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Chitosan as a Bioadhesive Agent Between Porphyrins and Phospholipids in a Biomembrane Model

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Porphyrins are currently used in photodynamic therapy as photosensitizers. In this paper we studied the interaction of two charged porphyrins, 5, 10, 15, 20-mesotetrakis(*N*-metyl-4-pyridyl) porphyrin, (TMPyP/chloride salt) cationic, and 5, 10, 15, 20-meso-tetrakis(sulfonatophenyl) porphyrin, ($TPPS_4$/sodium salt) anionic, nanoassembled in phospholipid Langmuir monolayers and Langmuir-Blodgett films. Furthermore, we used chitosan to mediate the interaction between the porphyrins and the model membrane, aiming to understand the role of the polysaccharide in a molecular level. The effect of the interaction of the photosensitizers on the fluidity of the lipid monolayer was investigated by using dilatational surface elasticity. We also used photoluminescence (PL) spectroscopy to identify the porphyrins adsorbed in the phospholipid films. We observed an expansion of the monolayer promoted by the adsorption of the porphyrins into the lipid-air interface which was more pronounced in the case of TMPyP, as a consequence of a strong electrostatic interaction with the anionic monolayer. The chitosan promoted a higher adsorption of the porphyrins on the phospholipid monolayers and enabled the porphyrin to stay in its monomeric form (as confirmed by PL spectroscopy), thus demonstrating that chitosan can be pointed out as a potential photosensitizer delivery system in photodynamic therapy.

Keywords: Phospholipids, Porphyrin, Chitosan, Photodynamic Therapy, Langmuir Monolayers, Langmuir-Blodgett films.

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1. INTRODUCTION

Porphyrin dyes and other closely related macrocycles, which may contain peripheral substituents or incorporated metal cations, have been used in different applications such as: catalysts in oxidation reactions as a mimicking agent of cytochrome P-450,[1] in medicine for tumour detection and arises as potential photosensitizers for photodynamic therapy of cancer.[2, 3] Photodynamic therapy (PDT) is a term used for a therapeutic modality in the treatment of a variety of oncological, cardiovascular, dermatological, and ophthalmic diseases.[4, 5] PDT is based on the use of photosensitizing chemicals, which preferentially accumulate in the target tumor cells, followed by local illumination with light in an appropriate wavelength to activate the specific drug.[6, 7] Upon photoactivation the generation of cytotoxic species, such as reactive singlet oxygen, leads to irreversible destruction of the treated tissues. However, many photosensitizers have some limitations, such as photo or structural instability, or a limited usable range of solvent conditions. In this sense, an important point that reflects the photosensitizer efficiency is related to its interaction with the biomembrane and its permeation and retention for the cell. This factor can be modulated by using specific carriers for drug delivery system and modified liposomes,[8] polymeric micelles[9] and nanoparticles[10] have been developed to encapsulate photosensitizers.[11, 12]

On the other hand, Langmuir monolayers of phospholipids constitute suitable biomembrane model system, since cell membranes can be considered as two weakly coupled monolayers.[13] The additional advantage of this model is the possibility to investigate interactions between biological species in two dimensions with easy control of lateral pressure, molecular density, and composition. Moreover the transference of Langmuir monolayers to a solid support origins the deposited Langmuir-Blodgett (LB) films, allows additional characterization for studies of phenomena in biological membranes. The interaction of phospholipid monolayers with different kinds of porphyrins are reported in the literature and it was learned that the interaction depends on different

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parameters, such as porphyrin polarity.[14] However, in rare cases[15, 16] these studies are related with PDT. In order to incorporate the phorphyrin in liposomes or increase its interaction with biomembranes, chemical modifications are carried out, introducing hydrophobic substituents.[17] Otherwise, one could seek for a molecule with favorable features such as nontoxicity, biocompatibility and biodegradability, to actuate as a carrier for non amphiphilic porhyrins acting as photosensitizer. Chitosan, a natural polysaccharide obtained from chitin, gather together these characteristics.[18, 19] In addition one can expect an enhancement of the absorption and extent of drug trafficking *in vivo* because it promotes an intimate contact with the mucosal, due to its bioadhesive property.[20]

Therefore, in this study, the incorporation of two oppositely charged porphyrins, the cationic, TMPyP and the anionic, $TPPS_4$, in Langmuir monolayers and LB films of a negatively charged phospholipid were accomplished investigating chitosan as a mediator, aiming understand the role of the polysaccharide in this interaction.

The lipid monolayers were investigated by surface pressure (π)-area isotherms and dynamic dilatational surface elasticity measurements. Furthermore, the incorporation of the porphyrin into the films was characterized by quartz crystal microbalance and photoluminescence spectroscopy of corresponding LB films.

2. MATERIALS AND METHODS

2.1. Chemicals

The structures of the studied molecules are presented in Figure 1. In Figure 1(a) we present chitosan repetitive unity, and Figures 1(b) and (c) represents the porphyrins TMPyP and $TPPS_4$ respectively.

Dipalmitoylphosphatidylglycerol (DPPG/Figure 1(d)) and dimyristoylphosphatidic acid (DMPA/Figure 1(e)) and zinc acetate were purchased from Sigma® and used without further purification. Chitosan was obtained from Galena® with an acetylation degree of 15%, as determined using hydrogen nuclear magnetic resonance according to the method described in the literature.[21] The molecular weight, Mn (108 700 Da), and polydispersity index (6.2) were determined by size exclusion chromatography (SEC). Porphyrins were obtained from Mid Century®. All aqueous solutions were prepared using dust free Milli-Q® water (surface tension of 72.8 mN m^{-1} and resistivity of 18.2 MΩcm).

