Estudis d´interacció de tensioactius sintètics biocompatibles amb models de membrana. Potencial aplicació en medicina pulmonar

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Article I[1]

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Estudis d'interacció dels tensioactius derivats de diacilglicerols d'arginina amb monocapes de DPPC i DMPC. Relació amb l'activitat antimicrobiana

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Resum

Els productes antimicrobians tenen una gran importància pràctica en el sector de l'alimentació, així com en aplicacions farmacèutiques i cosmètiques per la prevenció de la contaminació microbiològica. El nostre grup d'investigació ha sintetitzat dos nous tensioactius catiònics d'estructura glicerolipídica derivats d'arginina denominats amb els acrònims de 1414RAc i 1212RAc. Per avaluar les propietats antimicrobianes d'aquests nous tensioactius, s'ha estudiat la seva interacció amb els fosfolípids 1,2-dipalmitoil-sn-glicero-3-fosfocolina (DPPC) i 1,2-dimiristoil-sn-glicero-3-fosfocolina (DMPC) com a models de membranes, així com amb organismes vius en presència i en absència de barreres externes, com són bacteris Gram-negatius, protozous humans de Leishmania i cèl·lules de mamífers. L'estructura i les característiques fàsiques de les monocapes mixtes s'han avaluat mitjançant l'anàlisi de l'elasticitat estàtica. La miscibilitat entre el tensioactiu i el fosfolípid en monocapes mixtes s'ha estudiat fent servir la regla d'addició i l'energia de Gibbs en excés de la mescla en funció de la fracció molar de fosfolípid, a diferents valors de pressió superficial. Per totes les mescles estudiades, les monocapes mixtes estan afavorides termodinàmicament excepte per la mescla 1212RAc/DMPC que presenta valors positius d'energia de Gibbs en excés.

La interacció dels tensioactius amb organismes vius només presenta efectes de toxicitat sobre les cèl·lules eucariotes degut a la permeabilització inespecífica de la membrana.

[1] En aquest manuscrit es van detectar errors que van ser esmenats després de ser publicat (veure Correcció de l'Article I, doi: 10.1016/j.colsurfa.2009.08.032).

Article II

Publicat en Colloids Surf. B, doi: 10.1016/j.colsurfb.2009.06.020

Estudi de la tensió superficial i de l'adsorció en mescles de tensioactius derivats de diacilglicerols d'arginina amb els fosfolípids DPPC i DMPC

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Resum

S'han estudiat les interaccions superficials de mescles binàries dels nous tensioactius derivats de diacilglicerols d'arginina amb fosfolípids (emprats sovint com a models de membrana) a 25ºC en solucions aquoses 0.1 M de clorur sòdic. Els tensioactius emprats són el clorhidrat de l'1,2-dimiristoil-rac-glicero-3-O-(N^{α}-acetil-L-arginina) (1414RAc) i el clorhidrat de l'1,2-dilauroil-rac-glicero-3-O-(N^{α}-acetil-L-arginina) (1212RAc), agents antimicrobians en potència i a més són biodegradables i presenten una toxicitat inferior a la d'altres tensioactius catiònics convencionals. Els fosfolípids utilitzats són l'1,2-dipalmitoil-sn-glicero-3-fosfatidilcolina (DPPC) i l'1,2-dimiristoil-sn-glicero-3-fosfatidilcolina (DMPC). La tensió superficial d'equilibri i dinàmica de cadascun dels tensioactius, fosfolípids i algunes de les seves mescles binàries s'han estudiat fent servir la tensiometria de bombolla en condicions d'àrea constant i a àrea polsant. A més, les densitats superficials a la interfase aire/aigua de les monocapes pures i mixtes s'han determinat mitjançant l'espectroscòpia d'infraroig de reflexió- absorció (IRRAS). S'ha observat un sinergisme (o antisinergisme en un dels casos) en la tensió superficial tant a l'estat estacionari com en condicions dinàmiques, així com efectes sinèrgics en l'adsorció de les dispersions mixtes estudiades. L'increment d'adsorció detectat amb l'IRRAS i la major disminució de la tensió superficial tant a l'estat estacionari com en condicions dinàmiques, evidencien una forta miscibilitat i l'existència d'interaccions atractives netes entre els dos components a la monocapa mixta.

