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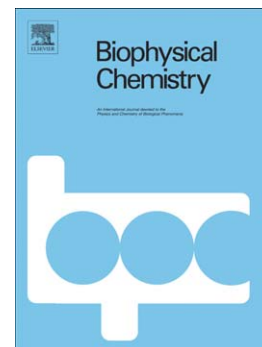
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**Influence of Steroid Hormone Progesterone on the Properties of  
Phosphatidyl Serine Monolayers and Thin Liquid Films**

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**Abstract**

In this work the capability of Progesterone (Prog) to penetrate to phosphatidyl serine (PS) monolayers (detected by equilibrium and dynamic surface tensions) and to induce rupture of PS thin liquid films (TLFs, known as foam films) in presence of  $\text{Ca}^{2+}$  ions is studied. TLF studies reveal that the presence of  $\text{Ca}^{2+}$  ions changes the type of PS films from thicker common black films to bilayer Newton black films and that the addition of Prog results in film destabilization and rupture. The effects of Prog in presence of  $\text{Ca}^{2+}$  ions were observed with the films consisting of negatively charged PS but not of neutral phospholipids. The results correlate with the proposed physiological role of Prog and  $\text{Ca}^{2+}$  in the acrosome reaction. The model of TLFs is used for the first time to study membrane fusion during acrosome reaction and proposes several new qualitative and quantitative parameters for studying of this reaction.

*Keywords:* Monolayers, Thin Liquid Films, Foam films, Progesterone, Acrosome reaction

## Introduction

The mammalian sperm acrosome reaction is one of the central events preceding fertilisation. The key molecular event during the acrosome reaction is the fusion of the outer acrosomal membrane with the overlying plasma membrane, resulting in formation of membrane vesicles and simultaneous release of the acrosomal content [1-3]. Numerous molecules, presenting *in vivo*, are proposed to be physiological initiators of the membrane fusion during the acrosome reaction, alone or in cooperation with each other [2, 4]. Such molecules are serum albumin, glucosaminoglycans, prostaglandins, steroid sex hormones and other pellucida components. Among all molecules, most controversial data are reported for steroid sex hormones (progesterone, estradiol and testosterone). Despite the results showing that progesterone (Prog) induced membrane fusion [2, 5, 6] during the acrosome reaction, in cell cultures and liposomes at concentrations corresponding to the physiological ones, the mechanisms of action and the physiological relevance remain still unclear. There are also data demonstrating that Prog effects are strongly increased in presence of divalent cations and especially of  $\text{Ca}^{2+}$  [7-10].

Recent studies showed that progesterone [6],  $\text{Ca}^{2+}$  ions or cooperation of the two agents [8-11] could drive induction of acrosome reaction *in vivo*. It is also proven *in vitro* that Prog could induce aggregation and fusion of membrane vesicles (the central event of acrosomal reaction) and this effect is strongly enhanced in presence of  $\text{Ca}^{2+}$  [2, 11]. Progesterone also induces coexistence of phases with different ratios of progesterone and phospholipid with distinctive molecular packing characteristics [12]; also it is well known, that fusion could be driven by the domain boundary tension in heterogeneous

membranes [13, 14]. These data suggest the possibility for direct induction of acrosomal reaction by interaction of progesterone and  $\text{Ca}^{2+}$  with spermatozoon membrane phospholipid components, working in parallel with the already reported receptor-mediated mechanisms [6].

The current work tests the effects of Prog and  $\text{Ca}^{2+}$  induced membrane aggregation and fusion with two well known model membrane systems at the air-water interface, namely phospholipid monolayers and Thin Liquid Films (TLFs). Phospholipid monolayer films (MFs) and TLFs, are preferred models for investigating *in vitro* the interactions between the membrane phospholipids and membrane active compounds present in the surrounding solution [15-17]. TLFs, being of several types, are composed of two mutually adsorbed, oriented “head-to-head”, phospholipid monolayers (Fig. 1 A-C), thus representing the contact area occurring between the *cis*-monolayers of two fusing membranes [18, 19].

