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Hemolytic Activity of a Bacterial Trehalose Lipid Biosurfactant Produced by *Rhodococcus* sp.: Evidence for a Colloid-Osmotic Mechanism

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A succinoyl trehalose lipid produced by *Rhodococcus* sp. behaves as a biological surfactant and also displays various interesting biological activities. Trehalose lipid has been shown to have a great tendency to partition into phospholipid membranes; therefore, the characterization of its interaction with biological membranes is of central importance. In this work, human red blood cells have been used as an experimental model. Trehalose lipid causes the swelling of human erythrocytes followed by hemolysis at concentrations well below its critical micellar concentration. Kinetic measurements show that, upon addition of trehalose lipid, K^+ release precedes that of hemoglobin. Osmotic protectants of the appropriate size added to the external medium make it possible to avoid hemolysis. The results indicate that trehalose lipid causes the hemolysis of human erythrocytes by a colloid-osmotic mechanism, most likely by formation of enhanced permeability domains, or "pores" enriched in the biosurfactant, within the erythrocyte membrane. Scanning electron microscopy shows trehalose lipid-induced spherocytosis and echinocytosis of red blood cells, which fits well within the framework of the bilayer-couple hypothesis. The presented results contribute to establishing a molecular basis for the biological properties of this trehalose lipid biosurfactant.

1. Introduction

The production of amphiphilic compounds with surface activity is widely spread among microorganisms. Thus, the term biosurfactant is commonly used to define surface-active amphiphilic compounds of biological origin. The intense investigation in this field is leading to the description of new chemical and biological properties; therefore, interesting potential applications are arising.[1−6] Biosurfactants present several advantages over surfactants of a chemical origin, particularly in relation to their biodegradability and environmental compatibility. An additional characteristic of some biosurfactants is the display of diverse biological activities, for instance, as antifungal or, in general, antimicrobial agents, which extends the field for future uses.[7−9] Some of these compounds can be produced through biotechnological processes in which the carbon source is provided by waste materials or side products from several industries, constituting an added value not only from an economic point of view but also for ecological reasons.[10] Thus, the identification of new biosurfactants and the characterization of their chemical and biological properties are the subjects of intense research activity.[5,6,11]

Among the various chemical structures found within biosurfactants, the most studied include lipopeptides, glycolipids, polymers, and others.[4] Because of their amphiphilic nature, biosurfactants, when added to biological systems, preferentially partition into biological membranes, altering their physicochemical properties and function.[12−14] Much recent work has been devoted to the study of the membrane action of various biosurfactants, including lipopeptides such as surfactin or iturin A[15−18] or glycolipids such as rhamnolipids[12,13,19,20] and trehalose lipids.[21−24] These compounds alter the physicochemical properties of the hydrophobic core of the bilayer as well as perturb the hydration status

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of the water/lipid interface,[12,19,22] and some of them induce membrane permeabilization in liposome systems[17,20,24] and/or hemolysis.[18,20,25−28] The surfactant-induced permeabilization of phospholipid membranes, either model membranes or biological membranes such as erythrocytes, may occur at concentrations below or above the critical micellar concentration (cmc), which depends on the chemical nature of the surfactant. However, membrane permeabilization can take place concomitantly with the solubilization of the membranes or without altering the membrane integrity, depending on the molecular mechanism.

Trehalose-containing glycolipid biosurfactants constitute an emerging group of interesting compounds.[29] These biosurfactants are mainly produced by rhodococci and present interesting physicochemical and biological properties.[30] Trehalose lipids significantly reduce the surface tension of water[31] and form microemulsions.[32] Thus, several possible applications have been proposed for these compounds.[30] As to their biological properties, succinoyl trehalose lipids have been found to induce the differentiation of leukemia cell lines[33] and to inhibit protein kinase activity.[34] Given its surfactant nature, with marked amphiphilic character, it is most likely that a majority of the biological actions of these trehalose lipid biosurfactants are related to their interaction with the phospholipid bilayer. It is therefore of great interest to characterize the kinetic and molecular mechanisms underlying such actions, in particular, with regard to the capacity of trehalose lipids to permeabilize the lipid membrane. In the present study, the hemolytic activity of a succinoyl bacterial trehalose lipid biosurfactant produced by *Rhodococcus* sp. (Figure 1) is thoroughly characterized. Kinetic and osmotic protection experiments provide evidence to sustain a colloid-osmotic mechanism for hemolysis. These results add to recently published work on the trehalose lipid-induced permeabilization of model membranes[24] and help to explain the mechanism by which this biosurfactant may exert its membrane-perturbing actions.

