CHARACTERIZATION OF MIXED LANGMUIR MONOLAYERS OF CYCLOSPORINE A WITH THE PHOSPHOLIPID DPPC AT THE CHITOSAN SUBPHASE

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Abstract

The properties of one-component and mixed monolayers of phospholipid 1,2–dipalmitoyl–sn–glycero–3–phosphocholine (DPPC) and polypeptide cyclosporine A (CsA) on the chitosan subphase were studied. DPPC is the main component that builds biological membranes, and CsA is an immunosuppressive drug typically used in medicine to prevent transplant rejections. The stability and reversibility of compression of these insoluble monolayers in the presence of chitosan (Ch) were examined by the Langmuir technique. The stability of the monolayers depended on the monolayer composition as well as the initial pressure (π_0) of the relaxation process. The smallest changes in the relative pressure as a function of time were obtained at π_0 = 10 mN/m. During compression–decompression cycles, the effect of chitosan was noticeable and caused isotherm shifts.

Keywords*: chitosan, cyclosporine A, Langmuir monolayer, monolayer stability*

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1. Introduction

The natural barrier that separates cells and intracellular compartments from their external environment is the biological membrane. It is mainly built of lipid molecules [1]. The action of all biologically active substances (including drugs) on living cells in the body begins with their interactions with the membrane. Therefore, it is important to study and better understand the type and magnitude of intermolecular interactions between the membrane and the drug.

Cyclosporine A (CsA) is a cyclic polypeptide produced by *Tolypocladium inflatum*. Its molecule possesses 11 amino acids that form a branched structure [2, 3]. CsA has strong immunosuppressive properties based on inhibition of T cell function and reduction of lymphokine production and secretion [4, 5]. Due to this activity, it is widely used in medicine, mainly against transplant rejections, and in the treatment of eye diseases, rheumatoid and arthritis [5]. However, due to its high molecular weight and hydrophobic nature, it is poorly soluble in water. As a result, its oral absorption is low, between 20 and 50% [4, 6]. In addition, it can cause nephrotoxicity, hepatoxicity and neurotoxicity with prolonged drug administration [5]. In order to avoid such serious side effects and increase CsA absorption, specialized drug release systems are designed that improve the bioavailability of the active substance [6]. One of the well-known compounds used to create controlled drugs release system is chitosan (Ch). Ch is a polysaccharide produced by *Mucor rouxii* and *Choanephora cucurbitarum* or by deacetylation of chitin. It is characterized by high biocompatibility and biodegradability and low toxicity. In addition, it is bactericidal and antifungal. Due to the afore-mentioned properties, it is used for the production of dressings, microspheres and microcapsules [7]. Chitosan capsules transport CsA very well, improving its bioavailability and permeability through cell membranes [6]. In addition, comminuted Ch molecules can increase the adhesion of CsA to surrounding tissues [8]. Ch can provide strong electrostatic interactions with the cell membrane [6]. However, to get better insight into the mechanism of a controlled drug release in the body, it is necessary to perform detailed analysis of the interactions of CsA and Ch with the cell membrane.

In order to realize this research aim, the phospholipid 1,2–dipalmitoyl–*sn*–glycero–3–phosphocholine (DPPC) was used because it perfectly simulates the behaviour of natural cell membranes. The Langmuir trough was applied to characterize a DPPC–CsA biomimetic monomolecular film on the Ch subphase. The Langmuir technique made it possible to study the changes in surface pressure as a function of time for the DPPC–CsA monolayers in the presence of Ch, as well as to determine hysteresis of the surface pressure versus mean molecular area during the compression–decompression cycle. The obtained results allowed drawing conclusions on the films' robustness and stability at different molar fractions of the monolayer components and provided information about reversibility of the compression process. From medical and pharmaceutical points of view, it is very important to obtain high-quality Langmuir–Blodgett films after transfer of the floating monolayer onto the solid support.

2. Materials and Methods

2.1. Material and Solution Preparation

In order to examine the interactions in the Ch/DPPC/CsA system, DPPC (semisynthetic, 99%, Sigma) and CsA (99+%, Alfa Aesar) solutions were prepared by dissolving the suitable weights in a 4:1 chloroform:methanol (v/v) mixture (methanol 99.8%, chloroform 98.5%, both purchased from Avantor Performance Materials Poland S.A.) to obtain a concentration equal to 1 mg/mL. Then, the mixtures with different molar

fractions of DPPC and CsA were prepared (χ_{CsA} = 0.25, 0.50 or 0.75) by mixing proper volumes of basic solutions. Solutions of DPPC or CsA alone as well as mixtures of them were tested in subsequent experiments. Moreover, Ch powder (MW 100,000–300,000 Da, degree of deacetylation 82%, Acros Organics) was dissolved in 0.1% acetic acid (diluted from concentrate, 99.5%–99.9%, Avantor Performance Materials Poland S.A.) with water from a Milli-Q Plus 185 system with a specific resistivity of 18.2 MΩcm. The above procedure provided the 0.1 mg/mL Ch solution.

