPPC/PS with Ca^{2+} at 10^{-5} M of the mixture. In the presence of gentamicin, a similar plateau value is observed. However, the curve is shifted to lower concentrations but the effect is clearly detectable only at 10^{-6} M of the mixture.[3]

Comparing the adsorption monolayers formed by PS shown in Figs. 4 and 5, these can be arranged in ascending order of the corresponding plateau values: PS < PS/Ca^{2+} < PS/Ca^{2+}/gentamicin < PS/gentamicin. For the samples containing DPPC/PS, the order is DPPC/PS/gentamicin < DPPC/PS/Ca^{2+} = DPPC/PS/Ca^{2+}/gentamicin < DPPC/PS. The results showan increase in the plateau values for the samples containing gentamicin and PS. However, a decrease of plateau values is found for the mixture DPPC/PS in the presence of gentamicin. For both the samples, e.g., DPPC/PS and PS, the effects are sealed by Ca^{2+}. They are demonstrated by the equivalent γ_{eq} plateau values of the samples DPPC/PS/Ca^{2+} = DPPC/PS/Ca^{2+}/gentamicin, and also by the order PS/Ca^{2+} < PS/Ca^{2+}/gentamicin < PS/gentamicin. Other evidence is the result showing that both curves containing DPPC/PS/Ca^{2+} and DPPC/PS/Ca^{2+}/gentamicin (Fig. 5) are almost identical—having differences only at 10^{-6} M DPPC/PS. A similar effect is found for the two curves representing PS/Ca^{2+} and PS/Ca^{2+}/gentamicin, respectively. Although the curves do not overlap statistically, the differences are much smaller compared to the same curves without Ca^{2+} shown in Fig. 4.

Data on dynamic surface tensions

Dynamic surface tension data were obtained by continuous and consecutive compression/decompression cycling of the already formed adsorption monolayers made of PS and of DPPC/PS mixtures. At the end of the compression part of a cycle, the γ_{min} value was measured; the data in the form of a γ_{min}/cycle number dependence are presented in Fig. 6. The graph shows the first three cycles per sample as the remaining ones coincide with the third one.

The curve depicting the DPPC/PS sample continuously decreases, with ca. 10 mN/m, with the increase of the cycle number. The same trend has the curve depicting DPPC/PS/gentamicin; however ,the γ_{min} value is ~5 mN/m lower for all the cycles under consideration. The values of γ_{min} at dynamic conditions are determined by at least of two processes: (1) disintegration and re-spreading of surface condensed phases during monolayer decompression, and (2) adsorption to the interface during monolayer decompression of the molecules which probably undergo desorption

[3] See error bars of the curves DPPC/PS/Ca^{2+} and DPPC/PS/Ca^{2+}/gentamicin at 10^{-6} M of Fig. 5.

into the subphase during compression (Adamson and Gast 1997). We consider the behavior (γ_{min} values) of the monolayers at dynamic (compression/decompression) conditions as a result of the combined action of the two above-mentioned interfacial processes, as their kinetics are comparable to the rate of the compression/decompression cycling (3 min/cycle).

In contrast to the continuous decrease of the γ_{min} values for the DPPC/PS monolayers (Fig. 6, dotted line), the minimal surface tension of PS adsorption films continuously increase during the compression/decompression cycling (Fig. 6, solid line). The curve, depicting pure PS monolayers, obtain a constant γ_{min} = 25 mN/m for the first and second cycles but increases with 5 mN/m for the third cycle. In contrast, the curve depicting PS/gentamicin continuously increases with the cycle number. The addition of gentamicin results in ~10 mN/m γ_{min} increase for all cycles.