Fig.1

The phospholipid TLFs have been successfully used as a model system to study membrane-membrane interactions, membrane fusion and adhesion, bio-surfactant behaviour at interfaces, lung surfactant, etc. [18, 20-25]. Phospholipid MF (Fig. 1, D) could be regarded as a “half” of the TLF and was used for a long time as a model system for studying the interactions between membrane components with different agents and ions presenting in the liquid subphase [15, 26-30].

The aim of the current work is to study the interactions and effects of the steroid sex hormone progesterone on the properties (stability, surface tension decrease, hydrodynamic behaviour etc.) of phosphatidyl serine (PS) monolayers and thin liquid films (TLFs) at air-water interface, in presence and absence of  $\text{Ca}^{2+}$  ions. Other aim is to estimate quantitatively the degree of penetration of progesterone to PS monolayers at equilibrium and at dynamic conditions (during monolayer compression and decompression). PS is chosen because it is known to be the representative phospholipid of the sperm plasma membrane and sperm acrosome membrane [2]. Progesterone is preferred among other hormones (testosterone, estradiol etc.) because, according to other studies [2], Prog showed strongest effects of aggregation and fusion on PS containing membranes.

## Materials and Methods

### *Materials*

Brain phosphatidyl serine (PS) and the steroid sex hormone Prog are purchased from Sigma.  $\text{CaCl}_2$  and NaCl are purchased from Merck and used without further purification.

Lipid dispersions are made with bidistilled water with conductivity less than  $1 \mu\text{S}$  and used further to form TLFs. Film forming dispersions used (stock solution with PS concentration =  $1000 \mu\text{g/ml}$ ) are with constant (evaluated with conductivity measurements) ionic strength  $I = 0.25 \text{ M}$ . For the experiments in absence of  $\text{Ca}^{2+}$  the desired ionic strength is reached by using NaCl solution only, while for the experiments in presence of  $\text{Ca}^{2+}$  (1mM), NaCl and  $\text{CaCl}_2$  solutions are mixed.

Progesterone is dissolved in chloroform, which is subsequently removed by at least 30 minutes evaporation under nitrogen stream and the sample is kept overnight under vacuum, as previously described [31]. The dry film obtained is hydrated at 70°C with electrolyte water solutions and the mixture is sonicated for breath periods (up to 2 min) until homogenous dispersion is formed which is not used for more than 2 h after its formation. Prog is involved in the monolayer studies by injection of 20  $\mu$ l into the monolayer subphase and of 5  $\mu$ l into the menisci of TLF-forming biconcave drop, as previously described [18, 19].

### ***Thin Liquid Films (TLFs)***

TLFs are formed by the method of Scheludko and Exerowa [20] using the modified measuring cell of Lalchev et al. [23]. A biconcave drop (50  $\mu$ l volume) of the phospholipid dispersion (pH=6.8-7.0; I=0.25 M in presence or absence of 1 mM  $\text{CaCl}_2$ ) is incubated in the measuring cell cylinder at  $T=37^\circ\text{C}$  (corresponding to liquid-crystalline phase state of PS) for 30 minutes. After sucking some volume of the drop solution, thick TLF (above 1000 Å) is formed (Fig. 1). Further the film spontaneously gets thinner and after a characteristic film thinning time,  $t_{0-1}$ , critical film thickness (ca. 300 Å) is reached. Then a black spot (local thinning in the film) appears (as schematically shown in Fig.1 A), expands with characteristics rate (black spot expansion time  $t_{1-2}$ ) and fills the whole film area (black film is formed). Two types of black films are formed: 150-200Å thick common black films (CBFs, Fig. 1B), with thin free liquid core between the monolayers, and ca.70 Å thick truly bilayer (without free liquid core) Newton black films (NBFs, Fig. 1C). All the films studied are with film diameter  $d_f=200\text{ }\mu\text{m}$ . The concentrations of film



forming PS dispersions used in our experiments were in the range 150-400  $\mu\text{g/ml}$  depending on the type of the films formed.

