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# Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties

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**Abstract**

New linear analogs of surfactin have been synthesized. Their physico-chemical parameters were determined. The results indicate that these linear products show surface activities although they are lowered compared to those of cyclic compounds. The hemolytic activities have also been assayed. In contrast with cyclic surfactins, no significant hemolysis occurs for the linear products in the range of concentrations tested. Moreover, a protective effect against Triton X-100 induced hemolysis has been highlighted for linear surfactins. The concentration at which this protective effect happens is correlated directly to the CMC, and inversely to the acyl chain length of the product. In a hypotonic medium, analogs having a long acyl chain tend to increase the hemolysis, meanwhile the product with the shortest chain tends to decrease it.


*Keywords:* Surfactin; CMC; Hemolysis; Surfactin analog

## 1. Introduction

Surfactin is a lipopeptide family excreted by *Bacillus subtilis*. Its structure is characterized by a heptapeptidic moiety linked to a beta hydroxyl-fatty acid. A lactone bridge between the beta-hydroxyl function of the acid and the carboxy-terminus function of the peptide confers a cyclic structure to this molecule [1–4] (see Fig. 1a). A natural diversity occurs, giving rise to homologs, differing from each other by the length (13 to 15 atoms of carbon) and the ramification of the fatty acid chain; and to isoforms, characterized by some differences in the peptidic sequence.

The increasing interest for these molecules is due to their amphiphilic character, which is responsible for their excellent surface-active properties [5–7]. In addition, surfactins exhibit diverse biological activities such as antiviral [8–11], antibacterial [12], antimycoplasma [8,9,13] and hemolytic activities [10,14,15]. However, this last property constitutes a drawback for medical applications.

The aim of this work was to use the possibilities offered by organic synthesis to vary the structure of surfactins in order to lower their hemolytic effect. Linear surfactins have been chosen as the first series of analogs. These molecules can be compared with a natural linear surfactin obtained by hydrolysis of the lactone function of surfactin. Some authors have already studied the surface-active properties [16,17]

*Abbreviations:* CHCA, α-cyano 4-hydroxy cinnamic acid; CMC, critical micellar concentration; DCM, dichloromethane; DIEA, di-isopropyl ethylamine; DMSO, dimethyl sulfoxide; $HC_{50}$, concentration inducing 50% of hemolysis; HOBt, n-hydroxybenzotriazole; NMP, N-methyl-pyrrolidone; $PC_{50}$, concentration inducing 50% of protection; RBC, red blood cells; SAL14, synthetic linear surfactin with an acyl chain of 14 atoms of carbon in which the carboxy terminus function has been amidated; SNC14, natural cyclic surfactin with an acyl chain of 14 atoms of carbon; SNC15, natural cyclic surfactin with an acyl chain of 15 atoms of carbon; SNL14, linear surfactin obtained by hydrolysis of SNC14; SSL10, synthetic linear surfactin with an acyl chain of 10 atoms of carbon; SSL14, synthetic linear surfactin with an acyl chain of 14 atoms of carbon; SSL18, synthetic linear surfactin with an acyl chain of 18 atoms of carbon; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TX-100, Triton X-100

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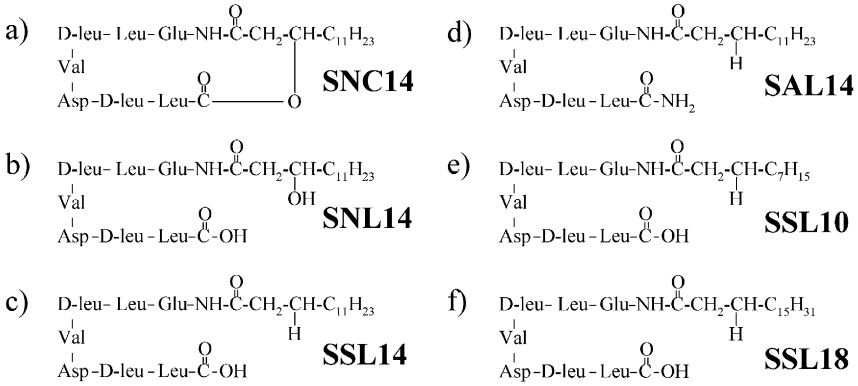


Fig. 1. Structure of the different molecules studied. (a) SNC14 or cyclic natural surfactin with a C14 acyl chain. (b) SNL14 or chemically linearised natural surfactin with a C14 acyl chain. (c) SSL14 or linear synthetic surfactin with a C14 acyl chain. (d) SAL14 or linear synthetic surfactin with an amidated carboxy-terminus function and a C14 acyl chain. (e) SSL10 or linear synthetic surfactin with a C10 acyl chain. (f) SSL18 or linear synthetic surfactin with a C18 acyl chain.

and the secondary structure [17] of a linear surfactin obtained by hydrolysis of natural cyclic surfactin. The linear surfactins used in this work are of synthetic or natural origins, have a unique and well-defined structure and differ from each other by the length of the acyl chain, or the number of ionisable acid functions.