The curves, depicting PS and PS/gentamicin monolayers, continuously increase (in γ_{min} terms) from 25 to 30 mN/m and from 29 to 40 mN/m, respectively, with the increase of the cycle number. In contrast, the curves for the monolayers formed from adsorption of DPPC/PS and DPPC/PS/gentamicin mixture, continuously decrease from 66 to 55 mN/m and from 62 to 53 mN/m, respectively. Another noticeable difference in Fig. 6, comparing the samples PS and DPPC/PS, is "the gentamicin effect"; the addition of this antibiotic to PS adsorption monolayers (solid line, open squares) results in a shift of the curve towards higher values in γ_{min} terms (full line, black squares), while gentamicin in the subphase of DPPC/PS monolayers (dotted line, open squares) shifts the curve to lower γ_{min} values (dotted line, black squares).

Adsorption kinetics and BAM

The adsorption kinetics and morphological properties of monolayers made from adsorption of PS and DPPC/PS in the presence of gentamicin and Ca^{2+} are studied and summarized in Figs. 7 and 8, respectively. It can be seen that distinct inflection point occurs in the continuous course of the $\pi(t)$ data. Vollhardt and Melzer (1997) used the term first-order phase transition to denote the changes in the slope of the surface tension transients during adsorption of lipids and the corresponding "changes in alkyl chain packing" observed in the surface film. Here we correlate the inflexion points in the surface tension transients to the surface morphologies monitored by BAM.

Figure 7 shows the pressure–time (π–t) "transients" in the kinetics of PS adsorption from a saline solution subphase: pure (PS bulk concentration C = 200 μg/ml) and in the presence of 1 mM Ca^{2+} (PS C = 360 μg/ml) or 10^{-6} M gentamicin (PS C = 210 μg/ml). PS concentrations for all the three samples represent the threshold concentration at

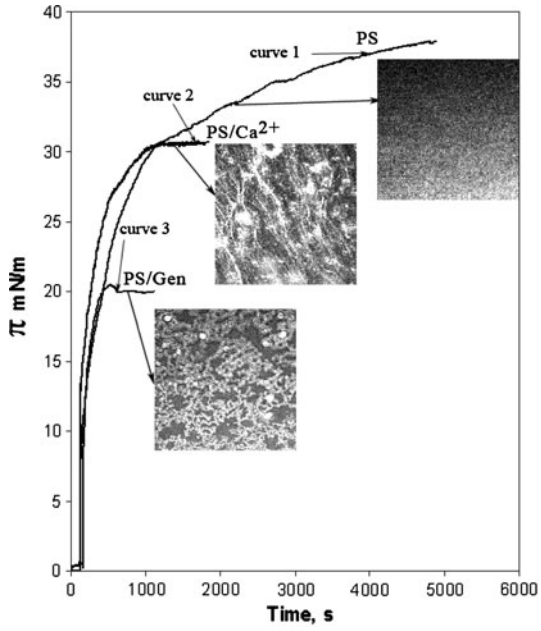

Fig. 7 Pressure–time (π–t) adsorption kinetics at bulk PS concentration C = 200 μg/ml (*curve 1*); at PS concentration = 360 μg/ml in presence of 1 mM Ca^{2+} (*curve 2*), and at PS concentration = 210 μg/ml in the presence of 10^{-6} M gentamicin (*curve 3*). PS concentrations for the three samples represent the threshold concentration at which stable black films are formed as shown in Fig. 2. The characteristic BAM images of the PS, PS/Gen, and PS/Ca^{2+} adsorption monolayers in the two-phase coexistence region at 37°C are inserted. The *arrows* indicate the times at which the BAM images were taken; the image dimensions are 1,200 × 1,200 μm. Experiments were conducted at C_{el} = 0.25 M and pH 6.8–7.0