2. Experimental Section

2.1. Materials. Sucrose and the various PEGs were from Sigma-Aldrich (Madrid, Spain). All the other reagents were of the highest purity available. Inorganic salts and buffers were of analytical grade. Purified water was deionized with a Milli-Q equipment from Millipore (Millipore, Bedford, MA) and had a resistivity of ca. 18 MΩ. Stock solutions of the trehalose lipid were prepared in chloroform/methanol (2:1) and stored at −80 °C. The buffer used throughout this work was 150 mM NaCl, 5 mM Hepes at pH 7.4 unless otherwise stated. Water and all of the buffer solutions used in this work were filtered through 0.2 μm filters prior to use. The osmolarity of all of the buffers and solutions was checked in an Osmomat 030 osmometer (Gonotec, Berlin, Germany).

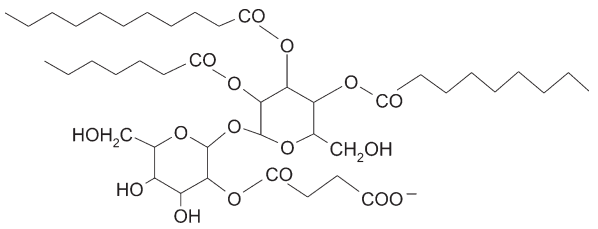

Figure 1. Chemical structure of the *Rhodococcus* sp. trehalose lipid.

2.2. Trehalose Lipid Production and Purification. Strain 51T7 was isolated from an oil-contaminated soil sample after culture enrichment with kerosene and was identified as *Rhodococcus* sp.[35] This strain was maintained by fortnight cultures on trypticase soy agar (Pronadisa, Spain) and preserved in cryovials at −20 °C. Biosurfactants were produced, purified, and characterized as described before.[21] The biosurfactant contains a succinoyl residue and three fatty acids with chain lengths ranging from 7 to 11 carbons. For the calculation of molar concentration, an average compound with heptanoyl, nonanoyl, and undecanoyl residues (molecular weight 877.09) was considered.

2.3. Hemolysis. Human erythrocytes were prepared right before the experiments from red blood cell concentrates supplied by a local blood bank. Cells were washed twice with buffer (150 mM NaCl, 5 mM Hepes at pH 7.4), and finally suspended in the same volume of buffer prior to use. All operations were carried out at 4 °C.

For the measurements of hemoglobin and K^+ release, the above erythrocyte concentrate was diluted with a 150 mM NaCl, 5 mM Hepes at pH 7.4 buffer to obtain a suspension with $A_{540} = 1$. Hemoglobin release was determined, upon incubation of red blood cells with trehalose lipid under different conditions, as indicated, by measuring the absorbance at 540 nm after pelleting the membranes by centrifugation for 2 min in a bench microfuge. The total amount of hemoglobin was established by lysing the erythrocytes with distilled water.

Leakage of K^+ from erythrocytes was measured using a K^+-selective electrode (Jenway, U.K.) as follows. Trehalose lipid was added from a 5 mM stock solution in buffer, and the potassium efflux was continuously monitored using a recorder. The total amount of K^+ was determined by disrupting the cells with sodium cholate. The solutions were continuously stirred using a magnetic device. Measurements were made in a jacketed vessel, and the temperature was kept constant using a circulating water bath.

2.4. Osmotic Protection Experiment. Erythrocytes were prepared as described above in a buffer containing 135 mM NaCl, 5 mM Hepes at pH 7.4, and 30 mM of one of the following substances: sucrose and polyethylene glycols 400, 600, 1000, 4000, 6000, and 10 000. Trehalose lipid was added to the different final concentrations indicated in each experiment from a 5 mM stock solution in buffer, and hemolysis was determined after incubation for 1 h at 37 °C. The following molecular diameters were used:[36] sucrose, 9.8 Å; PEG 400, 11.2 Å; PEG 600, 13.8 Å; PEG 1000, 17.8 Å; PEG 3350, 34 Å, PEG 6000, 54 Å; and PEG 10 000, 72 Å.

