**Rhamnolipids functionalized with basic amino acids: synthesis, aggregation behavior, antibacterial activity and biodegradation studies.**

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**Abstract**

Rhamnolipids have been intensively studied due to their remarkable properties; however, the biosynthesis of RLs cannot compete commercially with the production of synthetic surfactants. Here, novel cationic rhamnolipids (RLs) derivatives containing arginine and lysine were prepared for the first time using a straightforward synthetic procedure. The RLs used to prepare these new cationic derivatives were produced by Pseudomonas aeruginosa using waste frying oil as carbon source. It was found that the amino acid-based RLs form aggregates at very low concentrations, even below the CMC. Biodegradation studies indicate that these cationic RLs can be classified as readily biodegradable. Interestingly, the RL arginine conjugates exhibited notable DNA binding affinity and good antimicrobial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, which increases the potential applications of these compounds. Consequently, the use of low-cost substrates and the added value of the final product constitute a more cost-effective rhamnolipid production.

**Keywords:** Rhamnolipid production; used cooking oil; arginine and lysine derivatives; physico-chemical properties; antibacterial activity, biodegradation

**1. Introduction**

In the last decades, increasing environmental concerns have led to new protocols designed to reduce chemical pollution. With the needs of future generations in mind, the goal of green chemistry is to develop products that will reduce or eliminate the generation of substances harmful to health and the environment. In synthesis, the selection of a starting material may be one of the most significant factors in determining environmental impacts [[[1]](#endnote-1)].

The growing prominence of biosurfactants is based on their wide range of functional properties and renewable production routes based on microbes [[[2]](#endnote-2)]. Of all the varieties of microorganisms that produce these substances, those with the greatest potential are the species of the genus *Pseudomonas*, which typically produce a class of glycolipid known as rhamnolipids (RLs) [[[3]](#endnote-3)].

These biosurfactants have been intensively studied due to their remarkable properties: they are environmentally friendly compounds with excellent surface activity and low toxicity. Moreover, they can be prepared using low-cost renewables substrates [[[4]](#endnote-4),[[5]](#endnote-5)].The biosynthesis of RLs cannot compete commercially with the production of synthetic surfactants, which has inspired innovative approaches to enhance their multifunctionality and find novel applications [[[6]](#endnote-6)-[[7]](#endnote-7),[[8]](#endnote-8)]. In this context, the use of low-cost substrates can be an effective strategy to reduce the production cost and the preparation of cationic RLs offers an opportunity to extend the potential usage of these biosurfactants.

The combination of a positively charged head group and one or more hydrophobic alkyl chains gives cationic surfactants greater multifunctionality compared to anionics [[[9]](#endnote-9)]. They have been used in advanced medical applications: in gene therapy, because vesicles made of cationic surfactants have the ability to encapsulate RNA or DNA for cell transfer [[[10]](#endnote-10)]; as vehicles for certain drugs [[[11]](#endnote-11)], and as physicochemical and biological modifiers of biomaterials used in medicine [[[12]](#endnote-12)]. Moreover, some cationic surfactants show antimicrobial properties, which may contribute to resolving the acute problem of rapidly growing bacterial resistance to commonly used antibiotics. The interaction of cationic surfactants with the cell membranes of microorganisms has been harnessed in new therapeutic antimicrobial and antifungal applications [[[13]](#endnote-13)], with a mode of action that hampers the development of antimicrobial resistance.

In this study new cationic RL derivatives containing arginine and lysine (Scheme 1) were prepared by linking the free α-NH2 group of these amino acids to the terminal carboxyl of the RL. The primary aim of this modification was to increase the antimicrobial activity of RLs by introducing cationic charged head groups. The modified RLs were assayed to ascertain their surface activity, aggregation behavior, antibacterial activity, DNA binding properties and biodegradability. The novelty of this paper is the development of a new route for the transformation of anionic RLs in “green high-added value products”.

**(Scheme 1)**

**2. Experimental**

This section briefly explains the methods used in this work. Details of materials and experimental procedures are reported in the Supplementary Information.

*2.1. Production and purification of rhamnolipids*

The microorganisms used for RLs production were strains of *Pseudomonas aeruginosa* MB and the carbon source was waste frying oil. Using the procedure showed in the supporting information two different RL samples were obtained: the **RLmix** (mixture of monoRL and diRL) and the **monoRL** (isolated fraction containing mainly monoRL).

 *2.2. Synthesis of amino acid rhamnolipid conjugates*

The synthesis of the Arginine-based rhamnolipids was carried out in one step using H-Arg-OMe and RLmix or monoRL as starting materials and Diciclohexilcarbodiimida (DCC) with Hydroxybenzotriazole (HOBt) as the activating agent.. The Lysine-based rhamnolipids were prepared by means of a two-step procedure: the first step consisted of the preparation of RL-Cbz-Lys derivatives using Cbz-Lys-OMe and RLmix or monoRL as starting materials. After that, the target compounds were obtained by removing the Cbz-group under a H2 atmosphere. All the synthesized compounds were characterized by 1H and 13C nuclear magnetic resonance (NMR) analyses and Mass Spectrometry (MS). The supporting information contains the detailed description of the procedures used to carry out these syntheses as well as the NMR and MS spectra.