## 2. Materials and methods

### 2.1. Chemicals

Each of these products was purchased from the following companies: fmoc-leucyl wang resin and fmoc protected amino acid, from Advanced Chemtech, USA; HOBt, 98%, from Eurobiochem, Germany; TBTU, 98%, from Alexis, Switzerland; TFA, synthesis quality, from SDS, France; Tris, 99.8%, from Pharmacia Biotech, Sweden; CHCA, from Sigma, USA; TX-100, from Sigma, USA; milliQ water, obtained with a millipore synthesis A10 apparatus, from Millipore, USA. Capric anhydride, 98%; DIEA, 98%; stearic anhydride, 98%; and hydrochloric acid, 37% in water, were purchased from Acros Organics, USA. DMSO, 99.9%; myristic anhydride, 95%; and piperidine, 99%, were purchased from Aldrich, Germany. Acetonitrile, HPLC quality; DCM, multisolvant; methanol, multisolvant; NaCl, reagent grade; and NMP, for peptide synthesis, were purchased from Sharlau, Spain.

### 2.2. Production of surfactin analogs

Surfactin was extracted from a *Bacillus subtilis* S499 culture supernatant and purified by chromatography as previously described [18,19].

Natural linear surfactin with a fatty acid chain having 14 carbon atoms (SNL14, see Fig. 1 for the structure) was obtained by alkaline hydrolysis of pure natural cyclic surfactin having the same C14 fatty acid chain (SNC14) in methanol: 0.1 M NaOH 4:6 (v/v) at 37 °C during 18h, according to the procedure described by Morikawa et al. [16].

The production of the different synthetic linear analogs has been performed by the classical SPPS technique. An advanced chemtech 348 MPS (Advanced Chemtech, USA) apparatus was used. A wang resin, supporting 50 μmol of fmoc-protected leucine, was treated with piperidine 20% in NMP to remove the fmoc protecting group. Attachment of the next amino acid was performed by adding 1.5 ml NMP/DMSO/DIEA 82:16:2 (v/v/v) containing 150 μmol of the corresponding fmoc-protected amino acid, 150 μmol of HOBt and 145 μmol of TBTU. This coupling reaction was repeated twice. The procedure was the same for the attachment of each amino acid. At the end of its synthesis, the peptidic moiety was treated with 75 μmol of, capric-, myristic- or stearic-anhydride, respectively, in DCM, in order to obtain synthetic linear surfactins with C10, C14 and C18 chain length (SSL10, SSL14 and SSL18). This reaction was repeated fivefold. The lipopeptide was cleaved from the resin with TFA/DCM/water 49.5:49.5:1 (v/v/v). After filtration, the liquid phase was evaporated under nitrogen and dissolved in methanol. The lipopeptide was precipitated by dropwise addition of water.

Linear synthetic surfactin in which the carboxy-terminus moiety has been amidated (SAL14) was obtained using the same procedure on a rink amide resin which gives rise to amidated peptide once cleaved with TFA.

The purification of each product was performed by preparative HPLC (prep LC 4000, Waters, USA), using a 22×250 mm C18 or C4 column (Vydac, USA) with various gradients of acetonitrile/milliQ water 0.05% TFA. The obtained fractions were evaporated under vacuum, diluted with milliQ water and freeze dried. Identification and purity of the products were attested using infrared spectroscopy, amino acid analysis and rp-HPLC as described previously [18]. Mass spectra were taken from a solid spot containing the lipopeptide in a CHCA matrix with an MALDI-TOF mass spectrometer (ultraflex TOF, Bruker, Germany).

### 2.3. Determination of the surface active properties

A drop volume tensiometer (TVT1, Lauda, Germany) was used. Lipopeptides were dissolved in a 5 mM Tris buffer adjusted to pH 8.5 with HCl. Measurements were performed at 25 °C using the quasi-static mode in which drops are formed quickly in order to minimize the hydrodynamic effects [20]. Each drop measurement was repeated twice, and the whole experiment was repeated at least two times.

