Colloids and Surfaces B: Biointerfaces 166 (2018) 152–160

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

# Molecular features of nonionic detergents involved in the binding kinetics and solubilization efficiency, as studied in model (Langmuir films) and biological (Erythrocytes) membranes

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ARTICLE INFO

*Article history:*
Received 27 November 2017
Received in revised form 13 February 2018
Accepted 9 March 2018
Available online 10 March 2018

*Keywords:*
Erythrocyte membranes
Solubilization
Monolayers
Nonionic detergents
Brij

ABSTRACT

The effect of the nonionic detergents Brij-98 and Brij-58 over human erythrocytes was studied through quantitative hemolysis and in Langmuir films. Hemolytic tests revealed that Brijs are stronger membrane solubilizers than Triton X-100 (TX-100), with effective detergent/lipid ratios of 0.18 and 0.37 for Brij-98 and Brij-58, respectively. Experiments with Langmuir films provided significant information on the kinetics and thermodynamics of detergent-membrane interaction. The adsorption ($k_a$) and desorption ($k_d$) rate constants of Brijs were lower than those of TX-100. In the case of $k_a$, that is probably due to their larger hydrophilic head (with twice (20) the oxyethylene units of TX-100). As for the thermodynamic binding constant, the linear and longer hydrophobic acyl chains of Brijs favor their stabilization in-between the lipids, through London van der Waals forces. Consequently, $K_{b,m}$ values of Brij-98 (12,500 $M^{-1}$) and Brij-58 (19,300 $M^{-1}$) resulted higher than TX-100 (7500 $M^{-1}$), in agreement with results from the hemolytic tests. Furthermore, Brij-58 binds with higher affinity than Brij-98 to bilayers and monolayers, despite its shorter (palmitic) hydrocarbon chain, showing that unsaturation restrains the detergent insertion into these environments. Our results provide significant information about the mechanism of interaction between Brijs and membranes, supporting their distinct solubilization effect.

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## 1. Introduction

The aggregative properties of detergents have been considered as an extremely relevant factor for membrane solubilization [1–6]. In the model proposed by Le Maire et al. [7], the bilayer solubilization process that usually begins with destabilization of the lipid components, at detergent concentrations below the critical micelle concentration (CMC), and leads to a total membrane disruption, at concentrations close/above to the CMC. Therefore, solubilization of membrane lipid and protein is a complex process that depends on the physicochemical properties of the detergent as well as on the membrane composition.

Many studies have described that the classical detergent Triton X-100 (TX-100) acts on both lipids and proteins membrane [8–10]. Also, Brij detergents have been described for the disruption of cell membranes, with a reduced tendency to solubilize components from the inner leaflet when compared with TX-100 [11]. Indeed the data reported in our previous work suggested that Brij 58 and Brij 98 solubilize the erythrocyte membrane in a different way than that promoted by TX-100 [12]. This distinct effect should be a useful method to purify particular protein and lipids. However, the mechanism by which these detergents interact and selectively solubilize membrane components remains unclear.

Here, as a first approach, we analyzed the interaction of the nonionic detergents Brij 98 and Brij 58 (Fig. 1) with human erythrocyte membranes, using the quantitative methodology described by Lichtenberg [1] to lipid bilayers. The membrane solubilization potency of Brij detergents was determined by hemolytic

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https://doi.org/10.1016/j.colsurfb.2018.03.012
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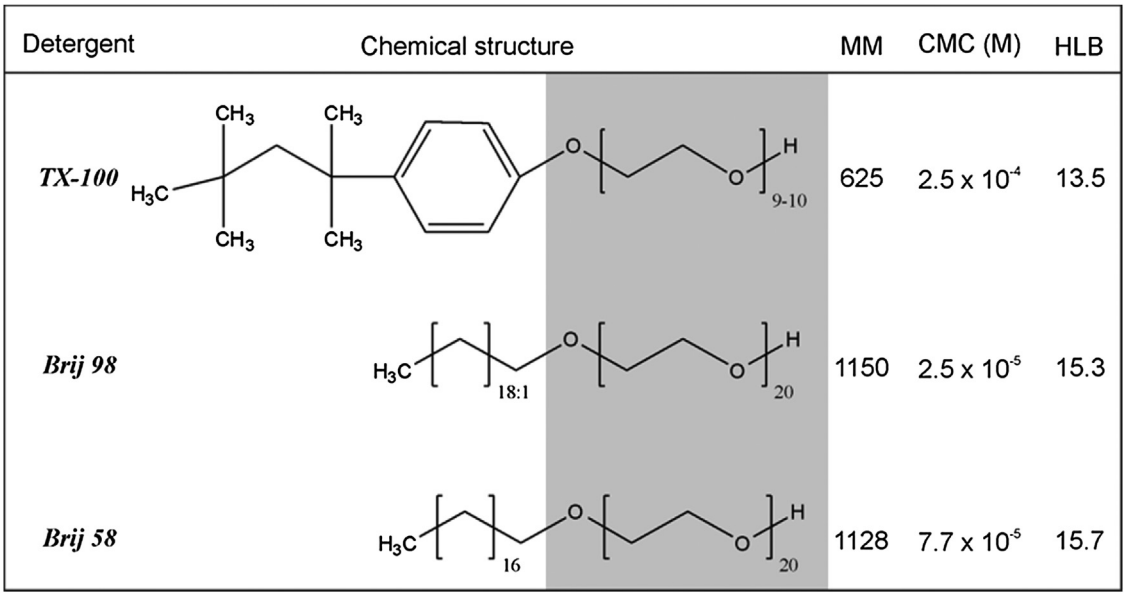


**Fig. 1. Chemical structure and some physicochemical properties of TX-100 (polyoxyethylene *t*-octylphenyl ether), Brij 98 (polyoxyethylene oleyl ether) and Brij 58 (polyethylene hexadecyl ether)**: molecular mass (MM), critical micelle concentration (CMC) and hydrophilic-lipophilic balance (HLB), according to Hait & Moulik [19].

experiment analysis [6,13], and compared to that of the classical detergent TX-100 [8].