which stable common black and Newton black films are formed (Fig. 2). The characteristic BAM images of the PS, PS/gentamicin and PS/Ca^{2+} adsorbed layers in the phase coexistence region at 37°C are inserted. The arrows indicate the times and π values at which the BAM images were taken. At conspicuous transition points, starting at 20 and 32 mN/m for PS/gentamicin and PS/Ca^{2+}, respectively, followed by a transition plateau regions, are observed. In the course of the experiments, the PS sample obtain the highest π values of 37.92 mN/m in 5,000 s, while PS/Ca^{2+} and PS/gentamicin reach, respectively, π values of 30.7 and 19.9 mN/m much faster, at 1,300 and 500 s, respectively. The morphological properties of the condensed phases formed in the two-phase coexistence region are characteristic for each sample. BAM micrographs of PS adsorbed layers show absence of condensed phase at this resolution; in contrast, PS/Ca^{2+} and PS/gentamicin monolayers show drastically increased amounts of condensed phase. In the case of PS/Ca^{2+}, extended condensed phase domains (ca. 100–130 μm) are formed; the PS/gentamicin micrograph represent a dense net of interconnected domains (ca. 20–40 μm) with more than one growth direction.

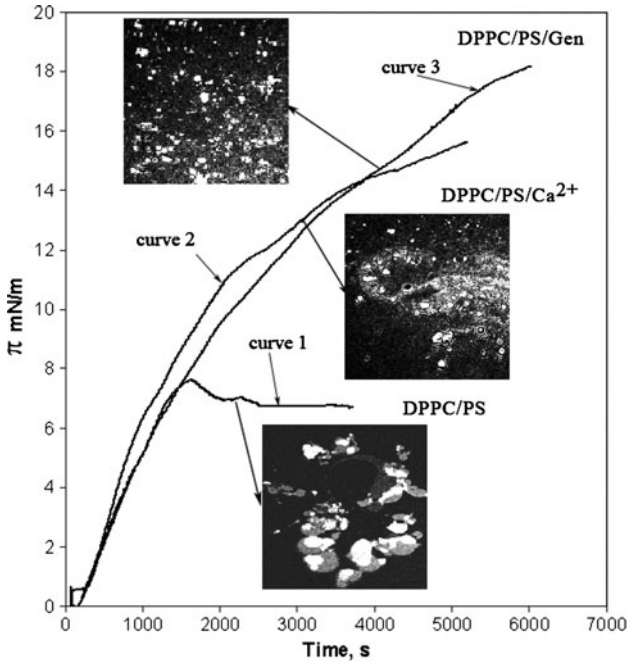

Fig. 8 Pressure–time (π–t) adsorption kinetics of DPPC/PS mixture in addition of Ca^{2+} and gentamicin. *Curve 1* shows the DPPC/PS sample, *curve 2* indicates Ca^{2+} presence in the film, and *curve 3* shows gentamicin presence instead of Ca^{2+}. DPPC/PS concentrations for the three samples represent the threshold concentration at which stable Newton black films are formed as shown in Fig. 3 (e.g., 200 μg/ml for *curve 1* and *curve 2* samples and 100 μg/ml for the *curve 3* sample). The *arrows* indicate the times at which the BAM images were taken; the image dimensions are 1,200 × 1,200 μm; experiments were conducted at 37°C, C_{el} = 0.25 M and pH 6.8–7.0

Figure 8 shows the pressure–time (π–t) "transients" of the kinetics of DPPC/PS adsorption from saline solution subphase: pure (phospholipid C = 200 μg/ml) and in presence of 1 mM Ca^{2+} (phospholipid C = 200 μg/ml) or 10^{-6} M gentamicin (phospholipid C = 100 μg/ml). DPPC/PS concentrations for the three samples represent the threshold concentration at which stable Newton black films are formed, shown in Fig. 3. The characteristic BAM images of the DPPC/PS, DPPC/PS/gentamicin, and DPPC/PS/Ca^{2+} adsorbed monolayers in the phase coexistence region at 37°C are inserted. The arrows indicate the times at which the BAM images were taken. In the course of the experiments, the DPPC/PS/gentamicin sample reaches the highest π values of 18.2 mN/m in 6,000 s, while DPPC/PS/Ca^{2+} reach π values of 15.6 mN/m and DPPC/PS/gentamicin reach π = 7 mN/m much faster, at 2,300 s.