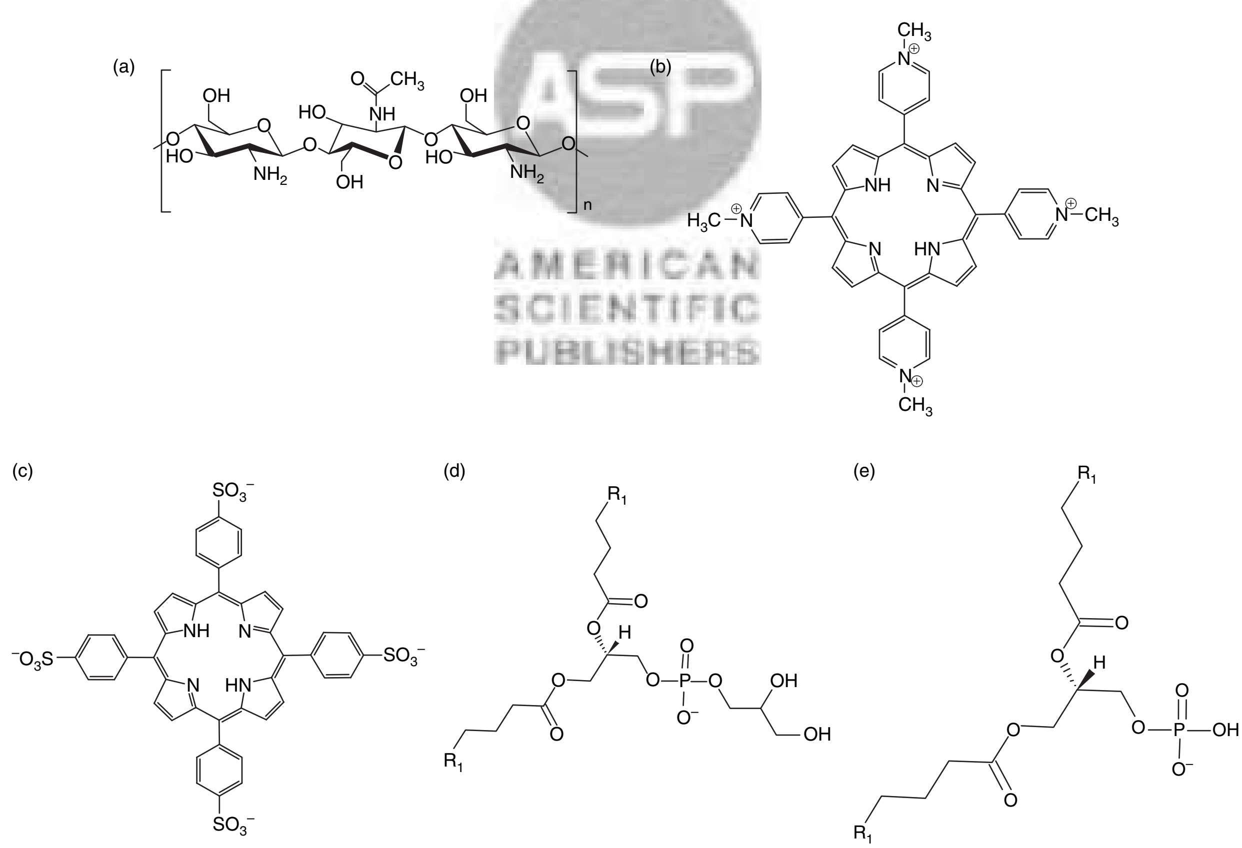

Fig. 1. Structures of (a) chitosan, (b) 5, 10, 15, 20-meso-tetrakis(*N*-metyl-4-pyridyl) porphyrin, (TMPyP), (c) 5, 10, 15, 20-meso-tetrakis(sulfonatophenyl) porphyrin, ($TPPS_4$), (d) Dipalmitoylphosphatidylglycerol, (DPPG), $R_1 = C_{11}H_{23}$ and (e) dimyristoylphosphatidic acid, (DMPA), $R_1 = C_{13}H_{27}$.

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2.2. Langmuir Monolayers

Langmuir monolayers were prepared in a 216 cm^2 Langmuir trough (Insight, Brazil). Aliquots of a chloroform:methanol (4:1) (J.T. Baker) 10^{-3} mol L^{-1} DMPA solution spread on an aqueous subphase containing 10^{-4} mol L^{-1} zinc acetate, or 10^{-3} mol L^{-1} DPPG solution on 0.05% (w/w) chitosan solution. The compression was performed with a speed of 10 mm^2 min^{-1} using movable barriers. Temperature was 23 ± 1 °C.

2.3. Dilatational Surface Elasticity

The dilatational dynamic elasticity of DPPG monolayers formed on pure water and on porphyrin aqueous solution was determined at $30 + 2$ mN m^{-1} (correspondent to the biomembrane lipid packing). It was measured by the axisymmetric shape drop analysis method (OCA-20, Dataphysics Instruments GmbH, Germany), with oscillating drop accessory ODG-20, as described in the literature.[22, 23] In this measurement, a DPPG chloroform solution (10^{-4} mol L^{-1}) was gently touched the surface of a reduced size drop, formed by pure water or 5.0 10^{-6} mol L^{-1} porphyrin aqueous solution. The drop was then rapidly expanded up to a predetermined drop area rendering the desired surface pressure. The dynamic surface elasticity data were obtained after the surface tension reached a constant value by using a periodic drop oscillation with an amplitude of 0.1 mm (relative area variation $\Delta A/A = 5.5\%$) and a frequency of 1.0 Hz. The viscous effect, related to the imaginary part of the elasticity modulus, was estimated from the phase angle.