*2.3. Surface tension measurements*

Surface tension measurements were measured by the Wilhelmy plate technique using a Krüss tensiometer.

*2.4. Fluorescence measurements*

The CMC was also determined by fluorescence measurements (Shidmadzu RF 540 spectrofluorometer). The fluorescence emission spectrum of pyrene in the 370-400 nm region depends on the polarity of the environment. The ratio of the first to the third vibronic peaks (II/IIII) in the fluorescence spectra was used to obtain the CMC.

*2.5. Size distribution analysis*

The size distribution profile of all RL formulations was determined by Dynamic Light Scattering (DLS) measurements using a Malvern Zeta Nanosizer. The size distribution profile was calculated using the correlation function. The Zetasizer (V6.20) software automatically determines the optimum parameters for the algorithm for monomodal and multimodal profiles.

*2.6. Etidium Bromide (EB) fluorescence*

The percentage of EB displaced from the DNA/EB complex due to the interaction of the nucleotide with the RL surfactants was calculated according to the following equation:

%EB = (I0 - I **/** I0 - IEB) x 100

where I0 and IEB are the maximum fluorescence intensities obtained for the EB and EB/DNA solutions, and I is the maximum fluorescence intensity obtained at different RL surfactant concentrations.

The results are represented based on the N/P ratios:

N/P = (mols of surfactants x Nº of charges) / (mols of DNA x Nº of charges)

*2.7. Agarose gel electrophoresis*

 Electrophoresis was carried out at 90 V in Tris-acetic buffer (pH 8.0) for 35 min. The DNA band was visualized under a transilluminator (Shimadzu-UV-160A).

*2.8. Antimicrobial Activity*

Antimicrobial activity was studied by determining the minimum inhibitory concentration (MIC) [[[14]](#endnote-14)], defined as the lowest concentration of antimicrobial agent that inhibits the development of visible growth after 24 h of incubation at 37 °C.

*2.9. Biodegradability*

The CO2 headspace test [[[15]](#endnote-15)] was applied to evaluate the biodegradability of the surfactants under aerobic conditions. This method allows the evaluation of the ultimate aerobic biodegradation (mineralization to carbon dioxide) of an organic compound in aqueous medium by measuring the net increase in total inorganic carbon over time.

**3. Results and discussion**

*3.1. Rhamnolipid production*

In order to reduce the cost of producing biosurfactants and use renewable materials, *Pseudomonas aeruginosa* MB was incubated in a culture medium containing waste frying oils as a carbon source. To increase the productivity, a 2% inoculum was used and four-phase culture was run. At the end of the fourth phase, a production of 19.17 g/L was achieved. In this work, two different RL samples were prepared: **RLmix** and **monoRLs**. The **RLmix** is the mixture of monoRLs and diRLs produced by the microorganisms. The **monoRL** sample, containing mainly monoRLs with alkyl chains of 10 carbon atoms, was obtained by purifying a sample of RLmix using silica gel column chromatography.

The composition of the two RL samples was evaluated by ESI-MS analysis (Figure S1). The **RLmix** showed two dominant peaks of m/z 503 and 649 (ratio 60/40), which are compatible with Rha-C10-C10 and Rha-Rha-C10-C10. Minor peaks corresponding to other homologues of mono- and di-rhamnolipids were also identified: Rha-C8:2 (m/z: 301), Rha-C12:2 (m/z: 357), Rha-C10-C8 (m/z: 475), Rha-C10-C12:1 (m/z: 529), Rha-Rha-C8-C10 (m/z: 621), Rha-Rha-C10-C12 (m/z: 677). The monoRL Rha-C10-C10 appeared as a predominant peak of m/z 503 in the purified **monoRL** sample This sample also contained minor peaks corresponding to other homologues such as Rha-C12:2 (m/z 357) and Rha-C10-C12:1(m/z 529) .

The ability of *Pseudomonas* species to produce biosurfactants has been extensively documented [[[16]](#endnote-16)]. Nitschke et al. [[[17]](#endnote-17)] carried out a study with *P. aeruginosa* LBI using several substrates as the carbon source and reported a significant RL yield of 11.7 g/L when using soy soapstock. Lan et al. [[[18]](#endnote-18)] obtained of 13.97 g/L when using 20 g/L of waste cooking oil. According to the literature, the nature of the homologue mixture depends on variables such as the strain type, carbon source, as well as the production strategy [[[19]](#endnote-19)]. Using glycerol and glucose, *P. aeruginosa* LBI mainly produced the diRL Rha-Rha-C10-C10, whereas when hydrophobic sources were used, the monoRL Rha-C10-C10 was predominant [17]. In contrast, using soapstock (a hydrophobic substrate) as the carbon source, a predominance of Rha-Rha-C10-C10 was found [[[20]](#endnote-20)].