Article III

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Propietats dinàmiques a la interfase aire/aigua de les mescles formades per un tensioactiu catiònic derivat del diacilglicerol d'arginina i un fosfolípid

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Resum

En aquest article s'han estudiat les interaccions binàries superficials del clorhidrat de l'1,2-dimiristoil-rac-glicero-3-O-(N^{α}-acetil-L-arginina) (1414RAc) amb el fosfolípid 1,2-dipalmitoil-sn-glicero-3-fosfatidilcolina (DPPC) en 0.1 M de clorur sòdic. El compost 1414RAc és un nou tensioactiu monocatiònic que presenta aplicacions potencials com a agent antimicrobià, és biodegradable i la seva toxicitat és inferior a la d'altres tensioactius catiònics comercials. El fosfolípid DPPC s'ha emprat com a component de models de membrana. S'ha avaluat la tensió superficial dinàmica de les dispersions aquoses de 1414RAc/DPPC, injectades a la subfase salina, per tensiometria,. La formació de monocapes dels sistemes binaris és sempre més ràpida respecte de l'adsorció de DPPC i, sorprenentment, la reducció de la tensió superficial és més ràpida i s'aconsegueixen valors més baixos de tensió superficial a concentracions de tensioactiu inferiors a la seva concentració micelar crítica (cmc). Les propietats reològiques dilatacionals a la interfase aire/aigua de les monocapes mixtes de deposició s'han determinat a partir del mètode oscil·latori amb la balança de Langmuir. L'efecte de la fracció molar de tensioactiu en els paràmetres reològics de les monocapes mixtes 1414RAc/DPPC s'han estudiat a amplituds relatives de deformació del 5% i a una freqüència de 50mHz. La viscoelasticitat de la monocapa presenta un comportament de mescla no ideal amb predomini de les propietats del tensioactiu. Aquest comportament no ideal és atribuït al predomini de les interaccions electrostàtiques.

Article IV

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Vesícules cataniòniques formades per tensioactius derivats d'arginina i la sal monosòdica de l'àcid 1,2-dipalmitoil-sn-glicero-3-fosfatídic

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Resum

En aquest article s'aporta informació de les mescles formades per tensioactius derivats d'arginina, tant monoacilats com diacilats, i un fosfolípid aniònic diacilat (sal monosòdica de l'àcid 1,2-dipalmitoil-sn-glicero-3-fosfatídic, DPPA). Aquestes mescles formen part de la família de mescles cataniòniques pseudo-tri- i pseudo-tetra-catenàries, respectivament. S'ha mesurat la dimensió de les vesícules i el potencial-ζ a diferents fraccions molars de mescla. També s'ha obtingut informació addicional respecte al grau d'associació del contraió, la dimensió de vesícula i la seva integritat a partir de mesures de potencial (elèctrode selectiu d'ions sodi), Cryo-TEM i SAXS. L'addició de tensioactius carregats positivament al fosfolípid DPPA provoca un increment en la dimensió de les vesícules. A més, el potencial-ζ presenta diferents tendències en funció de l'acidesa del medi emprat. En medi àcid, els valors del potencial-ζ s'aproximen progressivament a zero amb l'addició del tensioactiu derivat d'arginina. Sorprenentment, en aigua, el potencial-ζ presenta valors més negatius. Aquests resultats han estat discutits en termes de variacions del grau d'associació del contraió i de les dimensions de les vesícules.

Article V (Manuscrit)

Tensioactius derivats de diacilglicerols d'arginina. Propietats biològiques de les formulacions cataniòniques

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Resum

En aquest article s'ha assajat una estratègia de formulació com a vesícules cataniòniques amb l'objectiu de reduir la citotoxicitat dels tensioactius derivats dels diacilglicerols d'arginina 1414RAc i 1212RAc. S'ha estudiat el comportament d'aquests tensioactius purs així com el de les seves formulacions amb fosfatidilglicerol (PG), com a mescles cataniòniques pseudo-tetra-catenàries, i amb el fosfolípid fosfatidilcolina (PC), com a mescles catiòniques emprades com a control. La formulació tensioactiu/PG mostra una millora important en la selectivitat respecte a la del tensioactiu pur, conservant la seva activitat antimicrobiana en el bacteri S. Aureus i disminuint fortament la seva activitat hemolítica. Aquests resultats constitueixen una base sòlida per, mitjançant la formulació de sistemes cataniònics, reduir la citotoxicitat dels tensioactius, obrint així possibles aplicacions clíniques per tractaments de malalties infeccioses.