2.4. LB Films

In view of the fact that DPPG does not provide efficient interaction between successive layers, LB films were prepared by the asymmetric transference film methodology (Fig. 2). First, three layers of DMPA spread on 10^{-4} mol L^{-1} zinc acetate aqueous solution were transferred to the solid substrate, with a transfer ratio of 0.98.[24] DPPG monolayer was transferred as the fourth layer, by the interaction with the hydrocarbon tails of the lipids, with a transfer ratio of 0.038 mm s^{-1}. The quartz crystal microbalance (QCM) technique was used to determine the mass adsorbed per layer on LB films. In all cases the films were deposited on gold-covered quartz crystals (International Crystal Manufacturing, USA, 10 MHz, active area, A, of 0.662 cm^2), previously cleaned with chloroform, ethanol and toluene, and dried with a nitrogen flow. The oscillation frequencies (ICM Lever Oscilators-10 MHz-USA) were monitored (frequencymeter Lutron FC2700TCXO, China) until constant values. Under specific conditions, changes in the oscillation frequencies are proportional to the adsorbed mass, according to the Sauerbrey[25, 26] equation $\Delta F = (-0.26 \times 10_6 F_0 2\Delta m)/A$, where ΔF is the frequency change; F_0 is the initial frequency (without coverage); Δm is the deposited mass and A is the electrode area (0.662 cm^2). The chitosan samples were dissolved in water at 0,05% w/w concentration, $pH = 4.0$ and employed as subphase for the lipid monolayers. After LB film formation, they were immerged in a 5.0 10^{-6} mol L^{-1} porphyrin aqueous solution. The adsorption kinetics was studied monitoring frequency changes of the films after withdraw from the solution in determined time intervals, rinsing in water and dried by spontaneous water evaporation. The films were also analyzed by photoluminescence spectroscopy using a Jobin-Yvon Spex Fluorolog 3 spectrofluorometer. In addition to the monochromators, a filter set of 606 nm was used for selection of the emission wavelengths, in order to improve the quality of the spectrum.

3. RESULTS AND DISCUSSION

3.1. Porphyrins and DPPG Langmuir Monolayers

DPPG was chosen for this study because it is a natural lipid found in biological membranes. It is negatively charged and it is also able to interact by hydrogen bond due to the presence of hydroxyl groups. Figure 3 presents π-A isotherms for DPPG monolayers formed on pure water and on 5.0 10^{-6} mol L^{-1} TMPyP or $TPPS_4$ solution

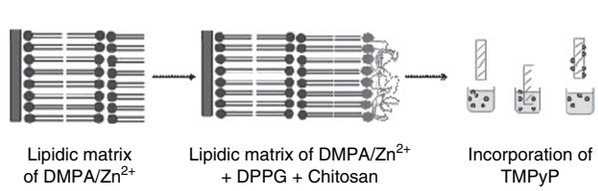

Fig. 2. Scheme of Asymmetric Film Transference methodology and porphyrin adsorption from solution.

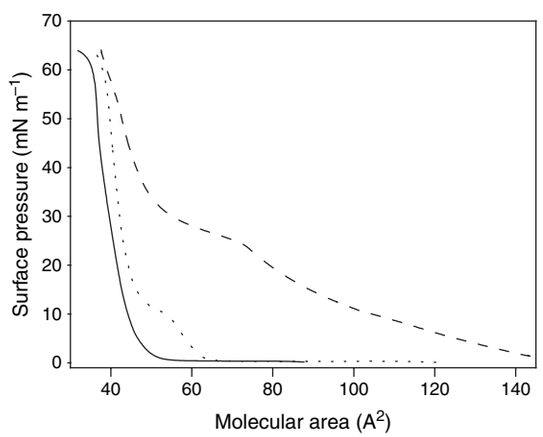

Fig. 3. π-A isotherms at $23 + 1$ °C of DPPG monolayer spread on: (__) pure water, (- -) TMPyP 5.0 10^{-6} mol L^{-1} and (...) $TPPS_4$ 5.0 10^{-6} mol L^{-1} aqueous solutions.