The equilibrium surface tension, $\gamma_{eq}$ was estimated by extrapolating the surface tension $t \rightarrow \infty$ in the $\gamma - t^{-1/2}$ curve. CMC was determined as the intersection of the linear part of this curve of equilibrium surface tension vs. the concentration (in logarithmic scale).

### 2.4. Red blood cell preparation

Peripheral blood was collected from sheep by venipuncture and 0.3% of EDTA was added. Red blood cells (RBC) were separated by centrifugation at $2000 \times g$. RBC were then washed once in PBS-EDTA 0.075% and three times in an isotonic buffer (10 mM Tris 150 mM NaCl adjusted to pH 8.5 with HCl). RBC were then suspended in this buffer at a cell density of $5 \times 10^8$ cells/ml.

### 2.5. Hemolysis assay

880 μl of isotonic buffer was added to 20 μl of a methanolic solution containing the lipopeptide. 100 μl of RBC suspension were added and the reaction was performed at 25 °C during 30 min. Unaltered RBC were then removed by a $10000 \times g$ centrifugation and the absorbance of the supernatant at 540 nm was compared with two control samples in order to determine the percentage of hemolysis. The first one (100%) was totally hemolysed with distilled water, and the second one (0%) contained 20 μl of pure methanol instead of the lipopeptide solution. Each experiment has been performed at least twice.

### 2.6. Protection against hemolysis

On one hand, the procedure described above was used to determine the effect of surfactin analogs against hypotonic hemolysis. The NaCl concentration of the buffer added to the methanolic solution containing the lipopeptide was in this case of NaCl 90 mM instead of 150 mM.

On another hand, the antagonism of lipopeptide against hemolysis caused by another surfactant was assayed by mixing 20 μl of lipopeptide in methanol to 10 μl of TX-100 or SNC14 in methanol. 870 μl of isotonic buffer and 100 μl of RBC suspension were added. Final concentrations are 160 μM TX-100 or 400 μM SNC14. The following operations were similar to the one described above.

The hemolysis percentage of each sample was reported to the hemolysis caused by isotonic conditions or by the other surfactant.

Each of these experiments was performed at least two times.

## 3. Results

### 3.1. Synthesis

The structural data collected by HPLC, amino acid composition analysis, infrared spectroscopy, and MALDI-TOF mass spectrometry are in accordance with the expected structure. The purity of all the purified products reaches at least 95% as determined by HPLC-UV at 214 nm (Data not shown).

The resulting molecules are represented in Fig. 1. SNC14 is the only molecule to possess a cyclic structure. SNL14 and SSL14 are both linear surfactins possessing three acid ionisable groups; but SNL14 possesses a beta-hydroxyl function due to its natural origin. SAL14 possesses only two ionisable groups, the third acid function being amidated. SSL10, SSL14 and SSL18 differ by the length of their acyl chain, constituted by 10, 14 and 18 carbon atoms, respectively.

### 3.2. Surface active properties

The surface tensions of various solutions of the different surfactants were measured as a function of the drop's lifetime. The linearity of the long time portion of the $\gamma$ vs. $t^{-1/2}$ curve means that the adsorption kinetics is controlled by the diffusion step [21]. It allows the equilibrium surface tension ($\gamma_{eq}$) to be estimated as the extrapolation of this linear segment to $t \rightarrow \infty$. An example of such curves is given in Fig. 2. The resulting equilibrium surface tensions are plotted as a function of the concentration in order to determine the critical micellar concentration. Standard deviation of each point is inferior to 1.5 mN/m. The curves are given in Fig. 3, and the values of the CMC are summarized in Table 1.

The comparison of SNC14 with SSL14, SNL14 and SAL14 shows that the loss of the cyclic structure does not suppress the surface-active properties, but only reduces them. Actually, both the values of concentration and surface tension at CMC are smaller for SNC14 (62 μM, 30 mN/m) than for the linear compounds (300–400 μM, 36–37 mN/m). The effect of the acyl chain length is very important on CMC value, as it decreases as this length rises.