*3.2. Rhamnolipid amino acid conjugates*

RL derivatization was carried out using the two RL samples previously prepared. Firstly we used the mixture of mono and dirhamnolipids (**RLmix**) produced by the microorganism. This sample was not purified because the aim of the work was to modify the rhamnolipid structure as little as possible using renewable raw materials. The second sample, **monoRL**, was purified to ascertain if the biological and physicochemical properties of the resulting surfactants changed meaningfully if the number of rhamnose moieties in the molecule varied.

The cationic derivatization of RLs was carried out by the preparation of arginine and lysine conjugates (Scheme S1). The design of these new amino acid-based RLs was based on the results of previous structure-activity relationship studies [13,[[21]](#endnote-21)]: 1) the introduction of cationic charges to the RL structures can enhance the antimicrobial activity of the precursors 2) compounds containing basic groups of the guanidine type attached to hydrophobic chains have excellent antimicrobial and antifungal properties 3) lysine and arginine RL derivatives can be prepared using simple and rapid chemical procedures, and 4) amino acid-based surfactants usually show high biodegradability and low ecotoxicity.

The two arginine derivatives were synthesized using RLmix or monoRL (Scheme S1). The guanidine group of arginine is an extremely strong base (pKa=12.5) that remains protonated under standard conditions for α-acylations, making it possible to work with an unprotected arginine side chain. The formation of an amide bond between the RLs and the amino group of arginine was achieved with different solvents and condensating agents, the best results being obtained using DCC with HOBt as the activating agent. Two RL samples functionalized with arginine were obtained: **RLmix-Arg** (from RLmix) and **monoRL-Arg** (from monoRL).

The procedure used to prepare lysine derivatives was performed in two steps (Scheme S1). The pKa of the lysine ε-amino group was lower than that of the guanidine group; therefore, for the lysine derivatives it was necessary to work with the ε-protected amino acid. The best results for this reaction were obtained using BOP (**b**enzotriazol-1-yl**o**xytris(dimethylamino)**p**hosphonium hexafluorophosphate) as the coupling agent. Two RL samples functionalized with lysine were also obtained: **RLmix-Lys** (from RLmix) and **monoRL-Lys** ( from monoRL).

ESI-MS studies confirmed the formation of the amino acid conjugates (Fig. S1). Both Rha-Rha-C10-C10-Arg (m/z 822) and Rha-C10-C10-Arg (m/z 675) gave the predominant molecular ion peaks in the RLmix-Arg sample, whereas only the peak corresponding to Rha-C10-C10-Arg (m/z 675) was observed for monoRL-Arg. ESI-MS also showed minor peaks corresponding to other RL derivatives. Similar results were obtained for the lysine derivatives; the predominant homologues in the RLmix-Lys were Rha-Rha-C10-C10\_Lys (m/z 793) and Rha-C10-C10\_Lys (m/z 647), whereas Rha-C10-C10\_Lys (m/z 647) was predominant in the monoRL-Lys sample.

The results indicate that it is possible to obtain the amino acid conjugates using a straightforward and highly efficient procedure. It should be emphasized that each reaction was performed with three replicates and the chromatograms obtained in the different reproductions always presented the same profile.

*3.3. NMR spectroscopy*

NMR spectra were used to confirm the chemical structure of all compounds (Figures S2-S7). The 1HNMR spectrum of the monoRL clearly shows the signals corresponding to the two main moieties of this molecule. The peaks at δ=0.90 ppm, δ=1.2 ppm and around δ =1.4 ppm indicate the presence of the terminal - CH3 group and the -CH2- of a straight-chain fatty acid. The 1HNMR spectrum also contains the typical signals of the rhamnose moiety; chemical shifts of δ=3.5-4.7 ppm correspond to the hydrogen atoms of the pyranose ring, and the peak of the -CH3 of pyranose was observed as a singlet at δ = 1.2 ppm. The 13CNMR spectrum also shows the presence of two alkyl chains and the rhamnose group: signals at δ=172.4 ppm and δ=174.2 ppm correspond to two carbonyl groups, signals at δ = 14.4 ppm and δ = 23.0-33.0 ppm to two alkyl chains, and signals at δ = 69.3-100.6 ppm confirm the presence of the rhamnose moiety. The 1HNMR and 13CNMR spectra did not identify the chemical shifts of all the protons and carbons of the sample, so 2D NMR spectra were also acquired. Table S1 contains the 1HNMR and 13CNMR data obtained from these experiments. The NMR spectra of the RLmix also support the proposed mixture. The region corresponding to the rhamnose carbons in the 13CNMR contains more signals than the spectra of the monoRL. The gHSQC shows three cross peaks corresponding to anomeric carbons of rhamnose (4.92/99.05, 4.78/100.5, 4.88/104.2); these signals clearly indicate the presence of the mono- and diRLs in the mixture. Using the mono- and bi-dimensional NMR experiments, the NMR data corresponding to the diRLs was determined (Table S2).