The probability ( $W$ ) for formation of stable black films depends strongly on the phospholipid concentration  $C$  [22-25] and can be calculated by the equation  $W = \Delta N / N$ , where  $N$  is the total number of trials (at least 20 for each concentration) and  $\Delta N$  is the number of trials in which stable black films are formed. Thus,  $W$  varies between 0 and 1 indicating respectively 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 stable black films are always (with  $W=1$ ) formed [16, 19, 20, 22, 23, 25].  $W(C)$  dependence and  $C_t$  value are extremely sensitive to the phase state of phospholipids used, composition of the film forming solution, temperature, presence of ions and different membrane-active agents in the bulk solutions [16, 18-20]. Thus in our study  $W(C)$  dependence of pure PS films is used as a control curve to estimate the effects on film stability of the interaction between the film building lipid (PS) and the added Progesterone (in absence or presence of  $\text{Ca}^{2+}$  ions). The changes in the film type, hydrodynamic behaviour and formation kinetics are measured by monitoring the film by inverted light microscope connected with digital photo camera, type Olympus C-7070 Wide Zoom. Thus the  $t_{0-1}$  and  $t_{1-2}$  values are determined.

### ***Monolayer Films (MFs)***

Spread monolayers of PS (with surface concentration corresponding to 80  $\text{\AA}^2$  area per molecule) are formed from PS solution in chloroform (stock solution of 1000  $\mu\text{g}$

PS/ml). Few  $\mu\text{l}$  of the solution are carefully deposited using Hamilton micro syringe at the air/water interface of the Langmuir trough. After that at least one hour is given for chloroform evaporation. Then the surface tension - time dependence is measured by the method of Wilhelmy with accuracy  $\pm 0.5 \text{ mN/m}$ , as previously described [15, 19]. After reaching the equilibrium surface tension ( $\gamma_{\text{eq}}$ ), five consecutive compression/decompression cycles from 100 % (recording  $\gamma_{\text{max}}$  value) to 50 % ( $\gamma_{\text{min}}$  value) of the initial monolayer area are conducted (only the results from the first three cycles are shown). Duration of one cycle is 3 minutes, 1.5 minutes for compression and 1.5 minutes for decompression. For each cycle the values of  $\gamma_{\text{min}}$ ,  $\gamma_{\text{max}}$ , and the cycle hysteresis area ( $A_{\text{H}}$ ,  $\text{mN}$ ) are measured. Experiments are made at  $T=37^\circ\text{C}$ ,  $\text{pH}=6.8-7.0$ ,  $I=0.25 \text{ M}$  in presence or absence of  $\text{CaCl}_2$  (1  $\text{mM}$ ) into the subphase.

## Results

### Monolayer Films

#### *PS pure monolayers*

As control experiments, surface tension measurements vs. time of PS spread monolayers ( $80 \text{ \AA}^2$  area per molecule) in presence or absence of 1  $\text{mM}$   $\text{Ca}^{2+}$  ions are conducted. After reaching the equilibrium surface tension ( $\gamma_{\text{eq}}$ ), five consecutive compression/decompression cycles are performed. It can be seen (Fig.2) that in absence of  $\text{Ca}^{2+}$   $\gamma_{\text{eq}}$  is lower than 50  $\text{mN/m}$  while in presence of  $\text{Ca}^{2+}$  ions  $\gamma_{\text{eq}}$  equals 53  $\text{mN/m}$ . It can be seen also, that PS monolayers spread in absence of  $\text{Ca}^{2+}$  much faster (reaching  $\gamma_{\text{eq}}$  in 5 minutes) in comparison to the films formed in presence of  $\text{Ca}^{2+}$ , where  $\gamma_{\text{eq}}$  is reached after 15 minutes.