2.2. Measurement of Isotherms

The surface pressure–area per molecule (π–A) isotherms for the insoluble DPPC–CsA monolayers on the Ch solution were obtained at 20°C by means of the Langmuir trough (KSV Nima). First, the trough was washed using methanol and twice rinsed with Milli-Q water. Then, the trough was filled with the Ch solution as a subphase. The surface tension was measured by the Wilhelmy plate method. Considering the DPPC, CsA or DPPC–CsA solution concentration (1 mg/mL) and the area of the trough (780 cm^2), the volume needed to obtain a monolayer in a gaseous state was determined, i.e. 30–60 μL, depending on the type of solution. The appropriate volume was placed onto the liquid subphase with a microsyringe (Hamilton–Bonaduz, Switzerland) by squeezing small droplets of the solution.

The system was left for 10 min for solvent evaporation. After that time, symmetrical compression was started using the barriers. The compression was conducted at a constant speed of 20 mm/min and lasted until the surface pressure reached the given value set in the operating programme. At the same time, the π–A isotherms were registered. Each experiment was performed at least three times, and the obtained isotherms were found to be reproducible within an error of ± 1 $Å^2$/molecule. Therefore, the molecular areas determined directly from the representative π–A isotherms are subject to the same error.

To examine the stability of the one-component and mixed monolayers on the Ch subphase, the surface pressure versus time (π–t) isotherms were determined. First, the monolayers were compressed to an initial surface pressure (π_0) equal to 5, 10 or 15 mN/m. In the case of the pure DPPC monolayer, a π_0 of 30 mN/m was also tested. The changes in surface pressure as a function of time over 1 h of relaxation were then measured.

The choice of surface pressure for the monolayer relaxation was dictated by the fact that CsA exhibits a collapse pressure above 25–26 mN/m, both in pure and binary systems [9]. Hence, the stability measurements were only conducted for the DPPC monolayer at the higher surface pressure (30 mN/m), i.e. the pressure of natural membranes. This was not possible for the other monolayers.

Compression–decompression cycles were also performed. For this purpose, the monolayers were compressed to a surface pressure of 10 or 15 mN/m, followed by immediate decompression. The former value (10 mN/m) was chosen because based on the π–t measurements, the monolayers were the most stable at this surface pressure. The latter value (15 mN/m) corresponds to the surface pressure of the packed monolayers in the conditions studied. Above that pressure, the CsA monolayer was neither kinetically nor thermodynamically stable [10]. The obtained compression–decompression curves provided detailed information about the monolayers' stability, potential loss of molecules from the interface to the subphase as well as reversibility of the process. All experiments were repeated at least three times; the most representative isotherms were considered.

3. Results and Discussion

3.1. Monolayer Stability Measurements

Fig. 1 presents changes in the relative surface pressure (π_t/π_0) as a function of relaxation time monitored for different initial pressures (π_0 = 5, 10 or 15 mN/m, as well as

30 mN/m for DPPC), corresponding to different stages of condensation. Table 1 shows the relative pressure (π_t/π_0) values for the DPPC–CsA monolayers on the Ch solution after 15, 30 and 60 min of relaxation depending on the initial pressure π_0 (5, 10 or 15 mN/m). These results, extracted from the stability curves, are shown for better comparison.

In general, there were changes in relative pressure (π_t/π_0) versus time, particularly within the first 15 min after starting the relaxation at the given surface pressure (Fig. 1). The absolute change in the relative surface pressure decreased with the increase of the