In three-dimensional systems such as liposomes and biomembranes, the organization of a particular membrane is defined by molecular aspects such as the degree of molecular packing, the geometry, and mobility of the molecular components [14]. Since these properties are affected by each other, effects on the degree of lipid packing or on the curvature of the membranes as those induced by the insertion of certain molecules into the membrane, cannot be separated. In a different way, the addition of amphiphilic compounds in self-organized monolayers at the air-water surface (Langmuir films) allows maintaining a constant flat topology and controlling the degree of molecular packing [15]. Thus, the Langmuir system constitutes an excellent model for the study of detergent-membrane interaction. However, most of the monolayer studies are performed with pure lipids or mixtures containing only the major lipid components, e.g. phosphatidylcholine (PC), sphingomyelin (SM) and cholesterol (Chol), and do not take into account the presence of minor but relevant lipids of biological membranes [16–18].

Thus, to investigate more in detail the lipid-detergent interaction, as a second approach we exposed monomolecular layers composed of lipids extracted from human erythrocyte membranes at the air-water interface and analyzed the effect of Brij 98 and Brij 58 detergents. The binding affinity of these detergents to the Langmuir film, as well as their effects on the stability of the whole molecular assemble (lipid/detergent) were studied through Gibbs adsorption isotherms. The resulting data allowed us to define the adsorption and desorption kinetics, the thermodynamic binding affinity constants and the effects of detergents on the membrane flexibility at different molecular packings. These results contributed to extending the analysis of the hemolytic action of detergents at a molecular level.

## 2. Materials and methods

### 2.1. Materials

Detergents TX-100 (polyoxyethylene *t*-octylphenyl ether), Brij 98 (polyoxyethylene oleyl ether) and Brij 58 (polyethylene hexadecyl ether) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

### 2.2. Purification of human erythrocytes and ghosts

Human blood from healthy donors was freshly collected and obtained from the blood bank (approved by Ethics Committee at Faculty of Medical Sciences, University of Campinas, São Paulo, Brazil – protocol n ° 227/2009). The erythrocytes were purified after three washes in 5 mM phosphate buffer containing 150 mM NaCl at pH 7.4 (PBS buffer) at 1000 g for 5 min. The erythrocyte suspension was then filtered through α-cellulose/microcrystalline cellulose to isolate erythrocytes from platelets and leukocytes [20] and subjected to a new centrifugation with PBS buffer.

For the preparation of erythrocyte *ghost* membranes (erythrocyte membranes without their intracellular content), the packed erythrocytes were mixed with 10 vol of hypotonic phosphate buffer (5 mM Na-phosphate, 0.5 mM EDTA, and 5 mM di-isopropyl fluorophosphate, pH 8.0) at 4 °C, followed by centrifugation at 26,000*g*; the supernatant was discharged and the washing repeated at least four times with the same buffer, according to Crepaldi Domingues et al. [21]. The final *ghost* pellet was suspended in isotonic PBS buffer for cell resealing.

Protein concentration was determined with bicinchoninic acid (Bio Agency Biotecnol., São Paulo, Brazil), using bovine serum albumin as a standard. Lipid extraction from *ghost* membranes was performed with a chloroform:methanol:water (2:1:1, volume ratio) solvent mixture [12].

### 2.3. Isotonic hemolytic assay

Variable TX-100, Brij 98 and Brij 58 concentrations were added to four erythrocyte suspensions (hematocrit, Ht = 0.15, 0.3, 0.45 and 0.6%); the samples were kept for 15 min at 37 °C and centrifuged at 1000*g* for 3 min. The concentration of hemoglobin (Hb) released in the supernatant was measured at 412 nm. The extent of hemolysis, expressed as relative hemolysis (RH), was determined on the basis of released Hb in the supernatant, according to Eq. (1) [22]:

$$\%\mathbf{RH} = \frac{(\boldsymbol{A_s} - \boldsymbol{A_{nc}})}{(\boldsymbol{A_{pc}} - \boldsymbol{A_{nc}})} \times 100 \quad (1)$$

where $A_s$, $A_{nc}$, and $A_{pc}$ are the absorbance of the sample, negative control (mechanical hemolysis of erythrocytes in PBS), and positive – 100% hemolysis – control (erythrocytes in water), respectively. Each experiment was run in triplicate and RH values represent the average of three independent experiments.

The solubilization process was quantified according to Lichtenberg [1]. The detergent concentrations required to induce membrane saturation ($C^{sat}$, onset of hemolysis) and total solubilization ($C^{sol}$, 100% lysis), were plotted as a function of membrane lipid concentration allowing determination of $R_e$, the effective detergent/lipid molar ratio for initial ($R_e{}^{sat}$) and total hemolysis ($R_e{}^{sol}$). The straight line obtained in each case is predicted by Eq. (2) [1]:

$$\mathbf{D_t} = \mathbf{R_e} \cdot \left[\frac{\mathbf{L}+1}{\mathbf{K_{b,e}}(\mathbf{R_e}+1)}\right] \quad (2)$$

where, $D_t$ is the total detergent concentration ($C^{sat}$, $C^{sol}$) and L is the lipid concentration in the membrane [13]. $R_e$ values were calculated from the slope of the resulting straight lines while the y-intercept corresponds to $D_w$, the concentration of free detergent in water. $K_{b,e}$ ($M^{-1}$), the molar binding constant of the detergent to the erythrocyte membrane, was derived from $R_e{}^{sat}$ and $D_w{}^{sat}$ values, according to Eq. (3) [1]:

$$\boldsymbol{R_e^{sat}} = \frac{\boldsymbol{K_{b,e}} \times \boldsymbol{D_w^{sat}}}{\left(1 - \boldsymbol{K_{b,e}} \times \boldsymbol{D_w^{sat}}\right)} \quad (3)$$

### 2.4. Langmuir monolayer

Monomolecular layers of erythrocyte membrane lipids at the air-water interface were prepared following the Langmuir technique. Aliquots of 5–30 μL of a lipid solution in chloroform were spread with a microsyringe onto an unbuffered aqueous surface in a Teflon trough (4.5 cm diameter and 0.5 cm depth,) with a 15.9 $cm^2$ available area; about 5 min was allowed for evaporation of chloroform. Lateral surface pressure ($\pi$) was measured by the Wilhelmy plate method using a roughened platinum plate (a platinized Pt foil 5 mm wide × 20 mm long × 0.025 mm thick).