as subphases. In the presence of both porphyrins, negatively and positively charged, it is observed an expansion of the monolayer promoted by the interaction of the lipid with the porphyrin. Analyzing the structures of DPPG and those of the two porphyrins it is expected two kinds of possible interactions between these molecules: electrostatic, dominant for DPPG and TMPyP, and hydrogen bond formation between the N–H groups of the porphyrins and O–H groups of the phospholipids. In fact, for the monolayer 8 formed in the presence of TMPyP (Figure 1(b)), a more pronounced expansion is noted compared with the monolayer formed in $TPPS_4$. A phospholipid monolayer expansion was also noticed[27, 28] in the case of the synthetic negatively charged DMPA in the presence of TMPyP lipid, and it was naturally attributed to coulombic interactions. Actually, DMPA has only a phosphate group and not the glycerol moiety. As the surface pressure initial lift-off occurs in an area approximately 2.5 times larger the one observed for the monolayer formed on pure water it is reasonable to presume the porphyrin molecules have some penetration within the DPPG monolayer. In this sense, the expansion of the monolayer is promoted by the adsorption of the porphyrin into the lipid-air interface. With further compression, however, the porphyrin and phospholipid molecules are gathering together and porphyring molecules may undergo some change in orientation, whereas phospholipids can seek for additional space. In the condensed state the difference in minimum areas for DPPG in the monolayer formed on pure water and on TMPyP solution is $\sim$15 Å.[2] Considering that, if all added porphyrin molecules would be at the interface, the porphyrin:phospholipid ratio would be higher than 10:1, and the area available for porphyrin molecule at the interface would be much lower than the area expected by TMPyP molecules lying parallel to the interface. Nevertheless, due to the solubility of TMPyP in aqueous solution it is not so straight this kind of assertion. Furthermore, for both porphyrins it is possible to observe a phase transition between liquid-expanded (LE) and liquid-condensed (LC) states, at about 25 mN m^{-1} for TMPyP, and 10 mN m^{-1} for $TPPS_4$. At the experiment temperature the LE-LC coexistence region is not observed for DPPG on pure water but it can be detected at 8 mN m^{-1} on PBS (phosphate buffer saline, pH 7.4 and 150 mM NaCl) buffer. At this condition DPPG presents the main transition close to 40 °C and the subtransition, solid-to-solid type at about 25 °C being highly dependent on the buffer condition; for instance in Tris-HCl, pH 7.4, it is about 17.7 °C. Nevertheless, this kind of compression phase transition was observed before for the negatively charged phospholipid, DMPA, with TMPyP,[27, 28] at about 27 mN m^{-1}, and with Mn(III)[TPP],[14] around 18 mN m^{-1}. Interestingly, this transition was not detected in the case of DMPA interacting with other positively charged more polar Mn(III)porphyrins. Pedrosa et al. also studied metalcomplex porphyrins at the air–water interface with lipids, and proposed different organization models depending of the coordination number of the metal ion.[29] In our case both are free base porphyrins and the electrical charge are localized at the peripherycal positive pyridyl or at the negative benzene sulphate rings. However, both have hydrogen atoms linked to the electronegative nitrogen able to establish hydrogen bonds with DPPG, within the center of the porphyrin ring. From former results reported in the literature and facts presented here, we deduce the assignment of this phase transition for DPPG in the presence of electrically charged solutes can be related to the change in the dissociation constant of the lipid due to the ionic strength of the subphase. However it is likely the huge difference of the isotherms of DPPG on TMPy and $TPPS_4$ is due to electrostatic interactions of the porphyrin with the negatively charged head group of the phospholipid. On the other hand the accommodation of phospholipid molecules at the free space existing between porphyring molecules, or on the top of the porphyrinic ring, can also have influence on this transition. Moreover, it can not be excluded the possibility of orientation change of porphyrin molecules caused by the confinement imposed by the compressing barriers.

Furthermore, based on π-A isotherms, reflection spectroscopy[27, 28] and Brewster angle microscopy,[30] different molecular organization of TMPyP dependent of the DMPA surface packing were proposed: around 8 mN m^{-1} TMPyP would be in its monomeric form, and with further compression, the presence of dimeric forms coexisting with monomeric ones was proposed around 24 mN m^{-1}. The presence of dimers was explained in terms of the strong tendency of the porphyrin to associate and to the accessibility of the negatively charged headgroups of the lipid monolayer. In our case the accessibility of the negative charge is however lower due to the additional OH group of glycerol moiety.

Adsorption kinetics of the porphyrins onto DPPG monolayers were monitored by surface tension changes in experiments using the pendant drop technique. The drop was formed from the porphyrin solution. A small aliquot of the lipid was spread on the surface of a small drop, which was rapidly expanded. Several preliminary attempts, combining volume and concentration of the lipid and expansion ratio of the drop, allow beginning the experiment from a surface pressure close of 30 mN m^{-1}, correspondent to the biomembrane lipid packing.[31] Images of the drop were captured and surface tension or pressure calculated from the retrieved images. Time "zero" was considered at the point that the drop was expanded up to the surface pressure of 30 mN m^{-1}. The results are depicted on Figure 4.

For TMPyP an exponential growth is observed and initial adsorption rates, $v_{ads}(0) = \lim_{t\to 0} (d\pi/dt)$, in addition to changes in surface pressure, ($\Delta\pi = \pi_{eq} - \pi_{in}$) indicate a more effective adsorption of TMPyP as compared with $TPPS_4$ (see Table I). Another 11 parameter that is worthwhile to compare is the time necessary for the system to

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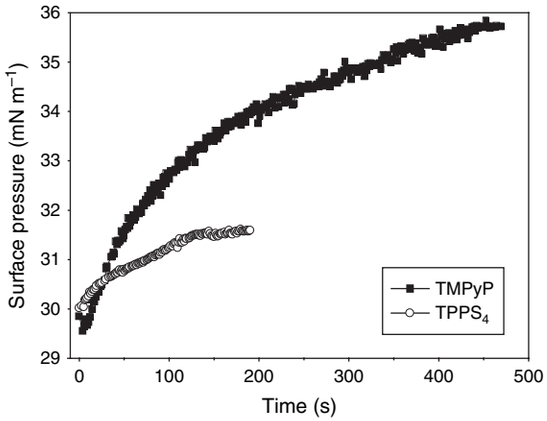

Fig. 4. Adsorption kinetics of the porphyrins onto DPPG monolayers at an initial surface pressure π_{in} of 30 mN m^{-1}.

attain the "adsorption" equilibrium. The change in surface pressure caused by the presence of $TPPS_4$ is less than 2 mN m^{-1} and occurs in less than 1.5 minutes. In the presence of TMPyP, instead, the equilibrium in reached after 7.5 minutes. As $TPPS_4$ and DPPG are both negatively charged, these results may simply be related to a rearrange of DPPG molecules at the interface caused by a disturbance of ionic species distribution within the electrical double layer. To discard this possibility and assume an adsorption process, one needs additional information on $TPPS_4$ incorporation/adsorption on DPPG films.

The surface compressional modulus, also referred as equilibrium in-plane elasticity (C_s^{-1}), were obtained from the surface pressure curves ($C_s^{-1} = -(d\tilde{\pi}/d\ln A)_T$), and compared with the dynamic dilatational surface viscoelasticity modulus (E). Whereas the former brings information of monolayer compaction in equilibrium conditions, the latter tell us about the ability that the bidimensional system has in reestablish surface tension conditions after a dilatational disturb, which can be related to changes in surface packing or effectiveness of the interaction between molecules at the interface. In order to measure E, first the kinetics of porphyrin adsorption was monitored as described above and, after the system reached the equilibrium, the drop was oscillated at a frequency of 1 Hz.