2.5. Scanning Electron Microscopy. Blood samples (1.5 mL) were mixed with 150 μL of 3% glutaraldehyde in phosphate buffer and left overnight. The cells were centrifuged at 1000 rpm for 10 min, and the supernatant was discarded. To the pellet, 2.5% glutaraldehyde was added and left for 4 h. The fixative was removed after centrifugation as above, and the cells were washed three times with distilled water. The final pellet was resuspended in a minimum volume of distilled water, and a drop was placed over

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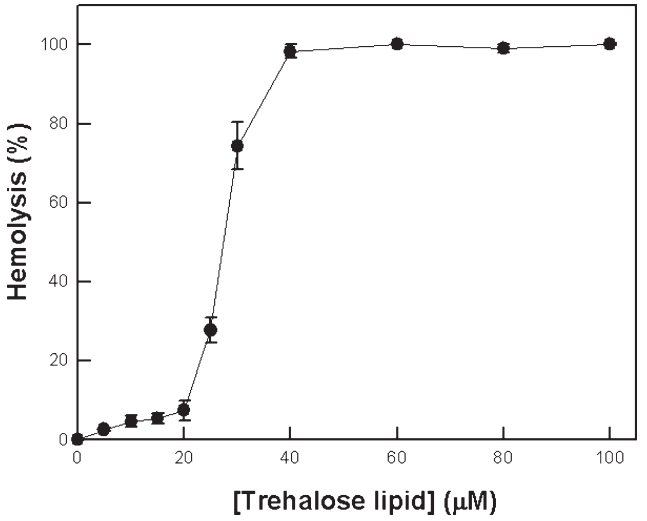

Figure 2. Dependence of human erythrocyte hemolysis on trehalose lipid concentration. Erythrocytes were incubated for 1 h at 37 °C at different trehalose lipid concentrations, and the amount of hemoglobin released was determined. The data correspond to the average of three independent experiments ± standard error (error bars).

a brass SEM stub and left to dry in a stove at 37 °C for 1 h. Samples were examined in a Jeol JSM-6100 scanning electron microscope (Tokyo, Japan).

3. Results

3.1. Trehalose Lipid-Induced Hemolysis. The dependence of hemolysis on the concentration of trehalose lipid is shown in Figure 2. In this experiment, hemolysis was determined at a fixed time, after 1 h of incubation in the presence of various trehalose lipid concentrations. Hemolysis varied with trehalose lipid concentration in a sigmoidal manner. At concentrations below 20 μM, the percentage of hemolysis was not significant; however, it increased sharply between 20 and 40 μM to reach essentially 100% hemolysis at this concentration.

Figure 3 shows the trehalose lipid-induced hemolysis of human erythrocytes as a function of time at various biosurfactant concentrations. All of the concentrations assayed were well below 300 μM, which corresponds to the value of the cmc of the trehalose lipid as previously determined.[24] Hemolysis was a relatively slow process, including the presence of an initial lag period in all of the curves. After the initial lag, hemolysis increased linearly with time. This steady-state rate of hemolysis was essentially the same for the various trehalose lipid concentrations assayed; however, the lag period and the time required to reach 100% hemolysis were considerably shortened as the concentration of the biosurfactant was raised. A general observation was that hemolysis was a rather slow process, on the minutes to hour scale depending on the concentration of the biosurfactant.

3.2. Leakage of K^+ from Human Erythrocytes. The kinetics of K^+ release from red blood cells, as compared to that of hemoglobin, usually sheds light on the underlying mechanism of hemolysis. Trehalose lipid-induced K^+ leakage from human erythrocytes was continuously monitored in a separate assay (Figure 4) under the same conditions as described above for hemolysis. It can be observed that K^+ ions started to leak after an initial lag period of 2 to 3 min. Interestingly, in this case the lag period seemed to be independent of the trehalose lipid concentration, different from that observed in the hemoglobin leakage curves, and the steady-state rates depended on the concentration