Aquests estudis han donat lloc a la sol·licitud d'una patent amb **Ref.: ES1641.261**, registrada el 13 de novembre de 2009 pel CSIC (veure l'*Apèndix I*).

Diacyl glycerol arginine-based surfactants: Biological properties of catanionic formulations

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Abstract

In this paper we report on a catanionic vesicles-based strategy to reduce the cytotoxicity of the diacyl glycerol arginine-based synthetic surfactants 1414RAc and 1212RAc. The behaviour of these surfactants were studied either as pure components or after their formulation as *pseudo-tetra-chain* catanionic mixtures with phosphatidylglycerol (PG) and as cationic mixtures with phosphatidylcholine (PC), used as control. The surfactant/PG formulation selectivity showed a significant improvement: the antimicrobial activity on *S. aureus* was maintained, concomitant to a strong decrease of hemolytic activity. The results constitute a proof of principle of our formulation strategy which reduces the cytotoxicity of many surfactants, opening their possible applications on clinical settings for treatment of infectious diseases.

Keywords: Amino acid-based surfactant, Antimicrobial agent, Phosphatidylglycerol, *Pseudo-tetra-chain* catanionic mixtures, *Acinetobacter baumannii, Staphylococcus aureus,* Hemolysis.

1. Introduction

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The interfacial and self-assembly properties of surfactants in aqueous environments have been investigated for many decades because of their basic interest in physical chemistry, biophysics and material science, as well as due to their enormous practical relevance (Jönsson B et al., 1998; Evans DF et al., 1999). Surfactants are used as additives in pharmaceutical and dermatological formulations. Thus, the concentration used in commercial formulations requires avoidance of adverse side-effects likes irritation and/or damage to skin, eyes, and mucoses. Therefore, developing new non-irritant biocompatible surfactants is of great interest.

Amino acids are not only essential components of living organisms, but also appealing raw materials for biocompatible surfactants. Acyl amino acids are particularly attractive because exhibit antibacterial activity (Infante MR et al., 1985; Xia J et al., 1995). In order to modulate their properties, a large number of N-acyl amino acids have been synthesized and tested for their surfactant properties and interactions with artificial membranes (Miyagishi S et al., 1989; Sanson A et al., 1987; Epand RF et al., 1998; Peypoux F et al., 2004). According to the information gathered through these experiments, N-fatty acid acylated amino acids deserve their classification into a separate class of lipids worthy of in depth.

In the last two decades, a number of papers addressing the synthesis and the study of properties of biocompatible cationic amino acid-based surfactants of different structures have been published (Infante MR et al., 1985; Seguer J et al., 1994; Allouch M et al., 1996; Perez L et al., 1996; Pegiadou S et al., 2000; Pinazo A et al., 2009). Acyl-glycerol amino acid conjugates constitute a class of specific lipoamino acids surfactants sharing properties with glycerides and phospholipids. They consist of one or two aliphatic chains and one amino acid polar head, linked together through ester bonds in the glycerol backbone (Moran C et al., 2001; 2002). The arginine glyceride conjugates were obtained in the quest to find improved and cheaper soft antimicrobial surfactants (Chart 1) (Perez L et al., 2004a; 2004b). Their use in food and cosmetic applications as well as topical disinfectants has been studied in Benavides T et al., 2004 and Vinardell MP et al., 2008. These compounds consist in a glycerol backbone esterified at positions 1 and 2 with aliphatic acid chains and with the carboxylic group of an arginine residue at position 3 (Chart 1) (Perez L et al., 2004a; 2004b; Perez N et al., 2005). The modulation of their physicochemical properties, hence of their

associated biological activities (Pinazo A et al., 2004; Lozano N et al., 2008), is achieved by tuning their charge-hydrophobicity balance, either by acetylation of the α-amino group or by variation of the length of the hydrophobic chains, giving rise to a wide collection of analogs with diverse behaviour in biological systems.