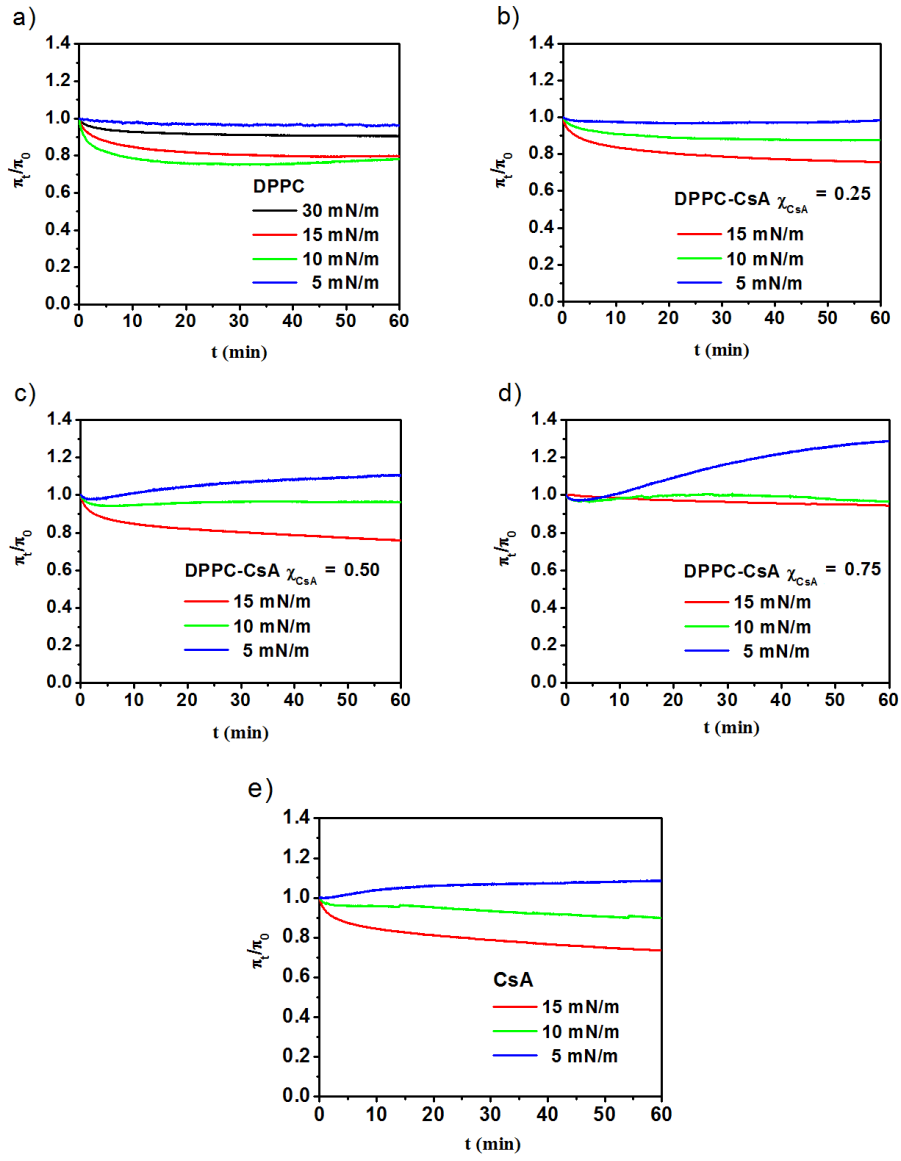

Figure 1. (a–e) Stability of the 1,2–dipalmitoyl–*sn*–glycero–3–phosphocholine (DPPC) – cyclosporine A (CsA) monolayers on the chitosan subphase obtained at the initial pressures (π_0) of 5, 10 or 15 mN/m, as well as 30mN/m for the DPPC monolayer.

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CsA concentration in the DPPC monolayer on the Ch solution at the given initial surface pressures (π_0) at which the barrier position is fixed. Excluding the pure DPPC or CsA monolayers, this trend was the same for all considered relaxation times (Table 1). The greater positive ($\pi_t > \pi_0$) and negative ($\pi_t < \pi_0$) changes in π_t/π_0 as a function of time indicate less monolayer stability. The deviations suggest that the monolayers are in a nonequilibrium state. Before compression, the CsA rings are arranged in the subphase plane while its side chains are in the gaseous phase. As the pressure increases during compression, the CsA molecules can adjust the vertical position and/or segments of CsA rings can undergo bending and partially penetrate the subphase. Due to over-compression, molecular reorientation or loss of molecules/segments to the Ch subphase, the monolayer relaxation at π_0 = 15 mN/m is related to the decrease in the surface pressure over time. Hence, the negative deviations in π_t/π_0 = f(t) appear. On the other hand, the positive deviations at π_0 = 5 mN/m can be associated with the fact that the folded CsA fragments return from the subphase to the interface and take on more spread conformations, parallel to the subphase plane [11]. The lowest stability was for DPPC–CsA (χ_{CsA} = 0.75) at π_0 = 5 mN/m, while the smallest changes in the surface pressure over time existed at π_0 =10 mN/m, proving the highest stability ($0.02 < \pi_t/\pi_0 < 0.25$).