The 1HNMR of the lysine and arginine derivatives also identified the target structures. The multiplet at around 4 ppm and the singlet at 3.7 ppm confirm the anomeric -CH- and the methyl ester group of the amino acid moiety. The 1HNMR was carried out using a delay of 25 s, which allows the integration of the proton signals to be compared accurately. Comparing the integrals corresponding to the –CH– and -CH3 of lysine or arginine with those of the - CH3 of the alkyl chains, it can be assumed that almost all RLs were amino acid derivatives. The 13CNMR of these derivatives also contain signals corresponding to the amino acid moieties: the anomeric -CH-, the -CH3 of the methyl ester group and the carbon atom of the guanidine group in the arginine derivatives. The 2D NMR spectra were also used for the complete assignation of all NMR data of these derivatives (Table S1 and S2).

3.4. Surface properties and self-aggregation

The critical micellar concentration (CMC) of RLs and their amino acid conjugates in water solution was investigated by steady-state fluorescence measurements using pyrene as a solvatochromic probe.

**(FIGURE 1)**

The I1/I3 ratio obtained at the diluted concentrations is about 1.6, which is comparable to that of pyrene in water (Figure 1A, 1B); a subsequent abrupt decrease in I1/I3 indicates that the pyrene is moving into the formed micelles. The data were fitted to sigmoidal Boltzmann-type curves, and the CMC values were taken as the middle point of transitions (Table 1). The CMC value of the monoRL samples is lower than that of the RLmix which can be ascribed to their hydrophobic nature. Although monoRL and RLmix contain the same hydrophobic group, monoRL can be considered a more hydrophobic as it contains only one rhamnose moiety.

Wide-ranging CMC values (1-400 mg/L) [[[22]](#endnote-22),[[23]](#endnote-23)] have been reported for mono- and diRLs and mixtures of both biosurfactants . For example, the CMC for mixtures of mono- and diRLs include 120 mg/L reported by Benicasa [20], 53 mg/L by Mata-Sandoval [[[24]](#endnote-24)], and 230, 106, 150 and 234 mg/L for RLs produced using soybean oil refinery wastes [[[25]](#endnote-25)]. Regarding the influence of the pH, the CMC of monoRLs was found to be 0.070 mM at pH=7.4 and 0.050 mM at pH=4 [[[26]](#endnote-26)], Chen [[[27]](#endnote-27)] found that for monoRL, the CMC increased as the pH decreased, from 0.18 mM at pH 7 to 0.36 mM at pH 9. Even when the same RL is used, discrepancies in CMC values can arise: using the same commercial RL of Jeneil, Zhong [[[28]](#endnote-28)] reported a CMC value of 0.12 mM for the monoRL fraction and 0.07 mM for the diRL fraction, whereas Chen et al. found a CMC of 0.18 mM for monoRLs and 0.11 mM for diRLs [27]. These different values can be attributed to the numerous factors that can affect the CMC: levels of sample purity, pH, ionic strength and the presence of unsaturated bonds. Moreover, given the possible coexistence of a variety of RL homologues, separating one can be challenging, and small quantities of other homologues can produce changes in the aggregation behavior.

The CMC of the arginine and lysine RL conjugates are of the same order. These results indicate that the hydrophilic/lipophilic balance of these surfactants is similar. Previous studies reported similar CMC values for arginine and lysine surfactants with the same alkyl chain length [13]. The CMC of the amino acid derivatives is higher than that of their RL precursors, which may be explained by their different ionic character. RLs can be neutral or negatively charged. The pKa values of purified mono- and diRLs were 5.9 and 5.6, respectively [[[29]](#endnote-29)]. This means that at a pH of 6.6 or higher, more than 97% of molecules are negatively charged, whereas at a pH of 4.6 or less, more than 97% of molecules will be non-ionic. At a pH between 4.6 and 6.6 water solutions of these compounds contains anionic and non-ionic species. Chen [27] found that the CMC of monoRLs did not change after the addition of NaCl, which confirms the low ionic character of these biosurfactants in water. The arginine RL conjugates have the cationic charge in the protonated guanidine group (pKa=12.5), while in the lysine derivatives the cationic charge is located in the ε-protonated amino group (pKa=10.5). The pKa of the basic amino group of amino acid is reported to decrease from 1 to 3 units when hydrophobic groups are linked to the amino acid [[[30]](#endnote-30)]. Taking into account these considerations and the pH of water solutions of RL and amino acid RL conjugates (pH values between 4.3-6.1), it can be assumed that the RL water solutions contain a high percentage of non-ionic molecules, whereas almost all the molecules in the water solution of the arginine and lysine derivatives are cationic. The high percentage of non-charged molecules in the RL water solution explains their lower CMC compared to the amino acid conjugates.