In an attempt to explore the potential applications of these arginine-based surfactants into applications close to clinical settings, a set of four arginine-based surfactants, 1,2-dimyristoyl-*rac*-glycero-3-O-(N^{α}-acetyl-L-arginine) hydrochloride (1414RAc), 1,2-dilauroyl-*rac*-glycero-3-O-(N^{α}-acetyl-L-arginine) hydrochloride (1212RAc), as well as the non-N^{α}-acetylated versions (1414R and 1212R, respectively) were synthesized, with a purity higher than 99%. Then they were assayed both against two pathogenic bacteria (*Acinetobacter baumannii* and *Staphylococcus aureus*) and against sheep erythrocytes, as a toxicity model for mammalian cells.

Catanionic vesicles have been extensively used as promoters for chemical and enzymatic reactions and, more recently, as a way to improve the delivery of either DNA or of other small size drugs into eukaryotic cells (Bramer T et al., 2007). The surfactant ions used in the preparation of catanionic mixtures can be *single* or *multiple chain*–ones. Mixing oppositely charged *single chain* surfactant ions gives *pseudo-double-chain* catanionic surfactants (Kamenka N et al., 1992; Silva BFB et al., 2005; Bonincontro A et al., 2006). However, a large amount of the literature deals with *single-double chain* surfactants, forming *pseudo-triple-chain* catanionic mixtures (Marques EF, 2000; Marques EF et al., 2002; 2006; 2008; Burgo P et al., 2007; Bonincontro A et al., 2008). Recently, Brito RO et al., 2009 has firstly reported on the relation between the acute toxicity and hemolytic activity/potential ocular irritancy of two types of *pseudo-triple-chain* catanionic vesicles using lysine- and serine-based surfactants. In that work, the micellization behavior of the pure surfactants was related with the spontaneous vesicle formation of the mixtures that occurs only for excess cationic surfactants.

The present work reports on the biological activity of two *pseudo-tetra-chain* catanionic systems based on the aforementioned monocationic arginine-based surfactants and phosphatidylglycerol (PG) formulations which fall into the category of *double-double chain* surfactants forming *pseudo-tetra-chain* catanionic mixtures (Caria A et al., 1996; Karukstis KK et al., 2003; Lozano N et al., 2009). The mixed

vesicle formation from individual cationic and anionic vesicles took place at every surfactant mole fractions assayed, regardless of the net charge of the catanionic vesicles. Increased selectivity of the biological selectivity against Gram-positive bacteria was observed for the PG formulations, mostly due to the strong decrease of hemolytic activity compared to the individual arginine-based surfactant.

2. Materials and methods

2.1. Materials

The monocationic (1414RAc, 747.5 g/mol; 1212RAc, 691.4 g/mol) and dicationic (1414R, 741.9 g/mol; 1212R, 685.8 g/mol) diacyl glycerol arginine-based surfactants were synthesized according to Perez L et al., 2004a and 2004b. Their purity, higher than 99%, was checked by elemental analysis and high-performance liquid chromatography (HPLC); see Table 1 for the analytical data. Phosphatidylglycerol (PG) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Sigma with a purity of 99% and used as received. Polymyxin E sulfate was purchased from Sigma (St. Louis, MO). Sodium chloride (>99.5% by weight) was purchased from Fluka. Water was obtained using a Synergy Ultrapure water system from Millipore (resistivity of 18.2 MΩ cm). HPLC-grade ethanol was supplied by Panreac (water ≤ 0.2%).

2.2. Preparation of vesicles

Previous to any manipulation, the surfactants and the phospholipids were sterilized by solubilization in ethanol and further evaporation in a cell culture flood hood under sterile conditions. Stock dispersions at a total concentration of 10 mM, of either pure surfactant, or phospholipids, or surfactant/phospholipid formulations at surfactant mole fractions of 0.2 and 0.8 were prepared by weight, with further hydration in water. All dispersions were shaken vigorously at room temperature for 5 min, and then sonicated at 50 ºC for 15 min to promote the formation of uniform vesicles. Lower concentrations were obtained by dilution from stock dispersions. Vesicle formation was checked by cryogenic transmission electron microscopy.