Table 1. The relative pressure (π_t/π_0) obtained for the 1,2–dipalmitoyl–*sn*–glycero–3–phosphocholine (DPPC)–cyclosporine A (CsA) monolayers on the chitosan solution depending on the initial pressure (π_0) and the relaxation time.

Time [min]	**5**	**15**	**30**	**60**	**π_0 [mN/m]**
Monolayer	**π_t/π_0**				
DPPC	0.99	0.97	0.96	0.96	**5**
DPPC–CsA χ_{CsA} = 0.25	0.98	0.97	0.97	0.98	
DPPC–CsA χ_{CsA} = 0.50	0.99	1.03	1.07	1.10	
DPPC–CsA χ_{CsA} = 0.75	0.98	1.05	1.17	1.29	
CsA	1.02	1.05	1.07	1.09	
DPPC	0.82	0.77	0.75	0.78	**10**
DPPC–CsA χ_{CsA} = 0.25	0.93	0.90	0.88	0.88	
DPPC–CsA χ_{CsA} = 0.50	0.94	0.95	0.96	0.96	
DPPC-CsA χ_{CsA} = 0.75	0.97	0.98	1.00	0.96	
CsA	0.96	0.96	0.93	0.90	
DPPC	0.88	0.83	0.81	0.80	**15**
DPPC–CsA χ_{CsA} = 0.25	0.87	0.82	0.79	0.76	
DPPC–CsA χ_{CsA} = 0.50	0.87	0.83	0.80	0.76	
DPPC–CsA χ_{CsA} = 0.75	0.90	0.86	0.85	0.84	
CsA	0.87	0.82	0.79	0.74	

3.2. Compression–Decompression Measurements

The second part of this study examined the dynamic compression–decompression of the DPPC–CsA monolayers at the Ch solution/air interface at the previously selected pressures, i.e. 10 mN/m (Fig. 2a) and 15 mN/m (Fig. 2b). The former was chosen due to fact that all monolayers were the most stable at this pressure, while at latter one, the monolayers were in the most packed state though less stable.

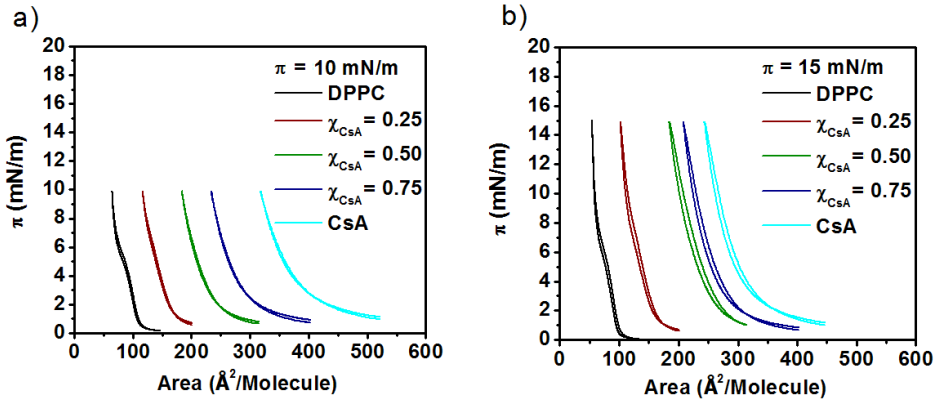

Figure 2. (a and b) Surface pressure–area isotherms for the compression–decompression cycles of the 1,2–dipalmitoyl–*sn*–glycero–3–phosphocholine (DPPC)–cyclosporine A (CsA) monolayers on the chitosan subphase.

In the first compression–decompression cycle (made at π_0 = 10 mN/m), small hysteresis loops were obtained for single and binary monolayers. The decompression branches of hysteresis loops were registered directly after the monolayer compression to the target pressure. The slight hysteresis indicates that the process is practically reversible without significant losses of the molecules from the Langmuir monolayer to the Ch subphase. In the case of π_0 = 15 mN/m, there were slightly larger hysteresis loops. This phenomenon may be associated with a change in the orientation of the CsA ring from the flat to perpendicular position in the DPPC monolayers. At 15 mN/m, the DPPC–CsA interactions are more repulsive than at 10 mN/m, a factor that can promote pushing molecules out of the monolayer [9]. It should be highlighted that despite the small hysteresis in the compression–decompression cycle, the phase transition and monolayers state are retained.