As already depicted for PS samples in Fig. 7, the morphological properties of the condensed phases formed in the phase coexistence region are characteristic for each sample. BAM micrographs of DPPC/PS monolayers formed from adsorption show condensed phase domains similar to the domains observed by other authors with the same mixture but using fluorescent microscopy and Langmuir (spread) monolayers (Jutila 2001). The domainsof the DPPC/PS monolayers are relatively large (ca. 100–150 μm) with a distinct roundish shape. In contrast, DPPC/PS/Ca^{2+} and DPPC/PS/gentamicin monolayers show smaller domains (ca. 50–70 μm), again with a distinct roundish shape. The DPPC/PS (Fig. 8, curve 1) domains seem to show evidence of bilayer formation or at least two distinct thicknesses in the film plane. This might be attributed to the fact that as the films are formed by adsorption of concentrated lipid dispersions the observed adsorption layers could contain various multilayer, vesicular, and multilammellar structures of different thicknesses as reflected by the variations of the gray scale intensity of the BAM images.

Discussion

The purpose of the current work is to examine the interaction of the cationic antibiotic gentamicin with the anionic PS (pure and mixed with DPPC) in thin liquid films. In contrast to the commonly used "normal" vesicular bilayers, TLFs (also known as foam films) represent inverted bilayers, i.e., they are composed of two oriented head-to-head phospholipid monolayers. Thus, TLFs permit the examination of the normal interactions between the *cis*-monolayers of the natural biomembranes and the changes induced by the interaction with gentamicin; a phenomenon closely associated with the toxicity of the antibiotic in vivo at various sites like the kidney plasma membrane, lysosomal membranes, cell membranes in the organ of Corti, vestibular maculae, etc. Adsorption monolayers are used in order to correlate the changes in the TLFs thermodynamic properties with the alteration in the lateral interactions between the lipid molecules caused by the drug. Calcium cations are used in part of the experiments as they are known to strongly affect the binding of gentamicin to the headgroups of anionic phospholipids.

The thick, 15 nm, phosphatidylserine CBFs (Fig. 1b) transform to the thinner, 12 nm, CBFs in the presence of gentamicin (Fig. 2) and to the even thinner (8 nm) NBFs (Fig. 1c) in the presence of gentamicin/Ca^{2+} and Ca^{2+} alone (Fig. 2). This can be interpreted in terms of decreased electrostatic disjoining pressure in the foam films due to the binding of the cations to the anionic PS headgroups, which results in neutralization of the negative surface charge. The result is reasonable as gentamicin and Ca^{2+} are known to avidly bind liposomes composed of acidic phospholipids resulting in charge neutralization and tightening of the lipid packing (Brasseur et al. 1984; Chung et al. 1985; Gurnani et al. 1995; Kubo et al. 1986), aggregation, and fusion (Aramaki and Tsuchiya 1989; Bambeke et al. 1995).

In addition to the changes of the film thickness and type, the stability of PS black films is also decreased by gentamicin and Ca^{2+}. The destabilization is manifested by the shift of BF formation probability (W) to higher threshold concentration values (C_t). The C_t value represents the minimal bulk surfactant concentration at which sufficiently dense interfacial molecular packing is achieved in order for stable black films to be formed (Exerowa and Krugliakov 1998). This finding strongly correlates with the tensiometric measurements. They reveal weaker surface adsorption (Fig. 7) and slower monolayer spreading (Fig. 6) expressed as higher γ_{min} values and increased loss of material during monolayer compression/decompression (the continuous increase of γ_{min} values during cycling).