In equilibrium conditions, C_s^{-1} follows the sequence: DPPG/$TPPS_4$ > DPPG > DPPG/TMPyP (Table I), indicating that $TPPS_4$-makes DPPG monolayer more rigid, whereas TMPyP should expand it, as it becomes more compressible. Comparing the dynamic and equilibrium data (Table I), one recognizes that C_s^{-1} and E coincides for DPPG monolayers formed on pure water. Also for this condition the phase angle is zero, indicating the monolayer is purely elastic. For DPPG monolayers formed on porphyrin solutions the results for E follows the same trend as that obtained in equilibrium conditions, in the sense that DPPG/$TPPS_4$ exhibits a higher viscoelastic modulus compared to DPPG/TMPyP. However, the absolute differences related to pure water subphase in dynamic conditions are less pronounced. Nevertheless, the drastic reduction of C_s^{-1} (85%) and of E (40%) for DPPG in the presence of TMPyP compared to the value obtained on pure water (see Table I), is interpreted as a decrease in surface packing of DPPG in the presence of TMPyP and also to a huge contribution of loss modulus (or surface viscosity) in the E value denoted by the higher value of E as compared to C_s^{-1}. In this case one does not have a true surface viscosity but it refers to an energy loss due to lipid-porphyrin interaction. On the other hand, the E value obtained for DPPG/$TPPS_4$ is contrary to that observed in equilibrium conditions: E does not surpass the value obtained for DPPG on pure water as C_s^{-1} does. Therefore, despite the porphyrins adsorption kinetics curves present a similar behavior, in the way that for both an increase in the surface pressure is recorded, the comparison between equilibrium and dynamic conditions show that different phenomena are occurring at the air/liquid interface for these two oppositely charged porphyrins. Whereas the kinetics adsorption curve for TMPyP represents the adsorption/incorporation of the porphyrin onto the lipid monolayer, the increase in π values promoted by $TPPS_4$ porphyrin, is much likely related to an effect in the electrical double layer of the DPPG monolayer, increasing the repulsion between DPPG molecules at the interface. In other words, this electrostatic repulsion acts preventing the molecules to come near the interface. This is the reason for an increase in surface pressure and not the incorporation of the $TPPS_4$ onto the interface. It is important to realize that in this case the decrease in E, as compared to E value for DPPG on pure water, is not accompanied by a huge contribution in surface viscosity: compare C_s^{-1} and E in this case. The decrease in E is then associated to a loss in freedom degree of the molecules at the interface caused by the electrostatic repulsion, which decreases the ability of the monolayer in recover surface tension conditions upon changes in area. These results put together indicate that the interaction of TMPyP and $TPPS_4$ with DPPG monolayers, used here as a

Table I. Initial adsorption rates of porphyrins (v_{ads}) as estimated from the slopes of the surface tension curves at the limit of $t \to 0$ and dilatational surface viscoelastic modulus (E) for DPPG monolayers formed on pure water and on porphyrin solutions, at 23.0 + 0.6 °C. The equilibrium compressional modulus C_s^{-1} is included for comparison.

	$v_{ads}(0)$ (mN m^{-1} s^{-1})	$\Delta\pi$ (mN m^{-1}) (±0.2)	C_s^{-1} mN m^{-1}	E (mN m^{-1})
DPPG (at 30 mN m^{-1})			210.3 ± 3.3	206.5 ± 4.0
DPPG/TMPyP	0.046 ± 0.003	5.8	30.4 ± 0.3	120.2 ± 2.5
DPPG/$TPPS_4$	0.020 ± 0.001	1.8	301.3 ± 5.9	157.9 ± 6.0

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biomembrane model, can be completely different indicating that one should expect a higher amount of the cationic porphyrin in the neighborhood of the interface.

3.2. LB Films

LB Films were prepared using asymmetric transference films methodology, as described in the Experimental Section. In this way, films presented a polar head ending. Quartz crystal microbalance (QCM) technique was used to determine the adsorbed masses. The template of the films, formed by DMPA/Zn_{2+} layers rendered a mass of 198 ± 2 ng per layer, in agreement with previous report.[24] The fourth layer, formed by DPPG, presented a mass of 170 ± 2.7 ng, in good agreement with the expected mass value, 180 ng, estimated from the π-A curve for DPPG. The LB films were then immediately immersed in a porphyrin solution, rinsed and dried at regular time intervals. After that, the frequency was recorded and the films were analyzed by emission spectroscopy. Several samples were prepared following this methodology with solutions of the two different porphyrins.

When the LB film, having DPPG as the top layer, was immersed in a 5.0 10^{-6} mol L^{-1} TMPyP aqueous solution, the deposited mass was 175 ± 10 ng. After rinsed, a strong desorption has occurred and the mass of porphyrin effectively attached to the film decrease to 75 ng. The larger initial deposition should be easily attributed to an excess of porphyrin forming multilayers weakly coupled that were washed out. Considering the number of DPPG molecules in the top layer and the molecular weight of TMPyP and $TPPS_4$ and neglecting the water co-adsorbed with the porphyrins, it is possible to estimate that DPPG:TMPyP ratios as 2.5:1. For $TPPS_4$, in the same concentration, a strong desorption is also observed, and the final adsorbed mass was about 47 ng (71 ng cm^{-2}), after four immersion/rinse cycles. On should be aware that for $TPPS_4$ one even would not expect that adsorption could take place, as from results for the air-liquid interface and because phospholipid and porphyrin present the same electrical charge. In fact, this surface mass density would be associated to a bilayer of water molecules adsorbed on the LB surface if one takes an average area per water molecule of 4 Å.[2,32] This estimative is only to confirm that the amount of adsorbed $TPPS_4$ onto DPPG LB films is in fact quite low. Instead of adsorb the porphyrin from solution, Prieto et al. have prepared DMPA:TMPyP as a mixed monolayer, forming LB films onto ITO and analyzing by UV–vis.[28] Comparing the recorded spectra with the one obtained from solution allow the authors deducing the adsorption of the porphyrin has occurred in one layer, but they have no quantification of adsorbed mass. The mixed 4:1 TMPyP/DMPA monolayer was also transferred onto hydrophilic glass at a surface pressure of 35 mN m^{-1}, by Martin and coworkers.[27] According to these authors, the absorption spectrum obtained for the mixed monolayer demonstrated that only the monomeric form was present on the substrate.