The surface activity of these biosurfactants was examined by surface tension measurements (Figure 1C and 1D). The surface tension decreased gradually with increasing RL concentration up to a certain value, the CMC, beyond which it remained almost constant. The CMC values obtained by surface tension did not agree with those determined by fluorescence (Table 1), especially for the amino acid RL conjugates. Similar results have been reported for cationic surfactants with two or more hydrophobic alkyl chains, including gemini and glycerolipid arginine-based [[[31]](#endnote-31)] and gemini pyridinium [[[32]](#endnote-32)] surfactants. This behavior has been ascribed to the formation of premicellar aggregates. Bhadani [32] used conductivity measurements to confirm the presence of premicellar aggregates in aqueous solutions of gemini pyridinium surfactants and Rosen also found an important difference between the CMC values achieved by conductivity or surface tension due to the existence of premicellar aggregates in gemini surfactants [[[33]](#endnote-33)].

Table 1. Surface active properties of RLs and their arginine and lysine derivatives at 25 °C. Critical micellar concentration (CMC), Surface tension at the CMC (γCMC) and pC20(–LogC20, Concentration required to reduce the surface tension of water by 20 mN/m)

|  |  |  |
| --- | --- | --- |
| Rhamnolipidsand their derivatives | Surface tension | Fluorescence |
|  | CMC (g/l) | γCMC (mN/m) |  pC20  | CMC (g/l) |  II / III |
| RLmix |  0,005  | 30.6 | 2.8 | 0.05 | 1.26 |
| RLmix-Arg | 0.016 | 31.1 | 2.5 | 0.18 | 1.26 |
| RLmix-Lys | 0.008 | 30.6 | 2.7 | 0.23 | 1.26 |
| monoRL | 0.010 | 28.8 | 3.0 | 0.014 | 1.26 |
| monoRL-Arg | 0.013 | 28.9 | 3.14 | 0.17 | 1.26 |
| monoRL-Lys | 0.015 | 28.3 | 3.11 | 0.14 | 1.26 |
|  |

The surface tension value at the CMC (γcmc) indicates how effectively the surfactant can reduce the solvent surface tension. The γcmc values obtained show that the studied RLs reduced the surface tension of water very effectively, the results being similar for all samples. The presence of two hydrophobic chains gives rise to hydrophobic interaction, which promotes a tightly packed monolayer of surfactants at the interface. Previously reported RLs [[[34]](#endnote-34)] and arginine-based surfactants with one arginine and two alkyl chains [13] also had low γCMC values. Moreover, all the RLs showed very good adsorption efficiency (pC20=-logC20) and very small quantities were required to reduce the surface tension of water.

*3.5. Sizes of Aggregates*

Dynamic light scattering (DLS) measurements were carried out to determine the hydrodynamic diameters of the aggregates formed by the RLs (Figure S8). Figure 2A shows the size distribution as a function of concentration for the monoRL and RLmix samples, which was similar for both products. For all concentrations tested, even below the CMC, scattering detected a bimodal distribution of sizes corresponding to small- (50-100 nm) and medium-sized vesicles (200-400 nm). The sizes remained roughly within the same range for all concentrations.

**(FIGURE 2)**

Formation of aggregates at very low concentrations, even below the CMC has also been described for other RLs. Haba et al. [[[35]](#endnote-35)] found a similar size distribution for RLmix produced by the same *P. aeruginosa* strain. Small aggregates were detected by Pornsunthorntawee at about one fifth the CMC and a bimodal population of vesicles of 50-250 nm above the CMC [[[36]](#endnote-36)]. Ikilier et al. [[[37]](#endnote-37)] reported that pure mono- and diRLs aggregated at a concentration one tenth of the CMC: aggregate size decreased up to the CMC and then increased up to 10 mM. The minimum concentration at which aggregation occurred was attributed to the transition from the liquid-solid coexistence in the monolayer to the ordered solid range. Zhong et al. [[[38]](#endnote-38)] also observed the formation of vesicles (50-60 nm) at one tenth the CMC, and a decrease in aggregate size beyond the CMC. Guo et al. [[[39]](#endnote-39)] reported a bimodal distribution of small and medium vesicles for a diRL-rich fraction; in this case, the vesicle diameter increased with the concentration. The discrepancies in aggregate sizes and trends are similar to the variability of reported CMC values and likewise can be attributed to differences in RL composition, the pH and sample preparation procedures.