### 3.3. Hemolysis

Each lipopeptide has been tested at various concentrations for its hemolytic activity. The results are given in Fig. 4 with their standard deviation. Natural cyclic surfactin SNC14 gives rise to a concentration dependent hemolysis. $HC_{50}$, which is defined as the concentration of surfactant that bursts 50% of the RBC, is equal to 300 μM (see Table 1). SNL14, SSL14 and SSL18 do not cause any hemolysis

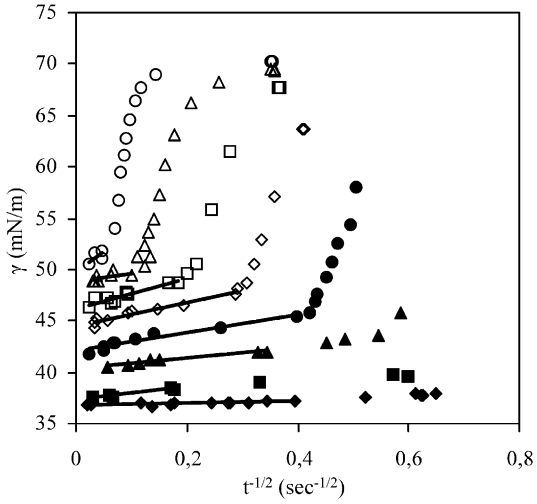


Fig. 2. Surface tension vs. inverse of time square root plots for SAL14. The concentrations are: (○) 6 μM; (△) 12 μM; (□) 24 μM; (◇) 49 μM; (●) 98 μM; (▲) 195 μM; (■)390 μM; (♦) 781 μ M. Linear segment of the curve in the region of small $t^{-1/2}$ value is used to extrapolate the value at $t \rightarrow \infty$. The measurements have been performed at 25 °C in 5 mM Tris pH 8.5.

in the concentration range used. The curve of SAL14 indicates that small hemolytic effects occur at high concentrations. SSL10 gives rise to a slight hemolysis at low concentrations, but this phenomenon decreases as the concentration rises and disappears at 50 μM.

### 3.4. Effect on hypotonic hemolysis

As several authors have highlighted the protective effect of different surfactants against hypotonic hemolysis [22–25], the different surfactin analogs have been assayed for this activity and were incubated at different concentrations with RBC suspended in a 90 mM NaCl buffer. In absence of surfactin, these conditions give rise to 30% of hemolysis. The results are shown in Fig. 5 with their standard deviation. When the concentration of SSL14, SAL14, SNC14 and SSL18 rises, the hemolytic activity increases up to several folds of the original activity. The magnitude of this enhancement is stronger for analogs having a long chain and for the cyclic analog.

Interestingly, SSL10 has a very different behavior and decreases the hypotonic hemolysis in a concentration-dependent way.

### 3.5. Antagonism with other surfactants

In order to determine an eventual protective effect of surfactin analogs against the hemolysis caused by other surfactants, RBC were incubated with both 160 μM TX-100 and various concentrations of surfactin analogs. In Fig. 6, the effect of each surfactant on the 160 μM TX-100 hemolysis is depicted as a function of surfactant concentration. The results clearly show that SSL14, SAL14, SSL10 and SSL18 have a protective effect against the hemolysis

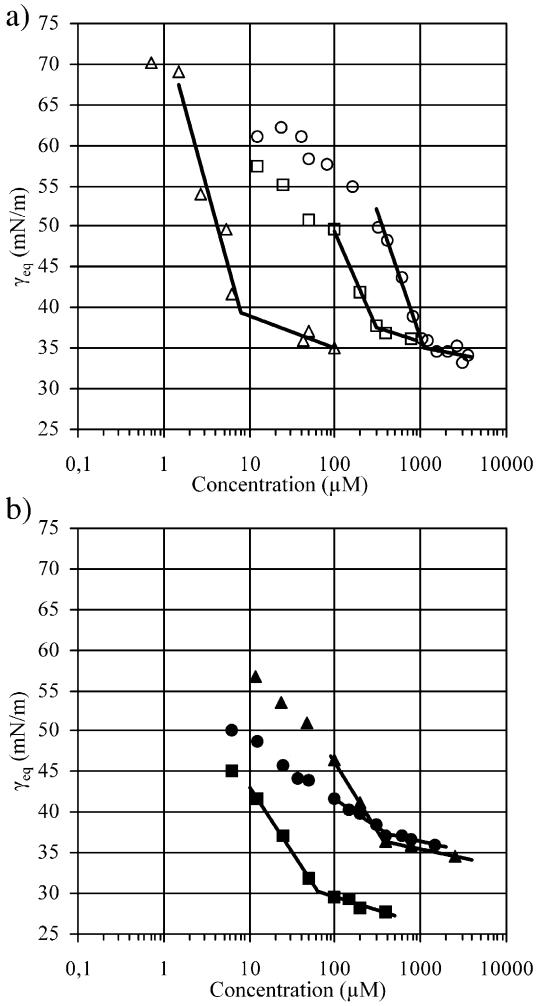


Fig. 3. Surface tension of various surfactin analogs solutions as a function of their concentration. These analogs are (a) (□) SSL14; (△) SSL18; (○) SSL10 for the upper panel; and (b) (●) SAL14; (♦) SNL14; (■) SNC14. The measurements have been performed at 25 °C in 5 mM Tris pH 8.5.

caused by TX-100. An eventual protective effect of SNC14 would be masked by the hemolytic activity of SNC14 itself.