In order to have some information about the organization of the porphyrin within the films they were also analyzed by emission spectroscopy. The recorded spectra obtained for these transferred films (Figs. 5(c–d)) were compared with those obtained for the photosensitizers in solution (Figs. 5(a–b)).

In a general way the spectra obtained for the porphyrins incorporated in DPPGLB films differ from those recorded for porphyrin solutions. It is also possible to infer that the porphyrin concentrations within the film is quite low, as from the noise noticed in the spectra. The spectrum obtained for $TPPS_4$ in solution (Fig. 5(a)), corresponds to the monomeric form of $TPPS_4$. The non-characteristic spectrum registered for $TPPS_4$ in DPPG film, Figure 5(c), can be attributed to porphyrin aggregation promoted by electrostatic repulsion between the photosensitizer and lipid, both negatively charged. Self-aggregation in aqueous systems is a common feature of water-soluble porphyrins carrying charged substituents. The polar groups (e.g., sulfonic, amino, or ammonium groups) that convert the porphyrin chromophore into a water-soluble molecule can exhibit a strong intermolecular interaction with the center of other porphyrin molecules for neutralizing a positive charge (metal cation or protonated nitrogens) or neutralizing a negative charge (deprotonated nitrogens).[33] Aggregation phenomena strongly affect the spectral and energetic characteristics of porphyrins, thus reducing quantum yields and excited-state lifetimes (singlet and/or triplet).[4] One possible solution to this problem is to encapsulate the porphyrin in biodegradable polymeric microspheres to provide an environment where the photosensitizer can be administered in a monomeric form.[34]

Even being oppositely charged, the electrostatic attraction does not provide an efficient binding of the TMPyP to the DPPG-LB film and a strong desorption occurs after rinsing. According to the literature, when a porphyrin is dissolved in water, this macrocycle can form noncovalent dimers or larger aggregates in bulk solution or on the surface of oppositely charged molecules (other macrocycles, cyanines, polypeptides, proteins, nucleic acids, or the components of mitochondrial membranes). This fact can explain the observed desorption, when the dimers or aggregates were removed during the rising. The emission spectrum of Figure 5(b) is typical of cationic porphyrins in polar solvents. The low resolution is caused by pyridinic groups that create a transference charge state very close to the first excited singlet state of these porphyrins, resulting in a mixture of both energy states. The proximity of these states is dependent of solvent polarity and rotation level of pyridinic groups.[35] In Figure 5(d) the TMPyP emission spectrum is well-resolved and it can be attributed to electrostatic interaction between porphyrin and lipid that promotes a decrease on pyridinic rotation level. In addition,