Aggregates were also observed at concentrations below the CMC with the lysine and arginine conjugates (Fig. 2B and 2C). The size distribution profiles of the monoRL-lys derivatives are similar to those of their RL precursors; a bimodal distribution of small and medium aggregates that remained fairly constant as the concentration increased. The RLmix-lys sample also showed a bimodal distribution and the size of the smallest aggregates did not change when the concentration increased, unlike that of the large aggregates.

Arginine RL conjugates showed different behavior. At low concentrations, medium-sized as well as large vesicles with hydrodynamic diameters higher than those found in the RL samples (between 350 and 750 nm) were observed. At the highest concentrations tested (0.150-0.3), three different size ranges of aggregates were found. The smallest had an average size of 4-9 nm and were most likely micellar structures; those of medium-sized were of 30-40 nm and large of 250 nm. Although the intensity of the peaks corresponding to the micelles (4-9 nm) is very low compared to those of the large aggregates, it should be taken into account that the latter are considerably amplified by the size distribution in terms of intensity.

The DLS studies indicate that all RLs formed vesicles at low concentrations. Vesicle formation has already been described for several cationic surfactants, including arginine- or lysine-based surfactants with two alkyl chains [13] and didodecyl dimethyl ammonium bromide, a cationic surfactant that also contains two hydrophobic chains [[[40]](#endnote-40)]. The type of aggregates formed by surfactants in aqueous solution can be predicted by the geometry of the molecule. Double chain surfactants have a cylindrical shape and tend to form bilayers through lateral van der Walls interactions of the alkyl chains and hydrogen bonds between the hydrophilic groups.

*3.6. DNA binding properties*

An important application of cationic lipids in modern biology is the transport of DNA or other genetic material through the cell. To determine if the new cationic amino acid-based RLs are suitable candidates for biomedical applications, their interactions with DNA were studied. The DNA binding affinity was evaluated with ethidium bromide intercalation and agarose gel electrophoresis assays.

The fluorescence intensity of EB, an aromatic cationic dye, increased drastically when the ethidium ion intercalated between the DNA base pairs (Figure S9). The addition of monoRL and RLmix to the EB-DNA complex (Fig. S9A and S9B) did not affect the stability of the DNA-EB complex, indicating that anionic aggregates do not interact with polyanionic DNA. The addition of cationic RLs (Fig. S9(C-F)) resulted in a quenching of the fluorescence emission intensity. The DNA was compacted by the cationic aggregates formed by the arginine- and lysine-based RLs, producing a gradual release of EB from the EB-DNA complex (Figure 3).

**(FIGURE 3)**

The results indicate that these cationic RLs possess good DNA binding capacity and that the most efficient quenching was produced by the arginine homologues. The higher DNA affinity shown by the arginine-based RLs could be ascribed to the nature of their cationic charge; it has been reported that arginine-based lipids also exhibit significant DNA-binding properties through the hydrogen bonds of the guanidinium group [[[41]](#endnote-41)]. The DNA affinity of the new RLs is similar to that displayed by monocatenary surfactants with long alkyl chains [[[42]](#endnote-42),[[43]](#endnote-43),[[44]](#endnote-44)].

Agarose gel electrophoresis was also used to study the interactions of the new RL derivatives with DNA (Figure 4). When an electric field is applied to DNA placed in agarose gel, the oligonucleotide replicates throughout the gel. As negative charges in DNA become partially or totally neutralized due to the interaction with other compounds, the migration of DNA though the gel is retarded or completely hindered.

**(FIGURE 4)**

No evident modifications in DNA mobility were observed in the presence of varying concentrations of monoRL and RLmix. As expected, anionic lipids did not interact with DNA, whereas it was confirmed by agarose gel electrophoresis that DNA forms significantly stable complexes with amino acid-based RLs. At low concentrations of amino acid derivatives migration of oligonucleotides was

not clearly modified. However, at higher concentrations, the loss of the fluorescence bands along the lines clearly indicates the complete complexation and neutralization of DNA by the tested compounds. The RLmix-Lys derivatives showed the lowest affinity to DNA, which agrees with the results of the EB fluorescence studies.

*3.7. Antibacterial activity*

The design and synthesis of new antimicrobial surfactants is of great interest in the fight against growing antimicrobial resistance. The MIC values of each compound against representative bacterial strains are presented in Table 2. Anionic RLs did not show antimicrobial activity at the concentration range tested. The results published in the literature concerning RL antimicrobial activity indicate that different bioactivity profiles can be obtained by changing the growth substrate, growth conditions or bacterial strain. Abalos [25] and Benicasa [20] reported MICs in the range of 4 to128 μg/mL against a variety of bacteria. Lotfabad [29] found that RLs exhibited activity only against some Gram-positive strains. On the other hand, different activity against bacterial and fungal species was described for two RL mixtures produced by two distinct Pseudomonas strains [[[45]](#endnote-45),[[46]](#endnote-46)]. In contrast, Aleksic et al. found no direct activity of natural RLs at concentrations of up to 500 μg/mL against Gram-positive and Gram-negative strains [[[47]](#endnote-47)] whereas synthetic RL amide derivatives were active against the Gram-positive bacteria tested.