2.3. Preparation of erythrocyte suspensions

To assess cytotoxicity, the hemolytic activity of the surfactants was tested as described below. Sheep erythrocytes from defribinated blood from Biomedix (Madrid, Spain) were washed twice and resuspended in Hanks medium (136 mM NaCl; 4.2 mM Na_2HPO_4; 4.4 mM KH_2PO_4; 5.4 mM KCl; 4.1 mM $NaHCO_3$, pH 7.2). Erythrocytes were resuspended in the same buffer at 2×10^7 erythrocytes/ml and incubated with the surfactant at concentrations ranging from 0 to 150 μM for 4 h at 37 ºC. All the samples were made by triplicate. Afterwards, cells were centrifuged, and the supernatant containing the released hemoglobin was collected, transferred into a 96 microwell plate and measured in a Bio-Rad 680 (Hercules, CA) microplate Reader at 550 nm. Full hemolysis was considered as that obtained with 0.1% Triton X-100. HC_{50} is defined as the surfactant concentration inducing 50% of hemolysis and was calculated using Sigma Plot, version 9.0.

2.4. Antimicrobial activity

The microorganisms used in this study were *Acinetobacter baumannii* ATCC 19606, *Staphylococcus aureus* CECT 240, *Bacillus Cereus LWLI* and *Brochothrix thermosphata CECT 847*. The antimicrobial activity of the surfactants was tested in 96 microwell plates by inhibition of bacterial growth in Müeller- Hinton Broth (Oxoid) at 37 ºC for 18 h with an initial inoculum of A_{600} = 0.005 (Saugar et al. 2006). Afterward, growth was measured by turbidimetry at 600 nm in a model 680 microplate reader (Bio-Rad Laboratories, Hercules. CA). Surfactants were assayed in a range of concentration from 0-250 μM. Minimum inhibitory concentration (MIC) was defined as the minimal concentration that inhibits bacterial growth measured after 24h. A suffix (MIC_x) stands for the concentration capable to inhibit the bacterial growth by a percentage of the suffix value under the same conditions (Saugar JM et al., 2006).

Data were expressed as the mean ± SD from triplicate samples. HC_{50}, MIC_{50}, and MIC_{90} were calculated with the Sigma Plot software 9.0.

Synergy with polymyxin E (PXE) was evaluated using the checkerboard method and measured by calculation of the fractional inhibitory concentration, (FIC), defined in Equation (1).

$$FIC = \left(\frac{MIC_{surf+PXE}}{MIC_{surf}} \right) + \left(\frac{MIC_{surf+PXE}}{MIC_{PXE}} \right) \quad (1)$$

FIC values were interpreted as synergism (< 0.5), additively (0.75-1.5) or antagonism (> 4).

3. Results and discussion

3.1. Hemolytic activity

A major concern about new antimicrobial agents is whether they fail to fulfill specificity requirements in order to spare eukaryotic host cells while killing the microorganism targeted. For surfactants this is usually reflected in an unspecific lysis. This undesirable lysis of eukaryotic cells can be easily measured by hemolysis assays, based on the release of hemoglobin from erythrocytes. To this aim, the diacyl glycerol arginine-based surfactants were incubated with sheep erythrocytes. As the hemolysis was linearly proportional to the erythrocyte concentration up to 5×10^7 erythrocytes/ml, the assay was routinely carried out with 2×10^7 erythrocyte/ml. Results are shown in Figure 1. The hemolysis by monocationic and dicationic arginine-based surfactants showed a sigmoidal curve against the surfactant concentration until 80 μM for monocationic and 100 μM for dicationic, concentrations beyond these values did not increase the plateau value obtained. The $HC_{50,}$ surfactant concentration causing 50% of hemolysis, were of 14 ± 2.0, 8.6 ± 2.0, 58.2 ± 7.0, and 160 ± 5.0 μM for 1414RAc, 1212RAc, 1414R, and 1212R, respectively. Full hemolysis was achieved at 80 μM for monocationic and at 120 μM for dicationic surfactants.