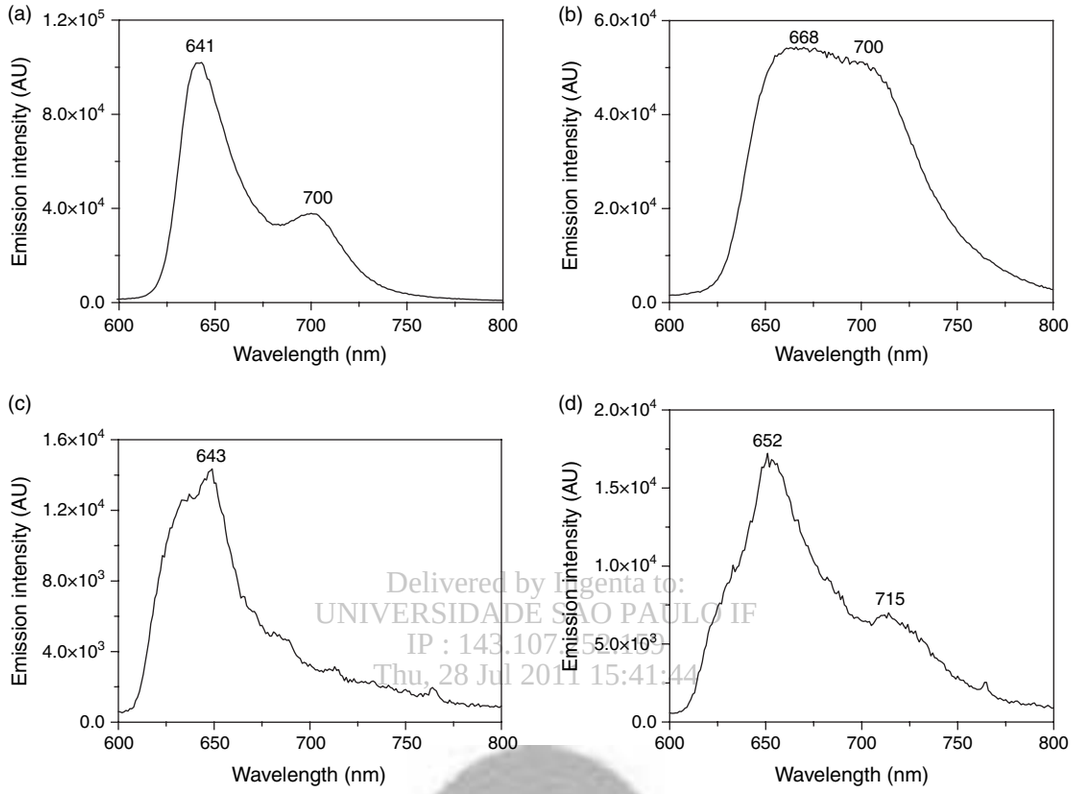

Fig. 5. Emission spectra for the porphyrins in aqueous solution: (a) $TPPS_4$ (1.9×10^{-6} mol L^{-1}) and (b) TMPyP (2.4×10^{-6} mol L^{-1}) and for the films: (c) LB-DPPG/$TPPS_4$ and (d) LB-DPPG/TMPyP films, $\lambda_{exc} = 515$ nm.

TMPyP is immobilized on the film and it also decreases the pyridinic group rotation.

In this sense, besides the feasibility of porphyrin incorporation onto the LB films, their concentration seems to be quite low and it is likely that the integrity of the photosensitizer should be preserved in order to increase its efficiency in PDT, which seems not to be the case for DPPG-$TPPS_4$. So, the next step was to evaluate chitosan as a possible drug delivery carrier analyzing its influence on porphyrin interaction with DPPG films, the biomembrane model used, since encapsulation in delivery systems shows other advantages like to increase the accumulation at the tumor site due to enhanced endocytotic activity and the photodynamic activity.[36–39]

3.3. Effect of Chitosan in the Porphyrin-Lipid Interaction

In order to analyze the effect of chitosan on the interaction of porphyrin and lipid, LB films were prepared in a similar way as described before, building a 3-layer DMPA/Zn_{2+} LB film. The top layer however, was formed by the transfer of a DPPG monolayer formed on a 0.05% (w/v) chitosan aqueous solution, pH 4.0. The deposited mass of the DPPG/chitosan layer, as assayed by QCM technique, was 481 ± 50 ng ($726.6 + 50$ ng cm^{-2}). This value apparently differs from the one obtained by Pavinatto et al.[40] of 809 ng for DPPG deposited from a 0.02% (w/v) chitosan aqueous solution at pH 3.0. Although pH and concentration may have influence on the deposited mass, these variables did not have significant role in our results. On the other hand, if one takes into consideration the surface mass densities obtained in both studies it will appear the values are equivalent, since the active area for the crystals used in the QCM technique by Pavinatto et al.[40] differs from the one employed in our experiments.

On the other hand, Messai et al.[41] investigated the influence of some physicalchemical parameters in the adsorption of chitosan on poly-(acid lactic) particles. The authors verified that the amount of adsorbed chitosan increased with the molecular weight. Their explanation was based on differences in the macromolecule conformation. Low molecular weight is associated to a rod conformation which can adsorbs flat onto the particle, occupying a large area. Increasing the molecular weight, flexibility was also increased and the polyelectrolyte adopted a coiled conformation. Thus, more chains could be accommodated on the surface reflecting in an increase in the amount of adsorbed polymer. The polydispersity index of chitosan used in this study is relatively high (PI = 6.2), which means the chitosan samples are composed by macromolecules with

different chain lengths. In fact, PI is a parameter often investigated in reports involving the interaction/adsorption of chitosan with different systems. For our system, the different chain lengths would probably lead to different magnitudes of adsorption. Previous studies involving the interaction between chitosan and lipids or proteins[39, 42–44] used the polysaccharide with similar PI values. Regarding the aim of this study, such differences are thought to be of minor importance for the final effect of adhesion, since one could consider as a comparison of an average behavior.

Figure 6 presents the adsorption kinetics of TMPyP and $TPPS_4$ 5.0 10^{-6} mol L^{-1} onto the LB film covered with chitosan. The deposition rates are similar for both porphyrins but TMPyP takes longer time to reach the equilibrium. Moreover, the total deposited mass is larger for TMPyP, 451 ng, as compared with $TPPS_4$, 249 ng, which reaches a saturation (represented by the plateau in Fig. 6) in less than 2 minutes.

In both cases, the immobilization of the photosensitizer mediated by the polysaccharide is larger, when compared with the experiment carried out in the absence of chitosan. The higher adsorption of the cationic porphyrin in the presence of chitosan, that also bears a small positive surface density, suggests that ion-dipole intermolecular forces are responsible for the adsorption. Moreover, the polysaccharide should provide a higher number of binding sites responsible for the increase in the surface mass density as compared to the adsorption carried directly on the phospholipid LB film.

Figures 7(a–b) show the emission spectra for the porphyrins adsorbed on DPPG LB films mediated by chitosan. Under this condition the $TPPS_4$ emission spectrum presented in Figure 7(a) looks like the one obtained for the porphyrin in solution (Fig. 5(a)). The red shift (around 13 nm) is probably a consequence of its interaction with the polysaccharide. This result attests that $TPPS_4$ remains in the monomeric form when the adsorption on DPPG LB