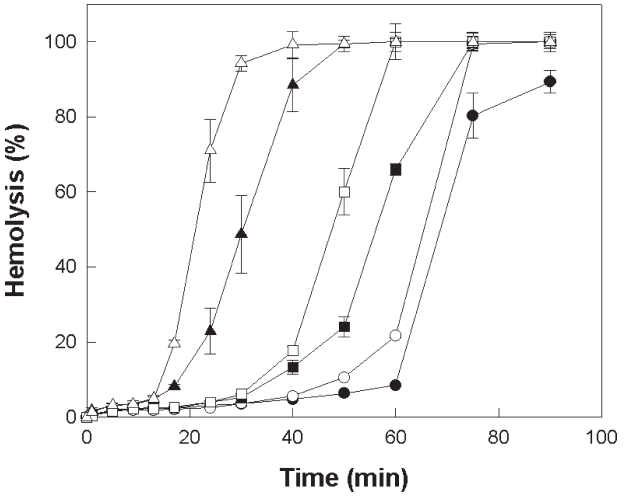

Figure 3. Trehalose lipid-induced hemoglobin release from human erythrocytes as a function of time. Trehalose lipid was added at time zero from a 1 mM stock solution to give final concentrations of 20 (●), 25 (○), 27 (■), 30 (□), 60 (▲), and 80 μM (△). Hemoglobin release was determined in a discontinuous assay at 37 °C, as explained under methods. Hemoglobin release of 100% was determined after lysing the cells with distilled water. The data correspond to the average of three independent experiments ± standard error (error bars).

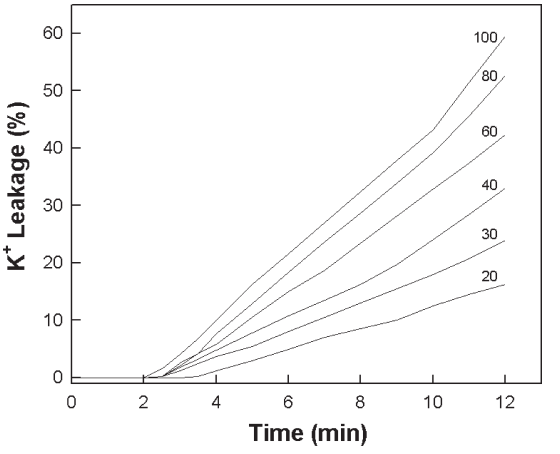

Figure 4. Time course of trehalose lipid-induced K^+ release from human erythrocytes. Trehalose lipid was added at time zero to the final concentrations indicated on the curves (μM) from a 1 mM stock solution. K^+ release was continuously monitored using a potassium-selective electrode at 37 °C. K^+ release of 100% was determined after disrupting the cells with sodium cholate.

of the glycolipid. Figure 5 presents the relative kinetics of K^+ and hemoglobin release obtained upon addition of 30 μM trehalose lipid to human erythrocytes. It is clearly observed that K^+ leakage was a much faster process, clearly preceding hemolysis. Thus, hemoglobin was not appreciably released until K^+ leakage reached values of ca. 70% of the maximum.

3.3. Osmotic Protection of Hemolysis. The different kinetic behavior of K^+ and hemoglobin leakage could be explained by the colloid-osmotic mechanism of hemolysis. To corroborate this hypothesis further, an osmotic protection experiment was conducted (Figure 6) that also allowed the determination of the size of the membrane pore by using protectants of different sizes. In Figure 6, the percentage of hemolysis is plotted against the diameter of the osmotic protectant present in each case. It was

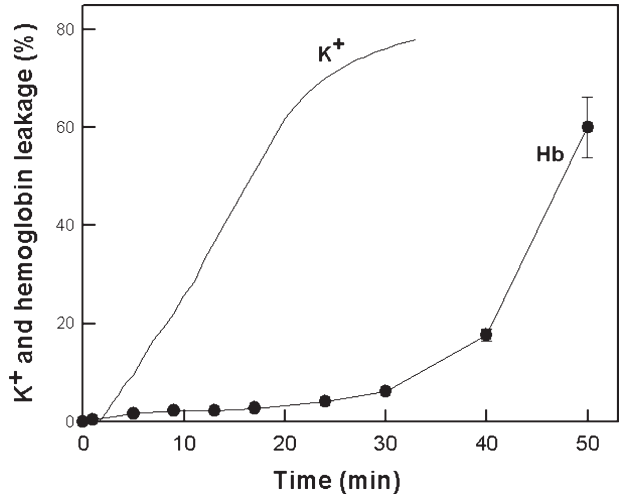

Figure 5. Relative kinetics of trehalose lipid-induced K^+ and hemoglobin release. Measurements were carried out at 37 °C in the presence of 30 μM trehalose lipid. The data correspond to one representative experiment.