Surfactin derivatives reduce the hemolysis caused by TX-100 in a concentration-dependent way. The curves have a sigmoid aspect. The maximum value of this curve arises at low concentrations and corresponds to the full effect of TX-100 that is 100% hemolysis. The hemolysis decreases with

Table 1
Main physico-chemical and hemolytic parameters of surfactin analogs

| Product | CMC (μM) | $HC_{50}$ (μM) | $PC_{50}$ (μM) |
|---|---|---|---|
| SSL10 | 1113 | – | 297 |
| SSL14 | 301 | – | 27 |
| SSL18 | 8 | – | 1 |
| SAL14 | 401 | – | 15 |
| SNL14 | 374 | – | ND |
| SNC14 | 62 | 300 | – |

CMC is measured at 25 °C in 5 mM Tris pH 8.5. $HC_{50}$ is measured at 25 °C in a 10 mM Tris 150 mM NaCl at $5 \times 10^7$ RBC/ml suspension at pH 8.5. $PC_{50}$ is measured at 25 °C in 160 μM Triton X-100 10 mM Tris 150 mM NaCl $5 \times 10^7$ RBC/ml suspension at pH 8.5.

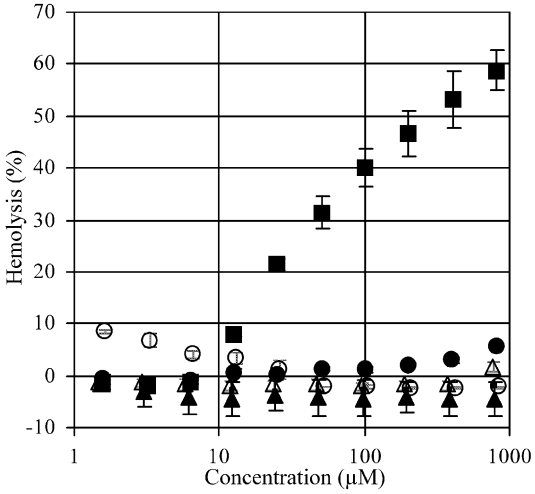


Fig. 4. Hemolytic activity of different analogs of surfactin as a function of their concentration. These analogs are: (□) SSL14; (△) SSL18; (○) SSL10; (●) SAL14; (▲) SNL14; (■) SNC14. The measurements have been performed at 25 °C in 10 mM Tris 150 mM NaCl pH 8.5. Standard deviation is represented by the error bar on each point.

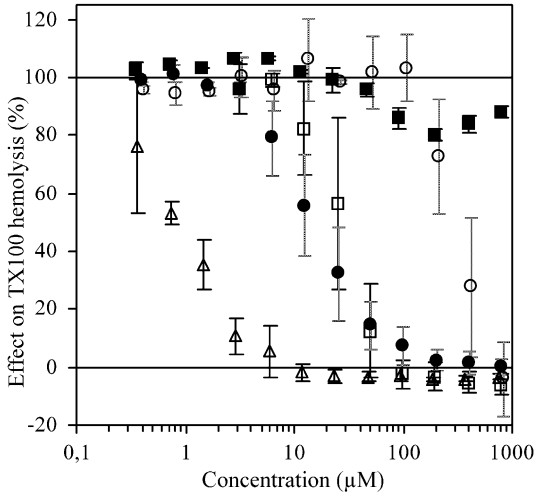


Fig. 6. Effect of different analogs on hemolysis caused by 160 μM TX-100. 100% refers to the Triton hemolysis in absence of surfactin. The analogs are: (□) SSL14; (△) SSL18; (○) SSL10; (●) SAL14; (■) SNC14. The measurements have been performed at 25 °C in 10 mM Tris 150 mM NaCl pH 8.5. Standard deviation is represented by the error bar on each point.

concentration, and at high analog concentration, it reaches 0%, which means that the analog completely inhibits the TX-100 hemolysis. The difference between the antagonist effects of these analogs is the concentration at which it occurs. It can be characterized by the concentration in surfactin that reduces the TX-100 hemolytic effect to 50% of its original value, $PC_{50}$. These values are 297 μM, 27 μM, 15 μM and 1 μM for SSL10, SSL14, SAL14 and SSL18, respectively, and are summarized in Table 1.