#### 2.4.1. Penetration

The initial surface pressure ($\pi_i$) was set between 0 and 40 mN/m (the latter is above the equilibrium lateral pressures of bilayers). The increase in $\pi$ as a function of time was measured up to a plateau ($\pi_{max}$) after injection of 1 mM TX-100, Brij 98 and Brij 58 in the subphase. Then $\Delta\pi_{max} = \pi_{max} - \pi_i$, was calculated, plotted against $\pi_i$ and a straight line was fitted; the maximal $\pi$ allowing reagent penetration ($\pi_{cut\text{-}off}$) was determined from the intersection of this line with the abscise axis.

#### 2.4.2. Kinetics

The kinetics of detergents incorporation in erythrocyte lipids monolayers was studied by recording the changes in $\pi$ versus time. The initial pressure ($\pi_i$) was 35 mN/m. These experiments were performed as described previously [23] within a wide concentrations range of TX-100, Brij 98 or Brij 58 above their respective CMC. The rate constants for the adsorption and desorption processes from the monolayer-water interface were determined from a plot of $\Delta\pi_t$ ($\pi_{max} - \pi_t$) *vs* t (the time elapsed after detergents injections in the subphase), and analyzed according to a single-exponential model represented by Eq. (4):

$$\Delta\boldsymbol{\pi_t} = \Delta\boldsymbol{\pi_{max}} \cdot \left[1 - \exp\left(-\boldsymbol{t}/\boldsymbol{\tau}\right)\right] \quad (4)$$

and the time constant ($\tau$) value could be determined for each individual detergent concentration in the subphase. Then the rate constants for the adsorption ($k_a$) and desorption ($k_d$) processes were obtained by fitting Eq. (5) to the plots of $\tau$ vs detergent concentration.

$$\frac{1}{\boldsymbol{\tau}} = \frac{1}{\boldsymbol{k_d} + \boldsymbol{k_a}\,[\boldsymbol{detergent}]} \quad (5)$$

Finally, Eq. (6) allowed calculation of the association binding constant of detergents to the monolayer ($K_{b,m}$), as follows:

$$\boldsymbol{K_{b,m}} = \boldsymbol{k_a}/\boldsymbol{k_d} \quad (6)$$

## 3. Results

### 3.1. Hemolytic experiments

Fig. 2A,B shows the hemolytic effect of Brij 98 and Brij 58, respectively, over erythrocyte suspensions in different hematocrits 0.15%, 0.30%, 0.45% and 0.60% at isotonic conditions, pH 7.4 and 37 °C. From these experiments it was possible to determine the concentration of detergent required for saturation ($C^{sat}$) and complete solubilization ($C^{sol}$) of the erythrocyte membrane (onset and 100% hemolysis, respectively) as shown the inset in Fig. 2A.

The hemolytic curves (Fig. 2A,B) displayed a sigmoidal behavior. Initially the incorporation of small amounts of detergent does not cause the disruption of the membranes; the beginning of lysis occurs only after $C^{sat}$ concentration is reached. Thereafter, the addition of more detergent molecules is accompanied by the membrane lysis, until its complete solubilization ($C^{sol}$), which is coincident with the release of 100% of the hemoglobin in the supernatant.

The sigmoidal curves observed upon membrane solubilization are typical both for osmotic [24] and detergent-induced hemolysis. Several authors have discussed about the mechanism of membrane solubilization by detergents [6]. However authors agree that the first step on surfactant-induced hemolysis is ruled out by detergent-membrane lipid interaction, osmotic lysis being a secondary/slower phenomenon [25]. Thus, the sequence of events from detergent-monomers partition into the membrane (below the CMC) to formation of mixed membranes (prior to membrane saturation, $C^{sat}$), and formation of mixed micelles (above $C^{sat}$) is responsible for the cooperative, sigmoidal behavior observed in Figs. 2A,B. The *all-or-nothing* hemoglobin release happens when membrane integrity is lost, caused by the movement of detergent and membrane lipids towards mixed-micelles [4].

Table 1 shows the $C^{sat}$ and $C^{sol}$ values determined for Brij 98 and Brij 58. Values previously obtained with TX- 100 are also shown, for comparison purposes [8]. The $C^{sat}$ and $C^{sol}$ values found for Brij 98 and Brij 58 were lower than those described for TX-100 [8] under similar experimental condition. Thus, $C^{sat}$ and $C^{sol}$ values reveal the high lytic power of Brij detergents, about 5–10 times greater than that of TX −100 (Table 1).

The $C^{sat}$ and $C^{sol}$ values obtained from hemolytic curves were plotted against the concentration of membrane lipids (Fig. 2C,D) allowing the determination of the effective molar ratio of detergent/lipid for the onset ($R_e{}^{sat}$) and complete solubilization ($R_e{}^{sol}$) of membrane (Table 2), and the association binding constant $K_{b,e}$, according to Eq. (2).

Also, $D_w{}^{sat}$ value (the concentration of free detergent in water, determined at the **y** intercept of plots such as those in Fig. 2C,D), that keeps relation to the CMC of the detergent, was determined. $D_w{}^{sat}$ values (Table 2) obtained for both Brij 98 and Brij 58 were smaller than their respective CMC (Fig. 1). These results show that the membrane lipids provide an additional contribution for the aggregation of the detergent, reducing the minimum concentration of monomers in water [26].

The determined values of $K_{b,e}$ reflect the higher membrane affinity and lytic potential of both Brij detergents over TX-100. Finally, $R_e$ values (Table 2) confirm that both analogs (Brij 98 and Brij 58)