The different levels of antimicrobial activity can be attributed to the variable homologue composition of RL mixtures and may also be seriously affected by pH. Ferreira et al. evaluated the effects of a RL on food pathogens under different pH conditions and observed activity only against Gram-positive bacteria, which decreased considerably at pH≥7[[[48]](#endnote-48)].

It is assumed that the breakage of the bacterial cell envelope requires an electrostatic interaction between the negative cell envelope and the RLs, but as RLs are often negatively charged, the chances of successful interaction are low. Therefore, it was expected that the introduction of the basic amino acids lysine or arginine could influence the permeability of the bacterial membrane, resulting in an antimicrobial effect. The obtained results show that arginine RL conjugates exhibited good antimicrobial activity against Gram-positive bacteria, with MICs ranging from 4 to 32 μg /mL. It is noteworthy that these compounds were also active against the Methicillin-resistant *S*. *aureus* (MRSA), the most common single multi-drug-resistant bacterium in Europe. These surfactants, with a positive charge in the guanidine group, showed an enhanced tendency to disturb bacterial membranes, as described for numerous cationic surfactants [13,[[49]](#endnote-49)[[50]](#endnote-50)-[[51]](#endnote-51)]. The arginine derivatives did not show activity against any of the Gram-negative bacteria studied. Gram-negative bacteria have a single outer membrane charged with lipopolysaccharides that functions as an efficient permeability barrier [[[52]](#endnote-52)]. Anti-bacterial activity was similar for the two arginine RL conjugates, indicating that it was not affected by the number of rhamnose moieties in the molecule. This result shows it is not necessary to purify the RL mixture, given the similar effectiveness ot the two arginine derivatives.

In contrast, the lysine derivatives did not show any activity, in accordance with the generally lower antimicrobial effects reported for lysine versus arginine derivatives [13,[[53]](#endnote-53)], which is attributed to the differences in the cationic charge. Arginine derivatives have a positive charge in the strongly basic guanidine group (pKa=12.5) whereas lysine derivatives have the positive charge in the ε-protonated amino group (pKa=10.5). The introduction of hydrophobic groups in the amino acid drastically reduces the apparent pKa; it has been described for lysine-derived surfactants that the pKa of the protonated amine group can be reduced by 2/3 units [30]. Assuming a reduction of 3 units, the pKa of the arginine RLs would be 9.5 while the pKa of the lysine RLs would be 7.5. Thus, at the pH of the antimicrobial assays (pH=7.4) the lysine derivatives were only partially protonated in comparison with the 100% protonation of the arginine derivatives. Therefore, the arginine based surfactants showed an enhanced tendency to disturb bacterial membranes, as described for numerous antimicrobial surfactants that feature strongly basic groups of the guanidine type attached to a fairly large lipophilic molecule [49,51]. These results also agree with the greater interaction of the arginine RLs with the DNA.

Table 2. Minimal inhibitory concentration (MIC) of RLmix and monoRLs, and their amino acid derivatives against selected Gram-positive and Gram-negative bacterial strains.

|  |  |
| --- | --- |
| **Bacteria** | **MIC (µg / mL)** |
|  | RLmix | MonoRL | RLmix-Lys | MonoRL-Lys | RLmix-Arg | MonoRL-Arg |
| *B. subtilis* | >250 | >250 | >250 | >250 | 16 | 16 |
| *S. epidermidis*  | >250 | >250 | >250 | >250 | 4 | 4 |
| *S. auereus*  | >250 | >250 | >250 | >250 | 8 | 8 |
| *S. aureus* (MRSA) \*  | >250 | >250 | >250 | >250 | 32 | 16 |
| *L. monocytogeneses* | >250 | >250 | >250 | >250 | 16 | 16 |
| *P. aeruginosa*  | >250 | >250 | >250 | >250 | >250 | >250 |
| *K. pneumoniae*  | >250 | >250 | >250 | >250 | >250 | >250 |
| *E. coli* | >250 | >250 | >250 | >250 | >250 | >250 |

This is the first report of chemically derivatized amino acid-based RLs with antimicrobial activities. Aromatic derivatives of RL [47], with antimicrobial and antibiofilm activity have also been described. Sophorolipids, another class of biosurfactants, have also been modified with amino acids. The best antimicrobial activity was obtained with the leucine conjugate, which had MIC values of about 1-2 μg/mL against *Moraxella sp*. and *S. sanguinis* [[[54]](#endnote-54)].Quaternary ammonium sophorolipids with antimicrobial activity have also been prepared recently [[[55]](#endnote-55)] .