The hemolytic behavior of the dicationic surfactants depend on the number of carbon atoms in the hydrophobic chains. Thus the surfactant with a 14 carbon length hydrophobic chains resulted with the highest hemolysis. When the charge of the polar head or the length of acyl chains was decreased, a stronger membrane

permeabilization was obtained. The results show the crucial role played by the charge of the polar head and the fatty chain length (hydrophobicity) in modulating the membrane activity.

3.2. Antimicrobial activity and polymyxin assays

Recently published results for diacyl glycerol arginine-based surfactants (Perez L et al., 2004a; 2004b), reported a moderated antimicrobial activity against a limited number of Gram-positive and Gram-negative bacteria. In order to get a better knowledge on the antimicrobial properties of these compounds, extended bactericidal activity was extended into four human pathogenic bacteria: the Gram-negative *Acinetobacter baumannii* and three Gram-positive: *Staphylococcus aureus, Bacillus cereus and Brochothrix thermosphacta*. MIC_X for the four of them were compiled in Table 2. Even at the highest concentration tested (250 μM), none of the surfactants tested reached full growth inhibition. Nevertheless MIC_{50} values inside the range were obtained for the monocationic surfactants 1414RAc (78.6 ± 4.2 μM for *A. baumannii* and larger than 250 μM for *S. aureus*), whereas for 1212RAc MIC_{50}s values were 44.4 ± 2.6 μM for *A. baumannii* and of 250 μM for *S. aureus*. For the two dicationic surfactants 1414R and 1212R, MIC_{50} were higher than 250 μM for the four bacteria tested.

Unexpectedly, for the dicationic compounds (1414R and 1212R) the substitution of the acetyl group for an amino group changes the antimicrobial activity dramatically. To understand the molecular basis of the differences in antimicrobial performance of monocationic and dicationic diacyl glycerol surfactants, some relevant insight may be formulated based on theoretical viewpoints and on our experimental observations. At one hand, in aqueous media the dicationic surfactants can dissociate in several species following an acid-base equilibrium (Pinazo A et al., 2004). They behave as monocationic depending on pH. In our studies, at culture pH media (~7), the α-amino group of the amino acid is deprotonated rendering monocationic molecules. Then the structure of these molecules, in regard to charges, is similar to that of the monocationic because only the positive charge of the guanidine group remains on the molecule but their antimicrobial activity is much lower (Table 2). In general, the antimicrobial activity is attributed largely to the net positive charge, our results

suggest that the relationship between charge and antimicrobial activity appears to be more complex (Papanastasiou EA et al., 2009; Valko EI et al., 1945). Our results agree with the complexity of physicochemical factors involved in the final antimicrobial outcome, described in the literature such as adsorption, hydrophobicity, aqueous solubility and diffusion in the test medium, for example (Denyer SP, 1995; Russell AD, 1995; Vieira DB et al., 2006).

The most likely hypothesis to account for the privileged lysis of the erythrocyte membrane over the bacterial one, obeys to the absence in these cells of external barriers (outer membrane, peptidoglycan wall), present in bacteria that prevents access of the monomer form, hence to the micellar form as well, of surfactant into the cytoplasmic membrane. In order to test this hypothesis, we carried out experiments of combination of polymyxin E (PXE) plus surfactants by the chequerboard method. Polymyxins are cationic antibiotic peptides active on Gram-negative bacteria. Its mode of action involves as an essential step it is binding to and permeabilization of the outer membrane, achieved by its privileged interaction with the anionic lipopolysaccharide major component of the external leaflet of the outer membrane (Landman D et al., 2008). The results on bacterial viability in presence of PXE and surfactant are compiled in Figure 2. At PXE concentrations higher than 0.5 μM, this peptide sensitizes the bacteria to lysis by surfactants at PXE concentrations scarcely active by the single surfactant by itself. Synergy with polymyxin E (PXE) was evaluated using the fractional inhibitory concentration, (FIC), defined by Equation (1). In fact, there is a strong positive synergy between both components, although we did not reach a real MIC value, taken the highest surfactant concentration tested as MIC, a virtual FIC calculation for 1414RAc and 1212RAc were 0.49 and 0.37, respectively. The positive synergism could be attributed to the formation of mixed aggregates with an undetermined stoichiometry.