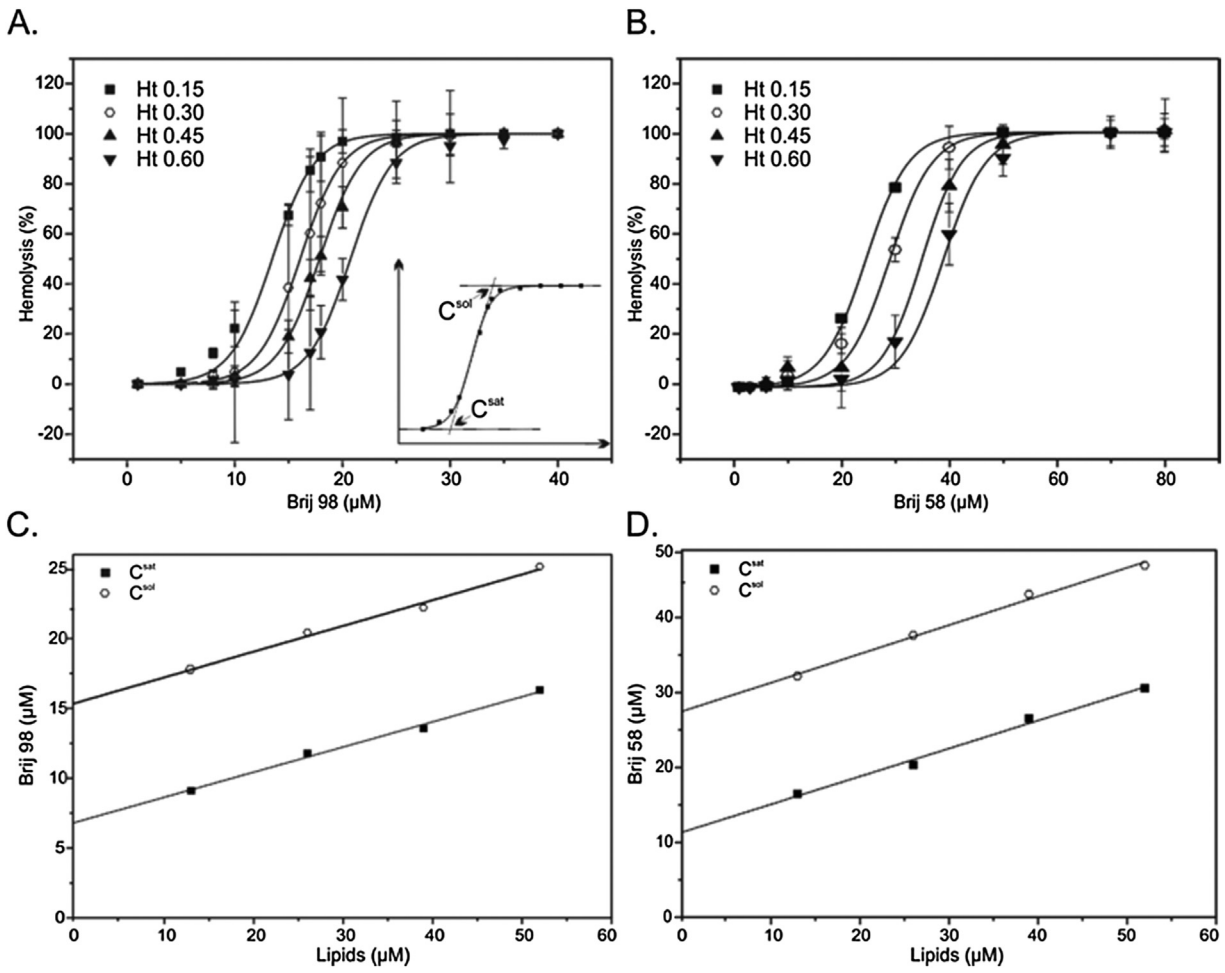


**Fig. 2. Hemolytic effect of Brij 98 (A) and Brij 58 (B) on human erythrocytes at different membrane (lipid) concentrations:** Ht = 0.15 (■), 0.30% (⬡), 0.45% (▲) and 0.60% (▼). Effective detergent/lipid molar ratios for erythrocyte membranes saturation (■) and solubilization (○) by Brij 98 (C) and Brij 58 (D) plotted against the lipid membrane concentration at the different Ht suspension curves. The inset on panel A represents a hemolytic curve with its respective $C^{sat}$ and $C^{sol}$ values. The results correspond to 3 independent experiments.

**Table 1**
Hemolytic effect on human erythrocytes induced by TX-100, Brij 98 e Brij 58.

| | | $C^{sat}$ | | | $C^{sol}$ | | |
|---|---|---|---|---|---|---|---|
| Ht | L (μM)[a] | TX−100 (μM)[b] | Brij 98 (μM) | Brij 58 (μM) | TX−100 (μM)[b] | Brij 98 (μM) | Brij 58 (μM) |
| **0.15%** | **13** | 131 | 9.1 | 16.5 | 222 | 17.4 | 32.2 |
| **0.30%** | **26** | 169 | 11.8 | 20.3 | 213 | 20.4 | 37.6 |
| **0.45%** | **39** | 184 | 13.6 | 26.5 | 255 | 22.2 | 43.1 |
| **0.60%** | **52** | – | 16.3 | 30.6 | – | 25.1 | 47.0 |

[a] L = lipid concentration in erythrocyte membranes at each hematocrit (Ht), calculated according to Malheiros et al. [13].
[b] Data from Preté et al. [8].

**Table 2**
Parameters for erythrocytes lysis induced by TX-100, Brij 98 and Brij 58.

| | TX−100[a] | Brij 98 | Brij 58 |
|---|---|---|---|
| $\mathbf{R}_e^{sat}$ | 1.58 | 0.18 | 0.37 |
| $\mathbf{R}_e^{sol}$ | 2.15 | 0.18 | 0.38 |
| $\mathbf{D}_w^{sat}$ | 100 μM | 6.8 μM | 11.3 μM |
| $\mathbf{K}_{b,e}$ ($\mathbf{M}^{-1}$) | 5900 | 22,302 | 23,957 |

[a] Data from Preté et al. [8].

solubilize biological membranes at smaller detergent/lipid molar ratios than those required by TX-100.

### 3.2. *Penetration of TX-100, Brij 98 and Brij 58 in Langmuir films of lipids extracted from erythrocyte* ghosts

The maximum $\pi_i$ value that allows penetration ($\pi_{cut\text{-}off}$) reflects the ability of the detergent to overcome the resistance imposed by the molecular packing of the monolayer as well as the stability of the resulting *ghosts*' lipid-detergent mixed monolayer. At the assayed detergent concentration (1 mM) the maximum pressure was generated instantaneously after an insertion of the detergents (time 0 s), and was kept constant during the 30 min (1800s) of the experiment (Fig. 3A–C). This is a common behavior observed in the partition of amphiphilic molecules into monolayers [26].