3.3. Effect of Chitosan

To evaluate the effect of chitosan on the DPPC–CsA monolayers, the difference in area per molecule for the films formed on chitosan and water subphase was determined ($\Delta A = A^{\text{Ch subphase}} - A^{\text{water subphase}}$) directly from the π–A isotherms at the given surface pressure. The results are shown in Table 2 for all examined monolayers at different surface pressures (5, 10 and 15 mN/m).

All ΔA values, except for the DPPC and DPPC–CsA χ_{CsA} = 0.50 monolayers, were positive. Thus, the average area per molecule in the DPPC–CsA monolayer on Ch subphase was greater than that on water, which suggests penetration of the Ch molecules between DPPC and CsA in the Langmuir monolayers. Such behaviour is reflected by a rightwards shift in the isotherms, i.e. towards larger areas. Due to Ch inclusion, the average distance between DPPC and CsA molecules increases, and thereby the average

Table 2. Differences in area per molecule (ΔA) determined for the 1,2–dipalmitoyl–*sn*–glycero–3–phosphocholine (DPPC)–cyclosporine A (CsA) monolayers on water and chitosan solution at different surface pressures.

Subphase	**Water**	**Chitosan**		
Monolayer	**Area per molecule A [Å^2]**	**Area per molecule A′ [Å^2]**	**A – A′ = ΔA [Å^2]**	**π_0 [mN/m]**
DPPC	80.72	72.52	**-8.20**	**5**
DPPC–CsA x_{CsA} = 0.25	136.90	147.01	10.11	
DPPC–CsA 0.50	212.93	196.10	**-16.83**	
DPPC–CsA 0.75	239.09	250.79	11.70	
CsA	290.33	309.04	18.71	
DPPC	57.04	55.64	**-1.40**	**10**
DPPC–CsA 0.25	108.09	119.12	11.03	
DPPC–CsA 0.50	181.12	168.56	**-12.56**	
DPPC–CsA 0.75	212.16	221.87	9.71	
CsA	257.58	275.85	18.27	
DPPC	53.14	53.17	0.03	**15**
DPPC–CsA 0.25	99.94	109.84	9.90	
DPPC–CsA 0.50	168.56	155.87	**-12.69**	
DPPC–CsA 0.75	196.78	204.20	7.42	
CsA	239.12	256.19	17.07	

area per molecule also increases. Ch penetrating the DPPC-CsA monolayer changes the orientation of molecular moieties, through electrostatic, hydrogen or hydrophobic interactions. As a result, the monolayer further expands. On the other hand, the increased thickness of the layers can be also observed [12]. The negative ΔA values for the binary monolayers highlight that the molecular area contracts in the presence of Ch, i.e. the isotherms shift to the left, towards smaller areas. This phenomenon is probably due to the strongest repulsive interactions that occur between DPPC and CsA molecules at χ_{CsA} = 0.50, as we had previously described [9]. This makes them prone to leave the monolayer as a result of the facilitated interactions with chitosan, thus decreasing the molecular area in the monolayers. The process can be reversible.

A similar mechanism was described by Camara et al. [13] for the spread of distearoyl phosphatidic acid (DSPA) on the Ch solution. The authors claimed that due to strong interactions between Ch and DSPA, i.e. hydrophobic forces and coulombic interactions among charged groups, the DSPA and Ch molecules were expelled from the interface into the subphase [13]. Therefore, the π–A isotherms of DSPA were shifted towards smaller molecular areas. An alternative reason for the isotherm shift could be attributed to reorganization of the hydrophobic chains in the DSPA monolayer in the presence of Ch, a phenomenon that made the film more condensed.

The above hypotheses can also be applied to the pure DPPC monolayer on the Ch subphase where the area contracted. It is known that DPPC, a zwitterion, has a positive charge on the quaternary ammonium group and a negative charge on the phosphate group.

Thus, it is likely that DPPC interacts with Ch via electrostatic forces and hydrogen bonds. These interactions affect the organization of DPPC molecules at the interface and provide smaller areas per molecule in the monolayers [14, 15].

4. Conclusions

In this study, the Langmuir technique was used to investigate the stability of the DPPC–CsA monolayer on the Ch solution/air interface. Based on the $\pi_t/\pi_0 = f(t)$ and compression–decompression measurements, the most stable monolayers were found at 10 mN/m. The presence of Ch affected the interactions in the DPPC–CsA monolayers, causing changes in its molecular organization that were revealed by isotherm shifts. Our investigations seem to be helpful to get better insight into the mechanisms governing peptide drug encapsulation, its release in the target site and Ch-based drug delivery systems.

5. References

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