Fig.2

For all compression/decompression cycles of PS monolayers (Fig. 3A), lower (with more than 7 mN/m)  $\gamma_{\max}$  values are observed in absence of  $\text{Ca}^{2+}$  ions than in presence of  $\text{Ca}^{2+}$ . The presence of  $\text{Ca}^{2+}$  ions also increased  $\gamma_{\min}$  values, with more than 11 mN/m at the 3<sup>rd</sup> cycle, compared to the value in absence of  $\text{Ca}^{2+}$ .

Fig.3 A, B and C

The results showing lower degree of spreading, delayed formation kinetics and higher surface tension values (at equilibrium and dynamic conditions) of PS monolayers in presence of  $\text{Ca}^{2+}$  (Figs. 2 and 3A) could be explained by the adsorption and adhesion of divalent  $\text{Ca}^{2+}$  ions to PS polar heads. The adhesion screens the head group negative charges, decreases electrostatic repulsion and the cohesive forces between the PS acyl chains became dominant in the film plane [15, 32-34]. This hypothesis agrees with the increase of compression/decompression hysteresis area (proportional to the cohesive forces between the lipid tails, according to Hills [35]) for PS films from 0.1 mN in absence of  $\text{Ca}^{2+}$  to 0.7 mN in presence of  $\text{Ca}^{2+}$  ions (data not shown).

#### ***Dynamic behaviour of PS monolayers at addition of Progesterone***

The dependencies of  $\gamma_{\max}$  and  $\gamma_{\min}$  during compression/decompression cycling of PS monolayers (in absence and presence of  $\text{Ca}^{2+}$ ) on the addition of Prog are shown in

Figure 3, panel B and C. The addition of Prog leads to decrease of  $\gamma_{\max}$  (Fig. 3 B) and the effect is stronger in presence of  $\text{Ca}^{2+}$  where  $\gamma_{\max}$  dropped from 58 mN/m to less than 50 mN/m. The decrease of  $\gamma_{\max}$  values could be a consequence of the steroid hormone penetration to the monolayers in agreement with the higher surface activity of Prog to PS monolayers in presence of  $\text{Ca}^{2+}$  ions (see Fig. 4 in next section). The values of  $\gamma_{\max}$  at dynamic conditions (3 minutes for cycle) are determined by at least of two processes: (i) disintegration and re-spreading of surface condensed phases during monolayer decompression and (ii) adsorption to the interface during monolayer decompression of the molecules which probably undergo desorption into the subphase during compression [36]. We consider the behaviour (hysteresis area,  $\gamma_{\max}$ ,  $\gamma_{\min}$  values etc.) of the monolayers at dynamic (compression/decompression) conditions as resultant of the kinetics of the above processes occurring at the surface, comparable to the rate of compression/decompression cycle (3 minutes/cycle) in our experiments.

The addition of Prog reveals an opposite effect on  $\gamma_{\min}$  values of PS films resulting in increase of  $\gamma_{\min}$  (Fig 3 C). The Prog influence is much stronger in  $\text{Ca}^{2+}$  presence, where  $\gamma_{\min}$  increase of more than 7 mN/m is observed (open symbols), comparable to 3-4 mN/m without  $\text{Ca}^{2+}$  (filled symbols). It is shown also, that  $\gamma_{\min}$  values of all films studied slightly increase with the cycle number. This  $\gamma_{\min}$  (cycle number) dependence can be explained with the loss of surface active material from the interface to the bulk phase at dynamic compression/decompression conditions. The increased slope of the dependence in presence of Prog and  $\text{Ca}^{2+}$  could be related to the enhanced stability of the PS aggregates in the liquid subphase, since  $\text{Ca}^{2+}$  ions neutralize the head group negative charge normally preventing the adhesion between PS molecules [20].