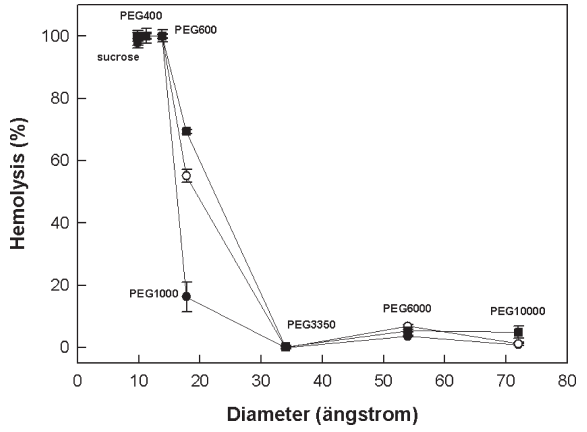

Figure 6. Trehalose lipid-induced hemolysis of human erythrocytes in the presence of osmotic protectants. Trehalose lipid was added to a human erythrocyte suspension at different final concentrations: 40 (●), 60 (○), and 80 μM (■). Measurements were made at 37 °C. The diameter of the protectants and the details of the hemolysis determination are given in the Experimental Section (2.4). The data correspond to the average of three independent experiments ± standard error (error bars).

found that hemolysis was avoided by compounds having a diameter larger than PEG 3350, independent of the concentration of trehalose lipid. This indicated that the size of the pore was close to the diameter of PEG 3350 (34 Å). We must consider the possibility that unspecific, strong binding of trehalose lipid to PEG would reduce the effective concentration of the biosurfactant, acting in the same way as the osmotic protectants. However, previous results showed that a related glycolipid biosurfactant, namely, dirhamnolipid from *P. aeruginosa*, caused hemolysis through a direct lytic mechanism instead of a colloid-osmotic mechanism, indicating the absence of glycolipid binding to PEG.[20] It is reasonable to expect similar behavior for the succinoyl trehalose lipid biosurfactant under study.

3.4. Trehalose Lipid-Induced Morphological Changes in Human Erythrocytes. The morphological changes produced in human erythrocytes upon incubation with trehalose lipid were followed by means of scanning electron microscopy. Figure 7

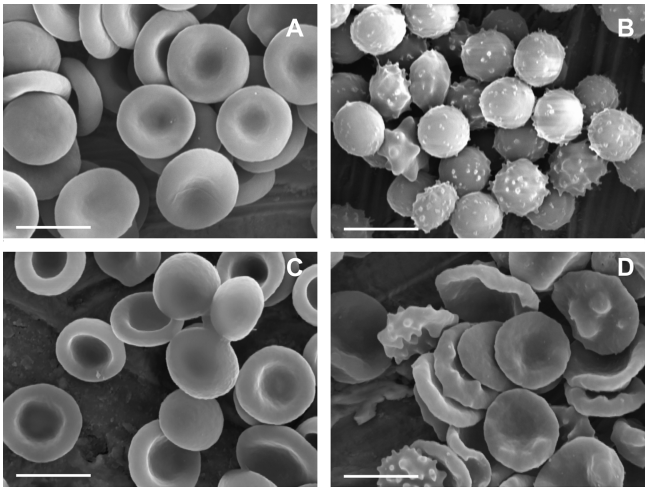

Figure 7. Effect of trehalose lipid on human erythrocyte morphology. Scanning electron micrographs are shown for control erythrocytes in the absence and presence of PEG 3350 (A and C, respectively) and erythrocytes incubated with 60 μM trehalose lipid in the absence and presence of PEG 3350 (B and D, respectively). Scale bars correspond to 5 μm.