The dicationic surfactants were considerably less hemolytic than the monocationic ones, although their poor bactericidal activity precludes their use for further experiments.

3.3. Catanionic vesicles

Taking into account the results exposed in the previous section and according to the preferential activity on erythrocytes, the most likely option to improve the therapeutic index obtained for surfactants as single components was to tune its controlled delivery. We formulated catanionic vesicles with the monocationic surfactants and the anionic phospholipid phosphatidylglycerol (PG) and cationic vesicles with the zwitterionic phospholipid (DPPC), used as control. Both were formulated at two different surfactant/phospholipid molar ratios of 0.2 and 0.8. In PG-based formulations, the diacyl arginine compounds formed positively or negatively charged vesicles, depending on the major component in the mixture (Lozano N et al., 2009).

As expected, once corrected for the real concentration of the surfactant, bactericidal and hemolytic activity of the DPPC-based formulations resulted in no significant differences with respect to the surfactant alone both for bactericidal and hemolytic activities (data not shown).

In the formulations with PG, when catanionic vesicles have negative net charge, activity versus *A. baumannii* falls slightly. And it was maintained when incorporated into positively charged vesicles. In contrast there was a significant improvement of the selectivity for the PG-formulation at surfactant mole fraction of 0.2 on *S. aureus* (Table 3), correlated with a strong decrease of hemolytic activity.

4. Conclusions

The use of catanionic vesicles with a net negative charge results in a reduction of both hemolytic and Gram-negative lysis activities while increasing against Gram-positive bacteria. Cationic vesicles as formulated with PC, however, results in negligible effect on the surfactant activities, that is, high hemolysis and activity against Gram-negative bacteria and moderate activity against Gram-positive. From these results, it is inferred that hemolysis and Gram-negative bacteria followed the same trend with the formulation effect. The reduction of hemolysis and activity against Gram-negative bacteria can be understood by considering the shielding effect of the formulation. These catanionic vesicles negatively charged will have a reduced affinity for negatively charged surfaces and conversely a higher affinity for positively or zwitterionic charged surfaces. The same effect could be responsible for the increased activity against Gram-positive bacteria. Although the surface charge of the bacteria is

also negative, the release of active surfactant imposed by the formulation will be probably under the form of neutral ionic pairs, through the peptidoglycan layer and while the cationic surfactant may still access the plasma membrane, the insertion of the negatively charged phospholipid will be probably more difficult.

Our results might be considered as a proof of principle of a strategy which may reduce the toxicity of many surfactants, opening possibilities into clinical applications. In these catanionic systems, the delivery rate was strongly dependent on the overall physical behaviour of the system and on the driving forces leading to formation of the aggregate, not only in vitro but also in the presence of biological fluids. Experiments aiming to improve these initial results testing more complex formulations of surfactant with two classes of phospholipids simultaneously, as well as improved new analogs are currently under progress.

Also, this study suggests that synthetic analogs are excellent candidates for developing new surfactants with tunable, well-defined properties for medical and biotechnological applications.

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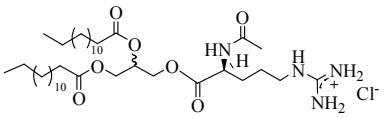
Chart 1. Chemical structures of the cationic diacyl glycerol arginine-based surfactants tested in this work.



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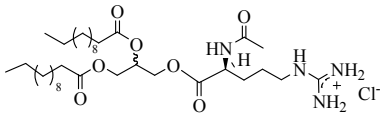

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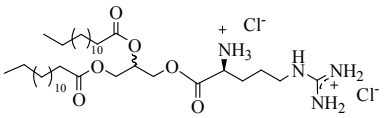

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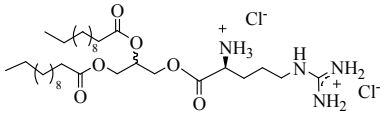

1212R

Table 1. Analytical data for the cationic diacyl glycerol arginine-based surfactants.