### ***Penetration of Progesterone to PS monolayers***

For studying the penetration of progesterone to the equilibrium monolayers in presence of  $\text{Ca}^{2+}$ , the hormone is added in the film subphase in concentration range  $1 \times 10^{-8} \text{ M}$  -  $1 \times 10^{-2} \text{ M}$  and the change of the equilibrium surface tension ( $\Delta\gamma = \gamma_{eq}^{PS} - \gamma_{eq}^{PS+Prog}$ ) is measured. As can be seen in Figure 4 the progesterone penetration to PS films is stronger in  $\text{Ca}^{2+}$  presence for all Prog concentrations up to  $10^{-3} \text{ M}$  at which  $\Delta\gamma$  reaches plateau value of 6.5 mN/m. The first  $\Delta\gamma$  change is detected at concentrations as low as  $10^{-7} \text{ M}$  Prog which proves very high surface activity and penetration capacity of Prog to the negatively charged PS monolayers.

Fig.4

### ***Thin Liquid Films***

In absence of  $\text{Ca}^{2+}$  ions pure PS formed stable common black films (CBFs, Fig. 1 B) at threshold PS concentration  $C_t = 200 \text{ } \mu\text{g/ml}$  ( $2.87 \times 10^{-4} \text{ M}$ ). The PS film thinning time,  $t_{0-1}$ , is 45 sec and the black spot expansion time,  $t_{1-2}$ , is 15 sec. The effect of Prog addition on film hydrodynamic behaviour is estimated by measuring  $t_{0-1}$  and  $t_{1-2}$  values (Fig. 5). An increase of  $t_{0-1}$  values from 45 sec to 60 sec (at  $4 \times 10^{-4} \text{ M}$  Prog) due to the addition of Prog is observed. In contrast, steep  $t_{1-2}$  decrease from 15 sec to 3 sec is measured with increase of Prog concentration in very narrow range ( $1.0 \times 10^{-4}$  -  $1.6 \times 10^{-4} \text{ M}$ ). When Prog concentration is higher than  $2.5 \times 10^{-4} \text{ M}$  strong destabilisation effect of Prog on the common black PS films is observed, resulting in film lifetime decrease from hours to 2-5 sec, after which CBFs always rupture (see also Fig.6). It is worth to be noted that in our

previous study on DMPC films with added lipid-linked PEGs [16] we showed good correlation between both  $t_{0-1}$  and  $t_{1-2}$  increase and stabilisation effect of added lipid-linked PEGs on DMPC films. Obviously, this was not the case for the system PS-Prog, where  $t_{1-2}$  values sharply decreased by addition of Prog, leading to film destabilisation and rupture.

Fig.5

The effect of Prog addition on the stability (in terms of  $W(C)$  dependence) of both PS CBFs and NBFs, in absence and presence of  $Ca^{2+}$ , is shown in Figure 6. We found that in presence of  $Ca^{2+}$  ions it is possible to form stable Newton black films (NBFs, Fig. 1 C) from PS. Bilayer NBFs of PS are formed at  $C_t = 355 \mu\text{g/ml}$  ( $5.30 \times 10^{-4} \text{M}$ ) as shown in Fig. 6. The values of  $t_{0-1}$  and  $t_{1-2}$  of PS NBFs were 35 sec and less than 1 sec respectively, both much lower than these of PS CBFs formed in absence of  $Ca^{2+}$  (Fig. 5). The acceleration of the film formation kinetics and the change of film type from thicker (ca. 150 Å) CBFs to truly bilayer (ca. 70 Å) NBFs in presence of  $Ca^{2+}$  ions could be explained with the neutralisation of PS head negative charge by  $Ca^{2+}$  ions, resulting in strong decrease of the electrostatic repulsion between the film monolayers. It can be seen in Fig. 6 that in absence of  $Ca^{2+}$  the addition of Prog (at concentration  $> 2.5 \times 10^{-4} \text{M}$ ) leads to rupture ( $W = 0$ ) of CBF at 200  $\mu\text{g PS/ml}$ , while at the same concentration the film without Prog is stable, ( $W=1$ ). Thus, an additional increase of PS concentration to 210  $\mu\text{g PS/ml}$  was necessary in order to form stable CBFs in presence of Prog (curve 1' in Fig. 6). Using the Boltzman equation,  $\Delta G = RT \ln (C_t^*/C_t)$ , as described in [19], we

calculated an increase of PS film free energy ( $\Delta G$ ) in presence of Prog, being with appr. 0.125 kJ/mol higher compared to pure PS film.