shows pictures of human erythrocytes before and after incubation with trehalose lipid at a concentration of 60 μM as well as the effect of the incorporation of osmotic protectant PEG 3350. Normal erythrocytes presented the usual morphology of a round disk shape depressed in the center, and the average size of the cells was 5 μm (Figure 7, panel A). Upon incubation with 60 μM trehalose lipid (Figure 7, panel B), erythrocytes were transformed into spherocytes (swollen cells) with the presence of crenates in the cell surface (echinocytes); the average size of the cells was essentially unchanged. The same experiments were carried out in the presence of PEG 3350, an osmotic protectant against trehalose lipid-induced hemolysis, as described above. PEG alone had essentially no effect on the morphology of erythrocytes, which maintained their normal discoid shape and size (Figure 7, panel C). In agreement with the hemolysis results presented above, the addition of trehalose lipid in the presence of PEG did not result in the formation of spherocytes (Figure 7, panel D) (i.e., osmotic swelling was impeded). Nevertheless, although in most of the cells the discoid shape was still patent, cells looked more irregular and the cell surface was not smooth but contained protrusions of different sizes. As compared to trehalose lipid-treated cells in the absence of PEG, the number of crenates was smaller whereas their size was in general larger.

4. Discussion

Biosurfactants, like chemical surfactants, penetrate biological membranes, altering their structure and function. The addition of a biosurfactant to red blood cells normally causes hemolysis because of either permeabilization or disruption of the membrane. This occurs at different biosurfactant concentrations, depending on the nature of the compound and its physiochemical characteristics, mainly its cmc. Given the important potential applications of biosurfactants, for instance, in cosmetics or as drug vehicles, the hemolytic activity of various representative examples has been investigated. These include lipopeptides such as surfactin[27,28] and iturin,[18,25] and glycolipids such as rhamnolipids produced by *Burkholderia pseudomallei*[26] or *Pseudomonas aeruginosa*.[20] Recently, we have published on the mechanism of model phospholipid membrane permeabilization by a bacterial trehalose lipid produced by *Rhodococcus* sp.[24] The results presented

here, using biological membranes, are complementary to that work and provide experimental evidence to explain the molecular mechanism of the trehalose lipid-induced hemolysis of human erythrocytes.

The hemolytic activity of surfactants, and consequently of biosurfactants, can occur through two different mechanisms.[37] Hemolysis may be caused by the direct disruption of the membrane through membrane solubilization, which normally occurs at high surfactant/membrane ratios, or may be due to enhanced permeabilization of the membrane to small solutes, which normally occurs at low surfactant concentrations, leading to osmotic lysis.

The sigmoidal pattern of trehalose lipid-induced hemolysis (Figure 2) is indicative of a complex process in which sufficient biosurfactant needs to accumulate in the target membrane to induce the osmotic lysis of erythrocytes. This occurs at relatively low concentrations in the range of 20 μM (i.e., well below the 300 μM cmc), in agreement with previous results showing that the trehalose lipid is a weak detergent that, in the presence of target membranes, prefers membrane partitioning over micellization.[24] The presence of a lag period, in the range of minutes, in the hemoglobin release curves (Figure 3) is also an indication that one or more previous steps are required before the erythrocyte membrane becomes permeable to hemoglobin. One of these steps is "pore" formation and the efflux of low-molecular-weight solutes, as discussed below. Altogether, these findings support the notion that hemolysis does not occur by the simple destruction of the molecular organization of the membrane but a more complex mechanism must be operating.

The much faster release of K^+ (Figures 4 and 5) suggests the trehalose lipid-induced early formation of permeability regions in the red blood cell membrane, allowing the comparatively rapid release of K^+, giving rise to osmotic changes across the membrane with hemolysis taking place by a colloid-osmotic mechanism. After the rapid leakage of K^+, water enters because of the osmotic gradient created by hemoglobin trapped inside, the erythrocyte swells, further damage occurs, and finally hemoglobin is released. It is interesting that the 2 to 3 min lag period observed in K^+ leakage curves (Figure 4) is within the same range as the 2 min lag period previously reported in the trehalose lipid-induced leakage of carboxyfluorescein in phosphatidylcholine unilamellar vesicles,[24] and in both cases, it is independent of the concentration of glycolipid. In that work, it was shown that two stages must lead to the proper insertion of trehalose lipid into the target membrane, before leakage is initiated: first, adsorption of the biosurfactant onto the outer leaflet, and second, the flip to the inner monolayer, with the latter being the rate-limiting step. The good agreement between experimental data in the model and erythrocyte membranes suggests that the same steps could be necessary in erythrocytes before K^+ leakage occurs. Nevertheless, the formation of crenated cells as discussed below indicates that there is a preferential partitioning of trehalose lipid in the outer versus the inner monolayer of the membrane. These results emphasize the main role of the lipid constituent of the erythrocyte membrane in trehalose lipid-induced hemolysis, which may also be the case for other biosurfactants.