The protective effect of SSL14 has also been assayed on the hemolysis due to 400 μM SNC14. As showed in Fig. 7, SSL14 is able to reduce hemolysis up to zero percent, behaving in a similar way than in the TX-100 system. Under these conditions, the concentration at which the hemolytic effect is reduced to 50% is equal to 111 μM SSL14.

## 4. Discussion

The formation of micelles from pure SNC14 has already been studied at 20 °C in 5 mM Tris pH 8.0, giving rise to the value of 33 mN/m and 65 μM at CMC [26], which is in agreement with the value of 30 mN/m and 62 μM measured in this work. Some authors have measured the CMC of the natural homolog mixture in presence of 100 mM $Na^+$,

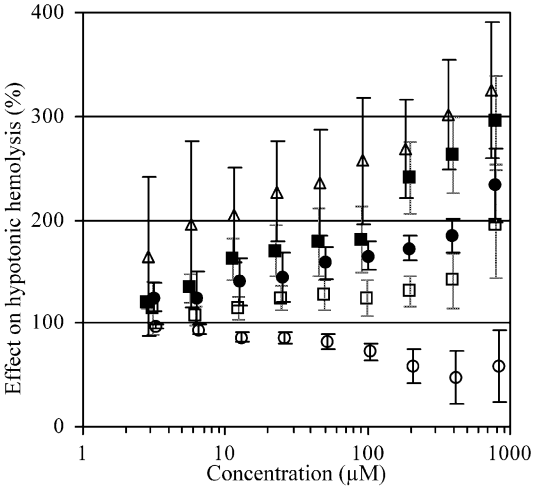


Fig. 5. Effect of different analogs on hemolysis caused by hypotonic conditions. 100% refers to the hypotonic hemolysis in absence of surfactin. The analogs are: (□) SSL14; (△) SSL18; (○) SSL10; (●) SAL14; (■) SNC14. The measurement has been performed at 25 °C in 10 mM Tris 90 mM NaCl pH 8.5. Standard deviation is represented by the error bar on each point.

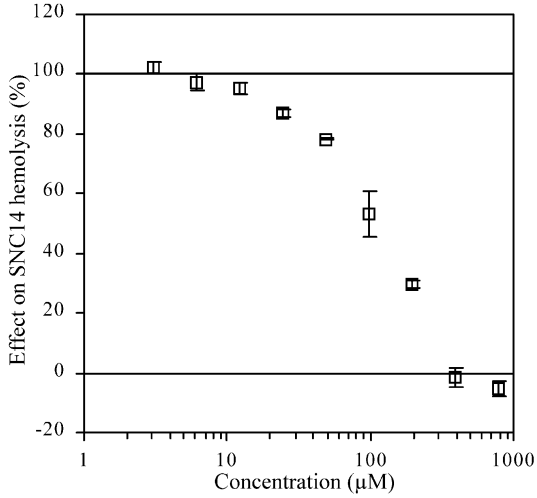


Fig. 7. Effect of SSL14 on hemolysis caused by 400 μM SNC14. 100% refers to the SNC14 hemolysis in absence of SSL14. The measurements have been performed at 25 °C in 10 mM Tris 150 mM NaCl pH 8.5. Standard deviation is represented by the error bar on each point.

giving rise to the values of 9 μM by surface tension [7,27] and 7.5 μM by isothermal titration calorimetry [28]. The CMC of the main constituent of this natural mixture, SNC15, is 20 μM and 12 μM, in absence and in presence of 100 mM NaCl respectively [26,29]. From these values, it appears that SNC15 has a predominant influence on the CMC of the mixture. Moreover, the presence of salt decreases the electrostatic repulsions and allows the molecules to associate at lower concentration.

Linear surfactin has already been studied as a hydrolysate of the natural homolog mixture. The CMC, measured in distilled water, is of around 70–80 μM [16]. This value is smaller than the 370 μM measured in this work probably because of the presence of the homolog possessing a C15 fatty acid chain that increases the hydrophobic effect and so the attraction between the molecules. Other authors have measured the CMC of this mixture of linear surfactin in PBS at pH 7.35 trough the use of fluorescent probes and a value of 12.8 μM was obtained [17]. This very low value can be explained by the presence of salt that decreases the electrostatic repulsions.