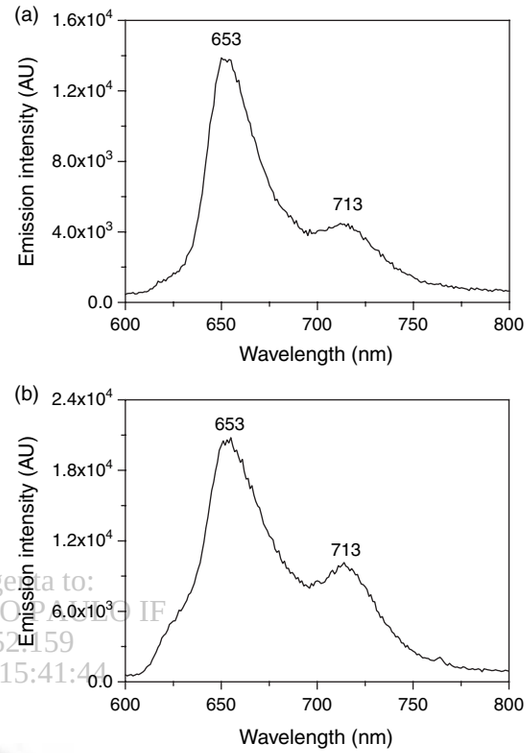

Fig. 7. Emission Spectra for (a) LB film with $TPPS_4$ and (b) LB film with TMPyP mediated by chitosan, $\lambda_{exc} = 515$ nm.

film is mediated by chitosan. The TMPyP emission spectrum presented in Figure 7(b) is considerably well-resolved as compared with the one recorded for TMPyP in solution (Fig. 5(d)). This result can be explained in terms of a decrease in the rotation level of the pyridinic groups due to the interaction between porphyrin and lipid mediated by chitosan. For the porphyrin that exhibited a higher adsorption, TMPyP, we have studied the influence of the immersion time on the porphyrin adsorbed mass. Two different immersion times were chosen: 30 seconds and 2 minutes.

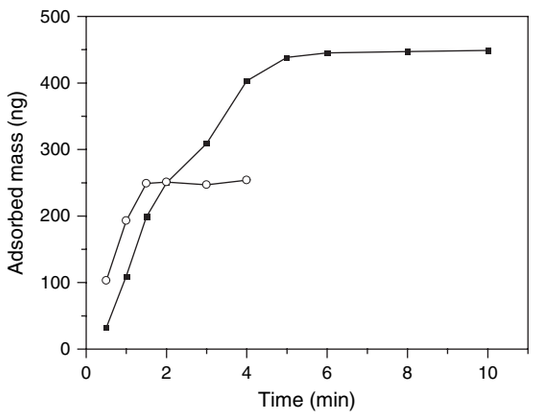

Fig. 6. Adsorption kinetics of TMPyP (■) and $TPPS_4$ (◯) onto the DPPG-chitosan LB films.

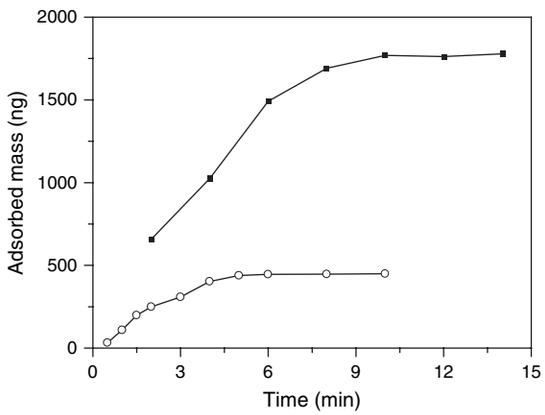

Fig. 8. Adsorbed mass of TMPyP on DPPG-chitosan LB films in intervals of (◯) 30 seconds and (■) 2 minutes.

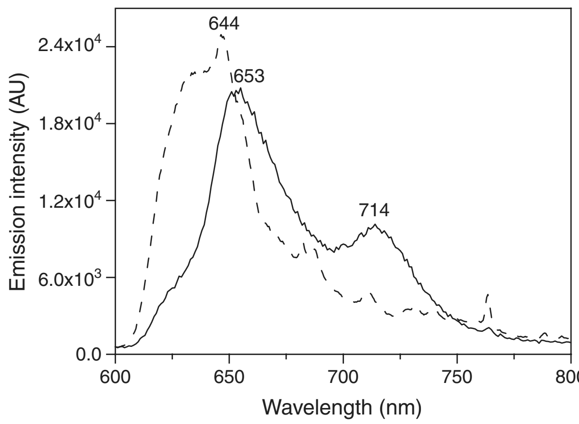

Fig. 9. Emission spectra of TMPyP on DPPG-chitosan LB film after 10 minutes in intervals of 30 seconds (__) and after 14 minutes in intervals of 2 minutes (- -), $\lambda_{exc} = 515$ nm.

The results are depicted in Figure 8, showing that the lower the time in which the films were immersed in porphyrin solution, lower is the deposited mass and time necessary to attain the adsorption equilibrium. As the film are dried between each time interval, it is likely the water withdraw may change the macromolecule conformation causing a decrease in the number of available/exposed binding sites, or may also change the aggregation state of porphyrin molecules present at the interface. In fact, the emission spectra from films prepared under the two different conditions were recorded (Fig. 9), indicating that the aggregation of TMPyP takes place in the film prepared at large immersion time, which explains the higher deposited mass in this case.

Previous report on the interaction between chitosan and DPPG monolayers studied by dilational surface elasticity indicated that the polysaccharide strongly interacts with the lipid monolayer, even not presenting surface activity. The study also evidenced that chitosan promotes alterations in the packing of the monolayer, showing an ability of chitosan to disrupt cell membranes.[39] This feature could result in a way to facilitate the penetration of the photosensitizer into the cell.

4. CONCLUSIONS

Considering the obtained results, we can conclude that the presence of the chitosan as a mediator of the interaction between the photosensitizer and the lipid monolayer, used as a cell membrane model, enable the porphyrin (specially $TPPS_4$) in its monomeric form (not aggregated) and increase its concentration at the interface. In this sense, the drug can be applied in photodynamic therapy more efficiently. Taking into account that the polysaccharide may destabilize the lipid monolayer, resulting in a way to facilitate the penetration of the photosensitizer into the cell, we conclude chitosan is a promising drug delivery for PDT.

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