Fig. 6

In presence of  $\text{Ca}^{2+}$  the addition of Prog at concentration of PS = 355  $\mu\text{g/ml}$  (where the NBF without Prog is stable) ruptures the films (Fig. 6), i.e. destabilisation effect of Prog on NBF is also observed as in case of CBF. It should be noted that in contrast to CBFs, where Prog concentration causing film rupture is higher than  $2.5 \times 10^{-4}$  M, the rupture of NBFs is observed at Prog concentration as low as  $10^{-8}$  M, i.e. the shorter distance between film surfaces the stronger destabilisation effect of Prog is observed. Other difference of Prog action on CBFs and NBFs is that the Newton black films rupture in Prog presence immediately at the moment of black spot formation ( $t_{1-2}$  is less than 1 sec), in contrast to much higher  $t_{1-2}$  values (3 sec, Fig. 5) for CBFs. The free energy difference  $\Delta G$  between NBFs in presence and absence of Prog is 0.037 kJ/mol, being significantly lower compared to Prog induced free energy increase observed for CBFs.

## Discussion

The aim of this study is to elucidate the effects of progesterone (Prog) and  $\text{Ca}^{2+}$  ions on some aspects of membrane fusion, which plays a key-role in the acrosome reaction. Phosphatidyl serine (PS) monolayers and thin liquid films (TLFs, known also as foam films) serve as models of sperm plasma and acrosome membranes. Negatively

charged PS is a representative phospholipid for these membranes.

The properties of phosphatidyl serine monolayer and bilayer films are strongly affected by  $\text{Ca}^{2+}$  ions and presence of Prog. Our experimental data are in agreement with the hypothesis [32-34] that  $\text{Ca}^{2+}$  adhesion to PS head group neutralizes the negative head group charge, decreases electrostatic repulsion and increases the magnitude of Van der Waals attraction forces in the films. Generally some of the results also correlate to the literature data [2, 8-11] about the non-additive synergetic action of Prog and  $\text{Ca}^{2+}$  on membrane fusion step of the acrosome reaction. However, in the frame of the models used we obtained some new quantitative and qualitative parameters and additional information for the fusion process.

The experiments with PS monolayers in presence of  $\text{Ca}^{2+}$  show delayed formation kinetics, decreased surface activity and increase of the compression/decompression cycle hysteresis area, due to enhanced acyl chains cohesion between PS molecules in the membrane plane (Figs. 2, 3A). Monolayer surface tension measurements demonstrate also easily penetration of Prog to the PS monolayers, stronger in presence of  $\text{Ca}^{2+}$  ions (Fig. 4). Monolayer experiments in this study serve (as monolayer film could be regarded as a “half” of the TLF, Fig. 1) practically only to describe some details on the main findings in this work revealed by the model of TLFs. The reduced by  $\text{Ca}^{2+}$  ions electrostatic component of the disjoining pressure resulted in decreased repulsion between the TLF monolayers and improved head to head adhesion, leading to additional thinning of the equilibrium black films (Fig. 6): from thicker CBFs (15 nm) to truly dehydrated bilayers, much thinner NBFs (7 nm). This film type transformation by  $\text{Ca}^{2+}$



ions is shown experimentally for the first time for the system PS TLF -  $\text{Ca}^{2+}$  and confirms that the fusion between the apposing surfaces requires closer contact [37].