Under the conditions applied during this work, the CMC and $\gamma_{CMC}$ of SNC14 are smaller than those of SNL14 and SSL14. The differences between these molecules are the number of ionisable acid functions (two for SNC14 and three for the others) and the cyclic/linear structure. As SAL14 has exactly the same number of ionisable carboxylic groups as SNC14 and behaves like SNL14 and SSL14, it can be concluded that the rise of the CMC and $\gamma_{CMC}$ values is due to the disruption of the cyclic structure. It confirms the hypothesis according to which the cyclic "horse saddle-like" structure of surfactin plays an important role in its surface active properties by favoring the existence of distinct polar and apolar domains [16,30].

The inverse correlation between CMC and the acyl chain length is due to the augmentation of cohesive hydrophobic forces with this length and has already been reported for surfactin [6,15] and other surfactants [31]. The ratio between the CMC of different linear analogs differing by the same number of atoms of carbon is not constant. This is also the case for cyclic surfactin [26] and iturins [29]. For example, the ratio between the CMC of different acyl maltose ester differing by two atoms of carbon in the chain is equal to ten and is constant [32]. In these last molecules, the headgroup is purely hydrophilic and does not participate to the attractive forces leading to the micelle formation. This is probably not the case for the different lipopeptides, and a more complex mechanism can be the reason for which the addition of two (or one) methylene group in the acyl chain has not a constant effect on CMC.

The ability of a surfactant to disrupt a membrane is related to two parameters. The first one is its capacity to penetrate into the membrane at low concentrations. This parameter is related to the association ability of the surfactant molecule such as CMC [28,32,33]. The second one is the molar ratio between surfactant and lipid, which must be reached in the membrane to give rise to its disruption. This parameter can be related to the shape of the molecule. Indeed, a conic molecule, with a large head group, introduces a more important constraint in the lipid packing and leads to earlier disruption of the membrane than rod like molecules [28,32].

On this basis, it can be supposed that the main reason for which linear surfactin are not hemolytic is that the loss of the cyclic structure decreases the hindrance of the head group and consequently the tendency to disrupt membranes. On another hand, SAL14, which possess the same linear structure than SSL14 shows a slight hemolytic activity. The difference between these molecules is their charge in a neutral buffer (−2 for SAL14 and −3 for SSL14 and the other linear analogs). As the RBC membrane is negatively charged, the third charge of SSL14 (and of other linear surfactins except SAL14) can lower the ability of these molecules to insert into the membrane. This explains the higher hemolytic activity of SAL14 compared with the other linear analogs. However, the important difference between the activity of SAL14 and SNC14 suggests that the charge contribution is of minor effect compared to these of the molecule's shape.

No obvious explanation was found for the slight hemolysis observed at low concentrations for SSL10. Further work with surfactants with shorter side chains could be helpful to understand this phenomenon.

During the hypotonic hemolysis, the concentration gradient exerts a driving force that tends to swell the cell. The membrane is then stretched until the cohesive forces are no more sufficient to ensure the membrane integrity, leading to pore formation. During the expansion of the membrane, the molecular area of the membrane constituents progressively increases. As the elastic energy cost for amphiphile insertion into a bilayer is inversely proportional to the area of the membrane constituents [34], the partition coefficient of amphiphiles can be improved under hypotonic conditions. Compared to the isotonic situation, molecules such as SSL14, SAL14 and SSL18 reach higher molar ratio in the membrane, sufficient to exert a hemolytic activity that is added to the hemolysis due to hypotonic conditions.

When the acyl chain length of the added surfactin analog decreases, the hemolytic activity under hypotonic conditions decreases. This tendency gives rise, in the case of SSL10, to a value inferior to the hemolytic activity in its absence. The direct correlation of hemolytic activity with chain length has already been highlighted for surfactin [10,15] and other surfactants [22].

It has been shown that a relationship exists between a low CMC and a high capacity of surfactant to insert into membranes [32,33]. This means that SSL18 has the best insertion properties and SSL10 the worst. As a consequence, following the positive action of hypotonic condition on insertion, SSL14 is able to reach a molecular ratio in the membrane sufficient to induce hemolysis; SSL18 reaches a

very high value of molecular ratio, giving rise to high hemolytic activity; and SSL10 does not reach a sufficient ratio to induce hemolysis. However, the molecules of SSL10 inserted in the membrane exert another kind of activity, which is a protective effect against hypotonic hemolysis. The relationship between chain length, CMC and hemolytic activity in hypotonic medium can be understood on this basis.

On another hand, it has been suggested [15,35] that the hemolytic activity of cyclic surfactin can be dependent of its capacity to self-associate inside the membrane. This is a second way by which hemolytic activity is related to CMC and thus to the chain length.