The decreased stability of PS/gentamicin TLFs and the aggravated adsorptive properties of PS/gentamicin dispersions (in the absence or presence of Ca^{2+}) when compared to the pure PS samples is associated with dramatic change in the morphology of the PS adsorption layers induced by the cations. The addition of gentamicin results in increase of the size of the condensed phase domains that merge into a net-like texture (Fig. 7). The observed effect can be explained by neutralization of the surface negative charges due to the binding of gentamicin to the anionic lipid headgroups, an interaction known to be of crucial importance for a wide range of in vivo processes: lysosomal phospholipases inhibition (Carlier et al. 1983; Mingeot-Leclercq et al. 1988; Mingeot-Leclercq and Tulkens 1999; Soejima et al. 2000), and inducement of phospholipidosis (Baronas et al. 2007; Hirode et al. 2008; Mingeot-Leclercq et al. 1989; Soejima et al. 2000) and cell apoptosis (Servais et al. 2008).

However, our results indicate another potential cause for the gentamicin toxicity—the change in the lipid phase state that might result in inhibition of various membrane proteins. This idea is exploited by a computer-aided conformational analysis on a simulated air–water interface, where gentamicin is shown to display a largely open crescent shape. When surrounded by phosphatidylinositol, it remained as such at the interface, which it locally *mis*-shapes, establishing a close contact with the negatively charged phospho-groups (Mingeot-Leclercq et al. 1989; Soejima et al. 2000). The changes in the phase heterogeneity of the PS adsorption layers caused by the antibiotic might explain the destabilization and rupture of the PS/gentamicin foam films. It is well known that the fusion could be driven by the domain boundary tension in heterogeneous membranes (Döbereiner et al. 1993; Georgiev et al. 2007; Lipowsky 1995). The capability of gentamicin to induce gelation of natural biomembranes is demonstrated by Kohlhepp et al. (1994) who measured a decrease in the diffusion coefficient of the fluorescent probe lipid NBD-PE in the membranes of LLC-PK1 cells grown in medium that contained the drug.

Another interesting result is the increase of the stability of foam films with low content of acid phospholipid. We observed an increase in the film stability of NBFs (Fig. 1c), made from adsorption of DPPC/PS (95/5, mol/mol) in the presence of gentamicin compared to the control DPPC/PS sample (Fig. 3). This result correlates with faster and enhanced surface adsorption (Figs. 5 and 8). In addition, an improvement of spreading was observed, shown by the lower γ_{min} values of the DPPC/PS mixture in the presence of gentamicin (Fig. 6). The spreading improvement results in faster formation of densely packed monolayers at the NBF's air–water interfaces crucial for the formation of stable black films (Exerowa and Krugliakov 1998). The NBF stability is also enhanced by the reduction in the size of the condensed phase domains formed in DPPC/PS samples containing gentamicin (compared to the control; Fig. 8), thus reducing the domain boundary tension. The physiological sense of the finding is rather unclear but suggests intensive surface re-organizations, resulting in better surface lipid packing in the presence of gentamicin. The latter processes apparently influence the microstructure and thus the function of membranes containing discrete domains with small amounts of acidic phospholipids.

A strong correlation was observed between the value of the threshold concentration (C_t) and the adsorptive properties and surface morphology of adsorption layers by the various compositions. When gentamicin and/or Ca^{2+} improves the adsorption of the dispersions and decreases the heterogeneity in the morphologies of the surface films (reduction in the size of the large condensed aggregates), the threshold concentration is lowered. This is the case for the mixture DPPC/PS (Fig. 3 compared to Fig. 8). In contrast, when gentamicin and/or Ca^{2+} inhibits the adsorption of the samples and increases the heterogeneity of the adsorption layer's morphology (increase of the size of the large condensed aggregates), the threshold concentration is increased. Such a trend is observed when films are formed entirely by the anionic PS (Fig. 2 compared to Fig. 7). Thus C_t, i.e., the minimal phospholipid concentration at which stable BFs are always ($W = 1$) formed, can serve as a sensitive parameter for rapid evaluation of the surface activity and the heterogeneity of surface layers formed by phospholipids in the presence of cationic drugs and other pharmaceutically applicable molecules.