The penetration of TX-100, Brij 98 and Brij 58 in the monolayers, expressed by $\Delta\pi$ values, decreased at increasing $\pi_i$ (Fig. 3D–F),

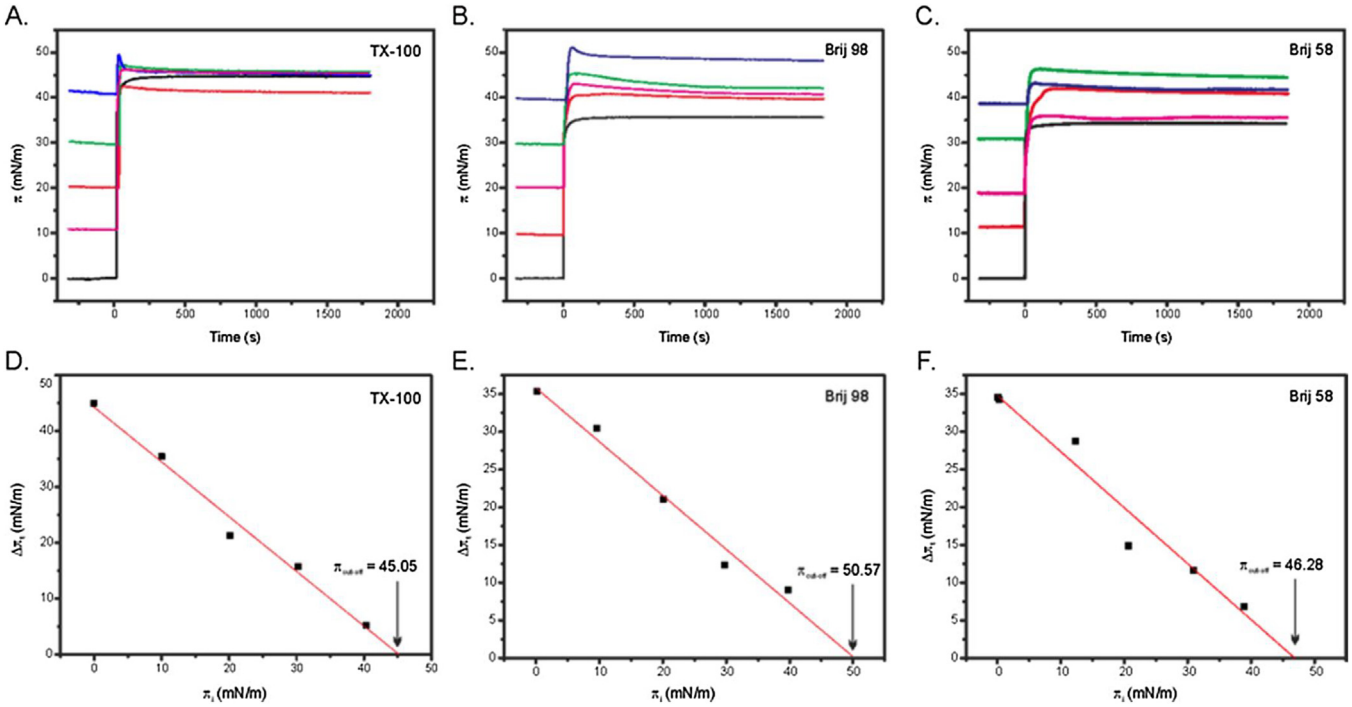


**Fig. 3. Effect of molecular packing on the penetration kinetics of detergents in Langmuir films composed of lipids from erythrocyte *ghosts*.** After the monolayer stabilized at the desired ($\pi_i$) pressure, detergents in aqueous solution (at 1 mM final concentration) were injected in the subphase. A–C, variation in surface pressure as a function of time for TX-100 (A), Brij 98 (B) and Brij 58 (C). D–F, maximal change in the lateral surface pressure ($\Delta\pi_t = \pi_{max} - \pi_t$) achieved after $\pi$ stabilization upon the injection of TX-100 (D), Brij 98 (E) and Brij 58 (F), determined from curves shown in panels A-C, at each initial surface pressure. The arrows point to the $\pi_{cut\text{-}off}$ values. Data correspond to the average of 3 independent determinations. The colors indicate different initial surface pressures for each detergent tested.

reflecting the growing resistance of the monolayer to the incorporation of the detergent due to the $\pi$-dependent increase in the molecular surface density. The $\pi_{cut\text{-}off}$ values determined for TX-100, Brij 98 and Brij 58 from the x-intercept of the $\Delta\pi$-$\pi_i$ curve were 45, 51 and 46 mN/m ($p < 0.01$, Student's T-test), respectively.

Since Brijs have a higher HLB than TX-100 (their polar portion has twice as many oxyethylene units as TX-100, Fig. 1) they are considered to be more hydrophilic so their insertion at the flat interface might be expected to be more favored. However, from the analysis of the $\Delta\pi_{max}$ values reached by TX-100 (~45 mN/m), Brij 98 (~35 mN/m) and Brij 58 (~34 mN/m) at $\pi_i = 0$, TX-100 resulted the most surface active of the three detergents (Fig. 3D).

### 3.3. Binding kinetics

The kinetics of the incorporation of TX-100, Brij 98 and Brij 58 detergents into *ghosts* lipid monolayers were also studied by the variation of lateral surface pressure ($\pi$) versus time (t) (Fig. 4). The concentrations of detergents used were close to their corresponding CMC (Fig. 1). The initial pressure ($\pi_i$) of the monolayer (adsorbent layer) was approximately 35 mN/m, which corresponds to the mean value of the surface pressure found in biological membranes [27].

In the presence of detergent, $\pi_t$ increases up to 46 mN/m (TX-100) and ~48 mN/m (Brij 98 and Brij 58), which are values close to their $\pi_{cut\text{-}off}$ (Fig. 3).

At concentrations closer or below the corresponding CMC, TX-100 (Fig. 4A) (e.g. ≤125 μM) exhibited a qualitative different penetration profile than at higher concentrations; Brij 98 (Fig. 4B) showed a slow penetration kinetics at 7 μM, and Brij 58 (Fig. 4C) at concentrations ≤15 μM exhibited a biphasic penetration kinetics. This strongly suggests that the arrangement (as monomer or aggregates) plays an important role in the way the detergent molecules access the lipid-water interface. See Discussion section for further analysis on this point.