The protective effect exerted by SSL10 has already been reported for different surfactants [22–25]. Three mechanisms can be suggested to explain this phenomenon. Firstly, the insertion of surfactant molecules into the membrane decreases the lateral tension in the membrane due to its stretching and allows the cell to swell more before the leakage [36,37]. In other words, the surfactant is used as a material that enlarges the membrane. The second mechanism is based on the fact that some surfactants can induce an increase in ionic permeability of membrane. This allows an equilibration of the concentrations inside and outside the RBC and a decrease of the hypotonic hemolysis [37,38]. The last explanation is that the elastic, mechanic and cohesive properties of the membrane can be improved by the insertion of amphiphiles. This last hypothesis can be related to the finding that surfactin film possesses good mechanic and rheological characteristics, giving rise to good foaming properties [6]. One or several of these mechanisms can be implicated in the decrease of hypotonic hemolysis exerted by SSL10.

Protective effect of linear analogs against hemolysis induced by TX-100 is surprising as the molecules having the lowest CMC and the longest acyl chain are the most efficient protectors, and inversely. This is opposite to the general tendency in which the molecules having the lowest CMC and the longest acyl chain are the more hemolytic agent, as observed in hypotonic system in the present study, and in other work with different cyclic surfactins [15].

As show in Fig. 8, there is a strong correlation between CMC of linear surfactin and the concentrations required to give rise to a protective effect. It has to be kept in mind that the exact concentration in surfactin analog at which micelles are formed under these conditions is different of the CMC measured in this work. Indeed, the presence of TX-100 has an influence to the CMC, and the presence of 150 mM NaCl decreases it. However, it can be supposed that, for a series of analogs having the same number of ionisable functions, the effect of salt on each analog is similar.

The inhibition of hemolysis due to a lytic surfactant by another surfactant has already been reported [39], and two hypothesis can explain this protective effect. In the first one,

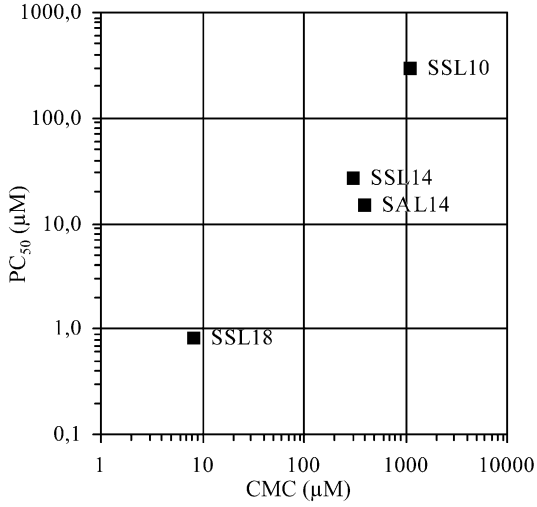


Fig. 8. Relation between the CMC of the different linear analogs ant the concentration required to give rise to 50% of their protective effect ($PC_{50}$). CMC have been measured in 5 mM Tris pH 8.5 at 25 °C. $PC_{50}$ values have been measured in a $5\times10^7$ RBC/ml suspension in presence of 10 mM Tris 150 mM NaCl 160 µM TX-100 pH 8.5 at 25 °C.

it has been suggested that the lytic surfactant is included in the micelles formed by the protective surfactant, thus forming mixed micelles [39]. This would decrease the number of lytic surfactant monomers available for insertion into the membrane and then for its leakage.

Another explanation is that the protective effect is exerted inside the membrane. As analogs having a low CMC insert in the membrane at lower concentration, the correlation between CMC and protective effect would be due to an insertion difference. This implies that both lytic and protective surfactants are inserted into the membrane and that the protective effect is correlated to the amount of inserted analog. Following the insertion of TX-100, an expansion of the headgroup region of the leaflet occurs, leading to a positive curvature and to membrane destabilisation. An inserted molecule that extends another region of the leaflet would balance this effect and decrease the TX-100 induced hemolysis. This could be a way for surfactin analogs to exert the antihemolytic activity. However, little is known about the location of the bulky part of linear surfactin in a bilayer and these molecules are more likely to be still cone shaped than inverse cone shaped.

To conclude, this work shows that linear surfactins have a non-aggressive behavior against RBC, and can even protect them against the action of other detergents. If other biological activities, such as antiviral, are maintained, linearisation of surfactin can be an interesting way to decrease the cytotoxic effect of this product. Avrahami and Shai [40,41] have recently reported the interesting ratio antibacterial effect-hemolytic effect of similar synthetic linear lipopeptides. Another potential use of linear surfactin is its incorporation into cyclic surfactin formulation in order to take advantage of their protective effect.

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