Of note is the lack of effect of gentamicin on the stability of NBFs formed by the neutral phospholipid DMPC (data not shown). This result is consistent with a previous report showing lack of gentamicin binding to membranes composed entirely of neutral lipids (Alexander et al. 1979). Similarly, there is no effect of gentamicin on the equilibrium and dynamic surface tensions of DMPC monolayers; therefore, we can conclude that the lipid/gentamicin interactions are mainly electrostatic for the systems in this study.

Ca^{2+} ions interact strongly with membranes made from PS as shown by a variety of methods including vesicle permeability, thermotropic phase transitions, and morphology determined by differential scanning calorimetry, X-ray diffraction, etc. It was shown, by Papahadjopoulos et al., that the concentration of 1.0–2.0 mM Ca^{2+} (1 mM in this paper) induces a highly cooperative phenomenon, resulting in increased vesicle permeability, aggregation, and fusion of phospholipid vesicles (Papahadjopoulos et al. 1977; Portis et al. 1979; Wilschut and Papahadjopoulos 1979). The cause is a phase change from a fluid to a crystalline gel state of the hydrocarbon chains in the lipid bilayers (Papahadjopoulos et al. 1977).

In this work, it has been shown that Ca^{2+} interacts avidly with CBFs made by the negatively charged PS, e.g., Ca^{2+} decreases the electrostatic component of the disjoining pressure in the film leading to the formation of thinner NBFs (Exerowa and Krugliakov 1998). This effect results in a strong shift of the phosphatidylserine C_t towards higher concentrations, e.g., Ca^{2+} itself ruptures films formed by PS (Fig. 2). The decreased stability of PS/Ca^{2+} samples (in comparison to the control PS films) correlates to: an increase of the surface tension plateau value (from 10^{-6} to 10^{-5}, compare Figs. 4 and 5); weaker surface adsorption (compare curves 1 and 2, Fig. 7);and formation of higher amounts of condensed phase with texture different from the one induced by gentamicin (BAM micrographs, Fig. 7, curves 2 and 3). Finally, we show that Ca^{2+} effectively deters the effect of gentamicin on pure PS bilayers (no change in the film type or stability is caused by the antibiotic), since the two molecules compete for the same membrane binding sites.

Conclusions

The interactions of the positively charged gentamicin with anionic phospholipid in the presence of Ca^{2+} were examined by the two model systems (TLFs and adsorption films) in order to clarify head-to-head interactions between lipid monolayers, which take place in numerous biological systems. The novelty of the results obtained by TLFsis that the presence of gentamicin and Ca^{2+} changes two properties of TLFs: (1) the film type and thickness (the type changes from CBFs to NBFs, the thickness from ca. 15 to 8 nm, respectively, Fig. 2), and (2) the film stability in terms of $W(C)$ dependence (increase of C_t in the case of single PS, Fig. 2, and decrease of C_t in the case of mixture DPPC/PS, Fig. 3).

Adsorption layers were studied (with surface tensiometry and BAM) as they represent half of the TLF (Lalchev 2007) and provided complementary information to the results obtained with foam films. By coupling of the data from the two experimental systems, strong correlation was obtained between the threshold concentration necessary for stable BF formation and the gentamicin- and/or Ca^{2}-induced changes in the adsorptive properties and the surface morphology of adsorption layers by the various compositions. Thus, C_t could be utilized as a sensitive parameter for rapid evaluation of the surface activity and of the heterogeneity of surface layers formed by phospholipids in the presence of cationic drugs and pharmaceutically relevant substances.

The strong effects of gentamicin/Ca^{2+} shown in this paper on the PS-containing foam films indicate the potential of TLFs as a novel tool to examine the interaction of lipids with phospholipidosis-inducing drugs. Further development of the methodology will be a primary task of our future research.

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