If hemolysis takes place through a colloid-osmotic process, then it should be protected by a solute of the appropriate size added to the outer aqueous solution. The rationale behind this consideration is based on the fact that if the osmotic pressure of intracellular hemoglobin is balanced with another solute added to the outer buffer, which cannot pass through the membrane pore, then hemolysis should not occur. The osmotic protection experiment shown in Figure 6 further confirms the colloid-osmotic mechanism suggested above. Thus, a pore size of ca. 34 Å diameter (corresponding to PEG 3350) should be sufficient to allow the free efflux of low-molecular-weight solutes, such as K^+ ions that have an ionic radius of 1.33 Å, as observed.

We have no experimental data to give any details about the possible structure of these enhanced permeability regions, or pores, in red blood cell membranes. They should consist of a defined number of trehalose lipid molecules strongly interacting with each other or, because of their amphiphilic nature, with phospholipid molecules in the vicinity. This is sustained by the great tendency to self-aggregate within the membrane of this glycolipid biosurfactant that forms domains, visualized by atomic force microscopy, in fluid phosphatidylcholine membranes.[21] This accumulation of glycolipid molecules in a defined region of the target erythrocyte membrane could locally enhance membrane permeability to small solutes (K^+ in this case), in the same way that it induces carboxyfluorescein leakage in phosphatidylcholine vesicles,[24] thus resulting in pore-type behavior.

As to the effect of trehalose lipid on erythrocyte morphology, the formation of swollen cells (spherocytes) and crenated cells (echinocytes) was observed. Because trehalose lipid is a negatively charged amphiphile at neutral pH, this scenario fits well within the framework of the bilayer-couple hypothesis,[38] which proposes that anionic drugs intercalate preferentially into the lipids present in the outer leaflet of the bilayer, expand that layer relative to the inner leaflet, and thereby induce the cell to crenate. In fact, it has been shown before that other anionic amphiphiles, such as sodium alkyl sulphates, are also potent echinocytogenic agents.[39] In the presence of PEG 3350, the trehalose lipid-induced formation of spherocytes is not observed, confirming the protective role of this PEG against osmotic lysis; however, echinocytosis does still occur. It is expected that, for a given relative cell volume, the number of echinocyte spicula increases while their size decreases as the number of intercalated amphiphilic molecules in the outer leaflet of the cell membrane is increased.[40] Our observation is that in the presence of PEG the number of spicula is smaller and the size is larger than in its absence. This could mean a smaller partitioning of trehalose lipid in the outer monolayer or a much higher ratio of biosurfactant in the inner versus the outer monolayer, as compared to the control. Finally, it has been suggested that in addition to the bilayer the cytoskeleton of the erythrocytes is also responsible for the formation of the echinocytic shapes, presumably because of the shear deformation of the skeleton.[41] Because we have observed substantial morphological differences between trehalose lipid-treated cells in the absence and presence of PEG 3350, the cytoskeleton-related effects of trehalose lipid cannot be discarded in our system.

5. Conclusions

A trehalose lipid biosurfactant produced by *Rhodococcus* sp. causes the hemolysis of human erythrocytes through a colloid-osmotic mechanism. This pore-type behavior can be explained by the formation of enhanced permeability domains in the erythrocyte membrane, as observed in model membranes. Kinetic

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measurements of hemoglobin and K^+ leakage correlate well with previous results on trehalose lipid-induced model membrane permeabilization, suggesting a similar mechanism and emphasizing the central role of the lipid constituent of the membrane in the mechanism of hemolysis. Morphological changes in red blood cells upon incubation with trehalose lipid include spherocytosis and echinocytosis and can be mostly explained by the bilayer-couple hypothesis.

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