In addition, the TLF experiments show for the first time destabilization effects (i.e. film rupture and free energy increase) on the black films induced by Prog (Fig. 6). These results correlate with data [12] that Prog induces gel/liquid crystal phase separation in the membrane plane [38]. We found stronger destabilisation effect of Prog (occurring at very low Prog concentration) on PS Newton black films (“dry” films, composed of mutually adhered monolayers) compared to that on CBFs (containing free liquid core between monolayers). Our results for detected increase of the minimal concentration for stable black film formation,  $C_t$  value for NBFs, agree also with the data [20] that the formation of Newton bilayers requires very dense molecular packing at the film surface (80 % of the maximal value), in contrast to CBFs (40 % molecular packing density). The data might account for the strong the strong cooperation between Prog and  $\text{Ca}^{2+}$  in fertilisation as the steroid hormone alone is incapable to induce transformation in the PS black film type (data not shown). Since dense molecular packing is decisive for stable black film formation [16, 19, 20] further investigations are necessary to clarify whether phase separation occurs in mixed Prog-PS films and its importance for TLF destabilisation. Obviously the negatively charged PS head groups play a major role in that process since no penetration to monolayers of neutral phosphatidyl cholines was observed, as shown in [17].

Finally, we found also that Prog influenced the hydrodynamic behavior of TLFs:  $t_{0-1}$  increase and  $t_{1-2}$  decrease in Prog presence (Fig. 5). The increase of  $t_{0-1}$  indicates decreased tangential mobility of film surfaces (formation of more rigid membrane

phases) and agrees with hypothesis for gel/liquid crystalline phase coexistence induced by Prog.

In order to estimate the physiological relevance of the obtained results in this paper one could compare the Prog concentrations used in our work to the hormone concentrations per spermatozoid observed *in vivo*. The PS concentrations used in our study were  $\geq 200 \mu\text{g/ml}$ , which corresponds to  $\geq 2 \times 10^7$  spermatozooids/ml [39]. Prog concentration necessary to rupture PS films was  $\geq 2.5 \times 10^{-4} \text{ M}$  and corresponds to  $\leq 2.7 \text{ ng Prog/spermatozoid}$  [40] which correlates to the values of  $5 \text{ ng Prog/ml}$  found in the Fallopian tube *in vivo* [41].

## Conclusion

Using the models of PS monolayers and TLFs some of the findings in this paper confirm our knowledge that  $\text{Ca}^{2+}$  ions screens the negative charge on PS and that the membrane fusion requires dehydration of the apposing surfaces and their close contact, which causes increase of membrane surface tension and membrane fusion [37]. The latter proved that we were able to choose an appropriate experimental approach, especially the model of TLF (composed of two mutually adsorbed, oriented “head-to-head” phospholipid monolayers, Fig. 1 A-C, thus representing the contact area occurring between the *cis*-monolayers of two fusing membranes), which is used for the first time to study the membrane fusion step of acrosome reaction. Proposed as a new experimental model, the TLFs reveal some new quantitative parameters for studying of this reaction, e.g. film thinning time, black spot expansion time, minimal concentration for stable black film formation, film stability in terms of film lifetime or film rupture (obligatory step for

the onset of membrane fusion), investigation of the effects of membrane active agents, e.g. Prog and  $\text{Ca}^{2+}$ , on TLF stability etc. In addition, the model of TLFs could provide direct visualisation of intermembrane adhesion (i.e. film type transformation), film formation kinetics and morphology by monitoring the film behaviour by inverted light microscope connected with photo camera.

In this paper we showed that Prog is able strongly to penetrate to PS monolayers and to destabilise PS TLFs.  $\text{Ca}^{2+}$  ions alone increases the threshold (minimal) concentration necessary for formation of stable PS TLFs. We proved that the simultaneous action of  $\text{Ca}^{2+}$  and Prog results in strong destabilisation of PS NBFs at very low Prog concentration, which correlates well to its values found *in vivo*.

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## Figure Captions

Fig.1. Schematic representation of phospholipid thin liquid films (TLFs) and phospholipid monolayer film formed at the air/liquid interface. Two types of black TLFs are shown: common black film - CBF (panel B) and Newton black film - NBF (panel C). Thick TLF with local thinning called black spot (panel A) is also presented. On panel D is shown the phospholipid monolayer, which can be regarded as a half of a thin liquid film.