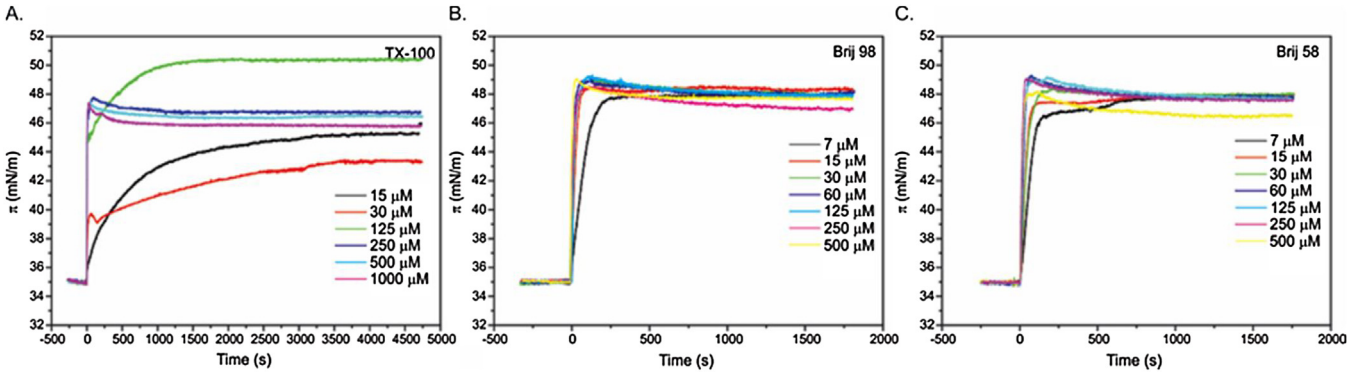


**Fig. 4. Binding kinetics of detergents to Langmuir films of lipids extracted from erythrocyte *ghosts*:** TX-100 (A), Brij 98 (B) and Brij 58 (C); temperature = 22 °C.

**Table 3**
Kinetic and thermodynamic parameters (adsorption – $k_a$, desorption – $k_d$ and binding – $K_{b,m}$ constants) for the incorporation of TX-100, Brij 98 and Brij 58 in *ghosts*' lipids monolayers.

| | TX-100 | Brij 98 | Brij 58 |
|---|---|---|---|
| $k_a$ ($M^{-1}$ $s^{-1}$) | 452.03 | 253.59 | 375.59 |
| $k_d$ ($s^{-1}$) | 0.06 | 0.02 | 0.02 |
| $K_{b,m}$ ($M^{-1}$) | 7500 | 12,500 | 19,300 |

Data from Fig. 4 analyzed under Eqs. (5) and (6) allowed calculating the rate constants of the adsorption ($k_a$) and desorption ($k_d$) processes and the thermodynamic binding constant ($K_{b,m}$) of the detergents in the *ghosts* monolayers(Table 3).

TX-100 showed higher $k_a$ and $k_d$ values and smaller $K_{b,m}$ values than Brij 98 and Brij 58. The faster apparent association of TX-100 is possibly due to the lower volume of its hydrophilic (PEG) head, allowing for its easier accommodation in the monolayer. The faster dissociation of TX-100 than Brij can be explained by the chemical structure of its hydrocarbon chain, short and with a cyclic group, which impairs its stabilization through London – Van der Waals forces.

In turn, $K_{b,m}$ reflects the balance between the ability of detergents to reach the membrane and to remain there. The determined $K_{b,m}$ values (Table 3) for Brij 98 and Brij 58 evidenced the high affinity of these detergents for the lipid monolayer.

## 4. Discussion

In the present paper, we investigated the effect of two Brij analogs and TX-100 on membrane solubilization (hemolytic effect on erythrocytes) and used Langmuir films (monomolecular layers at the air-water interface) to determine the kinetics and thermodynamics involved in the detergent-membrane interactions.

Previous studies from our laboratory using giant unilamellar vesicles (GUVs) prepared with lipids extracted from erythrocytes (erythro-GUVs) revealed remarkable differences in the solubilization induced by the Brij detergents and TX-100 [9]. This could be interpreted in terms of different ability of incorporation of each detergent in the bilayer and also different flip-flop rates [26]. The larger hydrophilic portion of the Brijs would favor its incorporation in the outer membrane leaflet (seen by the increase of the surface area and membrane curvature) leading to destabilization of the GUV, followed by an equilibrium of the detergent population between the two leaflets and rearrangement into several small vesicles, as first reported for SDS detergents [28]. Differently, the trans-membrane flip-flop rate of TX-100 seems to be fast, due to the low size of its head group [26,28]. Then, the addition of TX-100 to erythro-GUVs did not cause a clear increase of either the surface area or spontaneous curvature of erythro-GUVs. TX-100 partially solubilized and removed lipids from the inner and outer leaflets (mostly phospholipids) leaving an insoluble fraction of ca. 2/3 the area of the original giant vesicle [9]. Here, we used different approaches to study the membrane lipid-detergent interaction.

### 4.1. Hemolytic potency of the detergents studied

Recently we have proposed the use of $R_e$ values as a parameter to determine the strength of solubilization power of detergents [6]. Therefore, the $R_e$ values smaller than unity determined here, allow us to classify Brij 98 and Brij 58 as strong membrane solubilizing agents. Accordingly, hemolytic experiments showed that both Brij analogs saturate ($R_e^{sat}$) and solubilize ($R_e^{sol}$) biological membranes at smaller detergent/lipid molar ratios than those required by TX-100. Differently than TX-100, the solubilization process induced by Brij seems to occur as an abrupt process, since no significant differences were found between the Brij/lipid ratios for the onset and complete membrane solubilization ($R_e^{sat}$ and $R_e^{sol}$ values, respectively).

As for the analogs, the lower Re values of Brij 98 than Brij 58 were expected since the longer (oleic acid) hydrophobic chain of the first over Brij 58 (palmitic acid) should confer to it stronger hydrophobic interactions with the lipid bilayer. However, the association binding constant ($K_{b,e}$) of Brij 58 was found higher than that of Brij 98–a result also observed in monolayers ($K_{b,m}$), as discussed later in Table 3– showing that the saturated (palmitic acid) chain favors the insertion of Brij 58 in-between the lipids that are found in a highly ordered phase (segmental order parameters *ca.* 0.8) in the erythrocyte membrane [21,29].

Indeed the solubilization mechanism followed by Brijs and TX-100 seems to be different, as previously reported [12]. While Brij 98 immediately destabilized and restructured erythro-GUVs into several small vesicles of 1–2 mm diameter, TX-100 solubilized the membranes in a more continuous way [9]. Also, Sowmiya et al. [30] have found that the micellization of Brij (98 and 58) detergents is preceded by the formation of small aggregates known as premicellar aggregates; this phenomenon might explain the similar values of $R_e^{sat}$ and $R_e^{sol}$ in the Brij detergents.