*3.8. Biodegradation*

Biodegradation is an essential parameter to investigate, given that it is the best mechanism for the irreversible elimination of chemical products from aquatic and terrestrial environments. Indeed, “*Design for Degradation*” has been selected as the 10th Principle of Green Chemistry. European regulation requires that all surfactants used for domestic detergents are ultimately biodegradable. Only surfactants for special purposes can be simply primarily biodegradable, after having obtained a derogation based on risk assessment and benefit estimations. Biodegradability was evaluated by applying the ISO 14593-CO2 headspace test [15]. The stringent conditions of the test impose a low density of non pre-adapted bacteria, short duration, and absence of other sources of organic carbon. Compounds that exceed 60% biodegradation under these conditions within 28 days can be classified as readily biodegradable surfactants.

After 28 days, biodegradation percentages of RLs and amino acid-based RLs were higher than the 60% (Table 3). RL precursors were very easily degraded by aerobic microorganisms; 70% of biodegradation had occurred in only 7 days (Table 3). These good results match others reported in the literature. Mohan et al. [[[56]](#endnote-56)] found that RLs were biodegradable under aerobic conditions and also showed certain biodegradability under anaerobic conditions. Moreover, RLs may increase the biodegradation of many contaminants by influencing their bioavailability [[[57]](#endnote-57)]. The excellent RL biodegradation is consistent with what is expected from their chemical structure. The ester bond linking the two alkyl chains of RLs, as well as the chemical bond between the rhamnose moieties and the alkyl chains, can be easily broken, giving rise to the formation of three structural motifs, one rhamnose moiety and two carboxylic acids, which can be readily degraded by the microorganisms.

Table 3. Biodegradation percentages of reference substance (SDS), anionic (RLmixture and monoRL) and cationic (RLmix\_arg, monoRL\_arg and RLmix\_lys) rhamnolipids determined by the CO2 headspace test (95%CI of 28 –day results calculated from 4 replicates).

|  |  |
| --- | --- |
| Compounds of study | Biodegradation (%) |
|  | **7 days** | **14 days** | **21 days** | **28 days** |
| SDS\* | 66 | 85 | 90 | 90±1.5 |
| RLmix | 77 | 93 | 99 | 99±1.7 |
| MonoRL | 70 | 91 | 99 | 98±1.6 |
| RLmix\_arg | 59 | 78 | 90 | 85±3.6 |
| MonoRL\_arg | 43 | 71 | 82 | 79±3.9 |
| RLmix\_lys | 26 | 62 | 64 | 62±2.7 |
| MonoRL\_Lys | 20 | 56 | 61 | 61±0.2 |
| \*SDS (Sodium n-dodecyl sulfate) – Reference product |

Amino acid RL conjugates also showed high biodegradation levels. The amino acid functionalization slightly decreased the percentage of biodegradation, probably due to the presence of an extra amide bond for microorganisms to break down. Nevertheless, the biodegradation levels achieved were > 60% for all three derivatives and > 80% for the arginine derivative. In these RLs the amino acid was introduced through an easily broken peptide-like amide bond. The structural features of these amino acid derivatives were planned taking into account the guidelines for designing biodegradable surfactants: the use of natural building blocks, unbranched alkyl chains and the presence of hydrolysable groups (amide and ester groups). Moreover, given the assumed surfactant biodegradation mechanism, a safe biodegradable process is expected, as neither the initial products nor the intermediate metabolites (carboxylic acids, rhamnose, lysine or arginine) are toxic compounds. Similarly good levels of biodegradation have been obtained for arginine-based glycerolipic [[[58]](#endnote-58)] and N-acyl-lysine cationic surfactants [30]; both surfactant families also have ester or amide bonds linking the hydrophobic part with the polar heads. It has also been reported that the biodegradation of imidazolium-based ionic liquids increased significantly when an amino acid was introduced in their cationic moiety [[[59]](#endnote-59)].

**Conclusions**

Novel cationic biosurfactant conjugates were prepared from renewable starting materials (waste frying oil and amino acids) using a straightforward synthetic approach. The spectral characterization of the cationic derivatives confirmed that arginine and lysine amino acids were successfully introduced into the terminal carboxyl of the rhamnolipids. The amino acid RL conjugates as well as the precursors (RLmix and monoRL) showed very small CMC values, indicating that these surfactants form aggregates at very low concentrations. Moreover, they were highly efficient in reducing the surface tension of water. The arginine-based RLs exhibited good DNA binding ability, providing a basis for developing novel DNA-based materials. The arginine derivatives also exhibited considerably improved antibacterial activity compared to the precursors. The lysine derivatives did not exhibit antimicrobial activity, probably due to the different nature of their cationic charge. Interestingly, all RLs studied in this work showed high biodegradability levels and can be classified as readily biodegradable compounds.

The work confirms that a straightforward methodology can be used to transform waste materials into “high-added value products”.

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