Fig. 2. Dependence of the surface tension ( $\gamma$ ) on time (t) for PS spread monolayer films in absence ( $\blacklozenge$ ) or presence ( $\blacksquare$ ) of  $\text{Ca}^{2+}$  (1 mM  $\text{CaCl}_2$ ). Experiments are conducted at PS surface concentration of  $80 \text{ \AA}^2$  area per molecule,  $I=0.25 \text{ M}$ ,  $T=37^\circ\text{C}$ ,  $\text{pH } 6.8-7.0$ .

Fig. 3. Dependence of PS spread monolayers maximal ( $\gamma_{\max}$ ) and minimal ( $\gamma_{\min}$ ) surface tension on the compression/decompression (from  $80 \text{ \AA}^2$  to  $40 \text{ \AA}^2$  area per PS molecule) cycle number in presence or absence of  $\text{Ca}^{2+}$ . Panel A shows dependence of  $\gamma_{\max}$  (open symbols) and  $\gamma_{\min}$  (filled symbols) on cycle number of pure PS monolayers in absence ( $\blacksquare, \square$ ) or in presence of  $\text{Ca}^{2+}$  ( $\blacktriangle, \triangle$ ). Also shown are dependencies of  $\gamma_{\max}$  (Panel B) and of  $\gamma_{\min}$  (Panel C) on cycle number in absence (filled symbols) or in presence (open symbols) of  $\text{Ca}^{2+}$  for pure PS films ( $\blacktriangle, \triangle$ ) and for PS films with Prog added in the subphase ( $\blacksquare, \square$ ).  $\text{Ca}^{2+}$  ions were added as 1 mM  $\text{CaCl}_2$ . Experiments were conducted at  $I=0.25 \text{ M}$ ,  $T=37^\circ\text{C}$ ,  $\text{pH } 6.8-7.0$ .

Fig.4 Dependence of the equilibrium surface tension change ( $\Delta\gamma = \gamma_{eq}^{PS} - \gamma_{eq}^{PS+Prog}$ ) of PS ( $80 \text{ \AA}^2$  per molecule) spread monolayer films on progesterone concentration in absence ( $\diamond$ ) and presence of  $\text{Ca}^{2+}$  ( $\blacklozenge$ ).  $\text{Ca}^{2+}$  ions are added as 1 mM  $\text{CaCl}_2$ . Experiments are conducted at  $I=0.25 \text{ M}$ ,  $T=37^\circ\text{C}$ ,  $\text{pH } 6.8-7.0$ .

Fig. 5. Dependence of film thinning time ( $t_{0-1}$ ,  $\bullet$ ) and black spot expansion time ( $t_{1-2}$ ,  $\blacksquare$  and  $\square$ ) of PS thin liquid films on the progesterone concentration ( $C_{\text{Prog}}$ ). Dashed line ( $\square$ ) denotes the onset of formation of metastable common black films. Experiments are conducted at threshold concentration  $C_{\text{PS}}=200 \text{ \mu g/ml}$ ,  $d_f = 200 \text{ \mu m}$ ,  $T=37^\circ\text{C}$ ,  $I=0.25 \text{ M}$ ,  $\text{pH } 6.8-7.0$ .

Fig. 6 Dependence of black film formation probability (W) on PS concentration ( $C_{\text{PS}}$ ) in absence (1 and 1', CBF) and presence (2 and 2', NBF) of  $\text{Ca}^{2+}$  ions and W/C dependence after addition of Progesterone. Curves 1 and 2 – pure PS; dashed lines 1' and 2' - PS films with added Prog. PS concentration at which Prog is added ( $C_{\text{Prog}} = 75 \text{ \mu g /ml}$  for CBFs

and  $3.14 \times 10^{-3}$   $\mu\text{g/ml}$  for NBFs) is noted with arrows.  $\text{Ca}^{2+}$  ions are added as 1 mM  $\text{CaCl}_2$ . Experiments are conducted at  $d_f = 200$   $\mu\text{m}$ ,  $T = 37^\circ\text{C}$ ,  $I = 0.25$  M, pH 6.8-7.0.

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ACCEPTED MANUSCRIPT