#### 4.1.1. Langmuir films as membrane models

The use of Langmuir films composed of total lipid extract of erythrocyte membranes resulted in a good model to reveal details of detergent effect on those membranes. The $K_{b,m}$ values obtained in monolayers (calculated from the experiments in Fig. 4) and shown in Table 3 ($K_{b,m}$ = 7500, 12500 and 19300 $M^{-1}$ for TX-100, Brij 98 and Brij 58, respectively) were in very good correlation with the values of association binding constants determined by hemolytic experiments using whole erythrocytes ($K_{b,e}$, 5900, 22300 and 23957 $M^{-1}$ for TX-100, Brij 98 and Brij 58, respectively, Table 2), *i.e.*, in the presence of proteins. For TX-100, the $K_{b,m}$ resulted slightly higher than $K_{b,e}$. This is in accordance with observations of Kragh-Hansen et al. [31], who works with liposomes and endoplasmic reticulum membranes, showed that for solubilization to occur, cooperative binding was required and, in the case of nonionic detergents, this was achieved predominantly by their interaction with the lipid components of protein-containing bilayer membranes. For Brijs, the potential preference for their binding to lipids than for protein-rich domains may be overcompensated by the steric hindrance imposed by the big polymeric polar head to be accommodated in the flat surface of a monolayer. Furthermore, their values of $K_{b,e}$ higher than $K_{b,m}$ may also be reflecting a different degree of freedom found in those membranes (intact erythrocytes and Langmuir films). Therefore, erythrocyte membrane can offer the possibility of bending, as a mechanism to relieve the tensions triggered by the incorporation of detergent molecules, which is unlike to occur in monolayer films.

The reciprocal of $K_{binding}$ represents $K_{dissociation}$. The later parameter has the physical meaning of the detergent concentration required to produce half the maximal effect (either hemolysis or surface pressure increase, depending on the experiment). $K_{dissociation}$ calculated from the reciprocal of the corresponding $K_{b,e}$ (Table 2), resulted in 169, 44.4 and 41.7 μM for TX-100, Brij 98 and Brij 58, respectively, and the corresponding values determined in Langmuir films are quite similar. Thus, the half-maximal effect of all the detergents studied ($K_{dissociation}$) is around the respective CMC. It is important to note that the dissociation constants values derived from the present data (obtained through the measurement of two different effects) are within the magnitude order determined for other alkyl-ethoxylate detergents at exerting other phenomenon associated to a membrane binding process [32].

The CMC seemed to be a critical factor, which not only defines the onset of detergent aggregation but also marks a qualitative

change in the penetration kinetics of detergents. This was particularly evident with TX-100 in the π vs. time plots (Fig. 4A) performed at concentration ≤125 μM. In turn, Brij 98 (Fig. 4B) exhibited slow penetration kinetics at 7 μM, while biphasic penetration kinetics was observed for Brij 58 at low concentrations (Fig. 4C) (≤15 μM). The critical concentrations necessary to acquire the burst-like penetration kinetics were around 0.5, 0.28 and 0.19 times the respective CMC for TX-100, Brij 98 and Brij 58. This suggests that the pre-micellar [30] and micellar detergent aggregates follow an interaction mechanism with the monolayer that is different from that of the monomeric species. A careful look at the shape of the penetration curves obtained at detergent concentrations in the subphase above the CMC shows that π rises up to a maximum and then seem to relax slowly towards a plateau, which can be envisioned as a characteristic value of an equilibrium state. In conjunction, these results suggest that near the critical (CMC) values, detergent aggregating structures crush against the lipid-water interface, deforming the monolayers as far as allowed by its molecular packing, leaving monomers that result integrated to the monomolecular film.

The detergent disaggregation at the monolayer-water interface, in loosely packed monolayers, can be expected to be driven by the high surface tension (γ), and as a mechanism to relieve it. However, from the analysis of the $\Delta\pi_{max}$ values reached by TX-100 (~45 mN/m), Brij 98 (~35 mN/m) and Brij 58 (~34 mN/m) at $\pi_i = 0$ mN/m, TX-100, which has the highest CMC, resulted the most surface active of the three detergents (Fig. 3C–D). This suggests that detergent molecules, as monomeric species, insert into the membrane more efficiently than molecules released from the aggregate – membrane collision. Furthermore, it is possible that the stronger steric hindrance imposed by the bigger polar group of Brijs (20 oxyethylene units) compared with that of TX-100 (9–10 oxyethylene units) impairs the accommodation of their molecules side by side along the flat surface of the monolayers. In contrast, at increasing initial surface pressures ($\pi_1 \square 0$), the insertion becomes more favorable for Brijs than for TX-100 as shown by $\pi_{cut\text{-}off}$ values, suggesting a stabilizing effect of the longer hydrocarbon chains of the formers and the unfavorable effect conferred by the short, branched and benzene substituted hydrocarbon chains of TX-100 for its stabilization at the polar-apolar interface (Fig. 1).

*4.1.2. HLB, molecular structure and detergency potency*

The hydrophilic-lipophilic balance (HLB) expresses the mass ratio between the hydrophilic, and hydrophobic groups in the molecules. It was proposed by Griffin, late in the 1940s [33], being extensively used to describe detergent series. Preté et al. [8] showed that within the homologous $C_nE_m$ series, the greater the length of the hydrocarbon chain and the lower the HLB, the greater the lytic potential of the detergent. Similar results were shown with homologous series of Renex [6], ASB [5] and other detergents [6,34,35]. Considering only Brij detergents (Fig. 1), their HLB values and $C^{sat}$ and $C^{sol}$ values (Table 1), it can be seen that smaller concentrations of the more hydrophobic Brij 98 (HLB = 15.3) are required for membrane solubilization than for Brij 58 (HLB = 15.7). This reflects the importance of the hydrophobic moiety in determining the ability of the detergent to anchor to the membrane, such that, inside a series, the lower the HLB, the lower the detergent concentration required to destabilize the membrane.

In comparison to TX-100 (HLB = 13.5) the Brij detergents are more hydrophilic (their polar portion has twice the polyoxyethylene units of TX-100). Despite their lytic potential is much higher than the former. This indicates that the correlation between HLB and detergency may not be valid outside a homologous series. Hence, factors other than HLB are involved in the lytic process, favoring Brij insertion in-between the lipids (relatively to TX-100), such as steric match, length of the hydrocarbon chains and conformational states of the polymeric head. Brijs neither have the benzene ring – possibly localized at the polar-apolar interface – of TX-100, nor its short-branched hydrocarbon chains. On the contrary, Brijs have linear hydrocarbon chains. Additionally, the hydrocarbon chains of Brij 98 is 2-carbon longer than that of Brij 58 and carries one unsaturation that is absent in Brij 58. This may explain the order of monolayer binding affinity $K_{b,m}$ of the detergents studied (TX–100 < Brij 98 < Brij 58) based on the stability of their anchoring to the monolayer. The fact that $K_{b,m}$ and $K_{b,e}$ values were relatively similar also indicates that the lipids play a pivot role for detergent molecules to incorporate into the membrane. Nevertheless, the presence of proteins favored the solubilization action of Brij 98 upon erythrocyte membranes (as seen in Fig. 2 and Table 1). These results suggest that the longer unsaturated acyl chain of Brij 98 might incorporate into different membrane domains when proteins are present. Therefore, for these two Brij analogs, a higher association constant value is not directly related to a higher potency for biological membrane disruption.

Another important structural characteristic of the detergents analyzed in the present work is the length of their polymeric head, which may play an important role in their binding, stabilization and detergency mechanism.

It was proposed that linear polymers attached to interfaces can be found in two different organizational schemes depending on the packing density [36,37], i.e. a random coil (mushroom) conformation at a low molecular packing and a stretched (brush) conformation at higher molecular packing. A third conformation, the pancake, has also been predicted to exist in monolayers at the lowest surface pressures [38,39]. Moreover, on the basis of polymer physics [40] it was predicted that PEGylated phospholipids in mixtures with other phospholipids could achieve these two conformations ("brush" or "mushroom") not only in monolayers but also in liposomes [41]. Our experimental results with Langmuir films of dipalmitoylphosphatidyl ethanolamine (DPPE) covalently modified with polyethyleneglycols (PEGs) composed of 7, 23 and 113 oxyethylene units (PE-PEG$^n$) demonstrated that only those PE-PEG$^n$ with $n$~23 and higher could follow a polymeric transition [42]. So, this suggests that upon compression it would be expected to find a polymeric transition (pancake- mushroom or mushroom-brush) in Brijs but not in TX-100. It is important to recall that our experiments were performed at a fixed initial surface pressure ($\pi_i$) in conditions of a constant area, and that no external compression is imposed to the film other than that induced by the insertion of detergent molecules. So, it is unlikely for the hydrophilic head of these detergents to undergo a polymeric transition. At least in the case of Brijs, it is expected that the heads organize in a random coil (mushroom) conformation and only the first molecules that insert into the monolayers (only at very low π) may acquire a pancake conformation. The molecular area, which is defined by the volume of the polymeric head in mushroom conformation, restricts the number of molecules to be bound. At the same time, the polymer interaction through adsorption at the polar head groups of the monolayer-forming lipids may impose tensions, leading to a membrane bending [43] and increasing the membrane curvature induced by polymer adsorption. This mechanism may help to understand the results obtained during Brijs-induced solubilization process of GUVs [9]. This idea is reinforced by $^{31}$P NMR experiments with of 9:1 binary mixtures of DPPC/PE–PEG$^n$, which showed that when the polymeric layer bound to the lipid-water interface is thick enough the supramolecular aggregate does not acquire a bilayer structure but rather a micellar structure of high curvature [44]. Finally, the comparison of monolayers and natural bilayers allows realizing that while in the former the detergent-membrane binding process seems to end when the lipid-water interface is totally covered with the polymeric moieties of detergents, in intact erythrocyte ghosts, detergent binding goes ahead

with either detergent flip-flopping or membrane bending until membrane disruption.

## 5. Conclusions

Quantitative hemolytic experiments revealed that the nonionic detergents Brij 98 and 58 are strong membrane solubilizers ($R_e^{sat}$ values <1). Their solubilizing effect on erythrocyte membranes is higher than that of TX-100. Such experiments did not allow us to evaluate the kinetics of detergent insertion into the membrane, but the binding constants ($K_{b,e}$: Brij 58 > Brij 98 > TX-100) evidenced the higher affinity of the linear alkyl ether (Brijs) over the *t*-octylphenyl ether (TX-100). In turn, the binding constants determined in monolayers composed of lipids extracted from erythrocytes ($K_{b,m}$), showed good agreement with $K_{b,e}$.

Differences in the polar head groups and in the hydrocarbon chains of Brijs and TX-100 became clear in experiments in Langmuir monolayers. The kinetics of association from the monolayers was faster for TX-100, for which the length of the polar head group is half that of Brijs (20 polyoxyethylene units). The kinetics of dissociation, also faster for TX-100 than for Brijs, reflected the relevance of the linear and longer hydrocarbon chains of the latters compared with that of TX-100, in favoring their stabilization in-between the lipids, through London-van der Waals forces.

The profile of surface pressure evolution in detergent penetration experiments using packed monolayers at an initial surface pressure close to the equilibrium surface pressure of biological bilayers suggested that monomeric species (at concentrations below the CMC) were able to insert in the membrane more efficiently than molecules released from the collision of a detergent aggregate with the membrane surface.

In both (bilayers and monolayer) experiments the detergents were added to pre-formed membranes however, the organization reached by the inserted detergent molecules would be similar to what was observed with phospholipid-lipopolymer mixtures organized either in monolayers or dispersed in water. Thus, the random coil of the long polymeric heads of Brijs, but not the shorter heads of TX-100, adsorbed at the polar head groups of lipids would induce a membrane bending, which might trigger a budding process. This would explain the differential solubilizing mechanism of Brijs detergents observed previously in GUVs.

## Acknowledgements

This work was supported by grants from cooperation between Capes (Brazil) and Mincyt, SeCyT-Universidad Nacional de Córdoba, CONICET and Foncyt (Argentina); and also from CNPq (# 479993/2011-4). EMC and MAP are career members of CONICET. BRC and EP acknowledge their fellowships from CAPES and CNPq (# 308621/2013-1), respectively.

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