Surfactant	*Molecular formula (molecular weight, g/mol)*	*HPLC retention time (min)*	*Elemental analysis, calculated with 1.0[a] and 2.0[b] mol H_2O*		
			C (%)	H (%)	N (%)
1414RAc	$C_{39}H_{75}N_4O_4Cl$ (747.5)	24.0	61.19[a]	10.14[a]	7.32[a]
1212RAc	$C_{35}H_{67}N_4O_4Cl$ (691.4)	20.6	59.24[a]	9.73[a]	7.9[a]
1414R	$C_{37}H_{74}N_4O_6Cl_2$ (741.9)	19.3	57.71[b]	10.03[b]	7.20[b]
1212R	$C_{33}H_{66}N_4O_6Cl_2$ (685.8)	18.0	56.33[a]	9.67[a]	7.96[a]

Table 2. MIC_{50} and MIC_{90} (μM) for the cationic diacyl glycerol arginine-based surfactants.

Bacteria	***1414RAc***		***1212RAc***		***1414R***		***1212R***	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Acinetobacter baumannii ATCC 19606	78.6 ± 4.2	> 250	44.4 ± 2.6	> 250	> 250	ND	> 250	ND
Staphylococcus aureus CECT 240	> 250	ND	250	> 250	> 250	ND	> 250	ND
Bacillus cereus LWLI	> 250	ND	> 250	ND	> 250	ND	> 250	ND
Brochothrix thermosphacta CECT 847	> 250	ND	> 250	ND	> 250	ND	> 250	ND

Table 3. Inhibition of biological activity (% of control) by surfactants with or without inclusion in PG vesicles.

Cell assayed	*[surfactant], μM*	***1414RAc***		***1212RAc***	
		alone	*PG*[a]	*alone*	*PG*[a]
Acinetobacter baumannii ATCC 19606	0		2.8 ± 0.9		2.8 ± 0.9
	20	9.7 ± 1.8	0.9 ± 3.1	18.9 ± 3.3	0.8 ± 2.6
	80	49.6 ± 1.0	38 ± 0.8	52.4 ± 0.5	49.8 ± 1.2
Staphylococcus aureus CECT 240	0		3.3 ± 1.5		3.3 ± 1.5
	20	7.1 ± 1.6	52.2 ± 1.0	0.0 ± 0.0	58.4 ± 1.0
	80	15.3 ± 9.9	0.5 ± 1.7	6.6 ± 1.3	7.6 ± 1.6
Sheep erythrocytes	0		0.0 ± 0.0		0.0 ± 0.0
	20	68.5 ± 3.8	0.0 ± 0.0	82.3 ± 4.6	1.9 ± 0.9
	80	84.6 ± 8.0	78 ± 3.5	96.2 ± 4.8	73 ± 1.0

[a] *[PG] μM=100μM - [surfactant] μM*

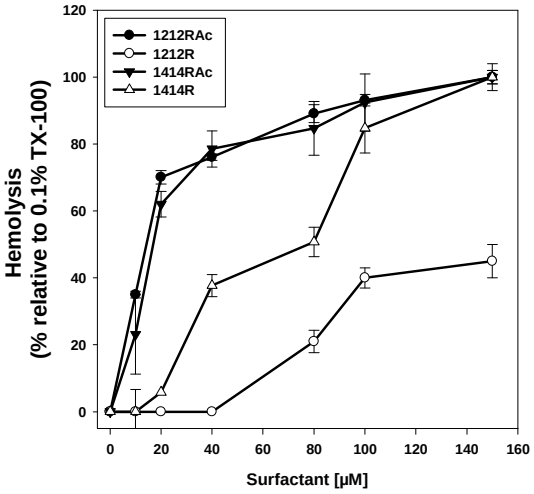

Figure 1. Hemolytic activity of the cationic diacyl arginine-based surfactants tested on sheep erythrocytes. Erythrocytes were incubated at 37 ºC with different concentrations of surfactants for 4h; the release of hemoglobin into the medium was monitored at 450 nm and referred as that obtained with 0.1% TX-100.

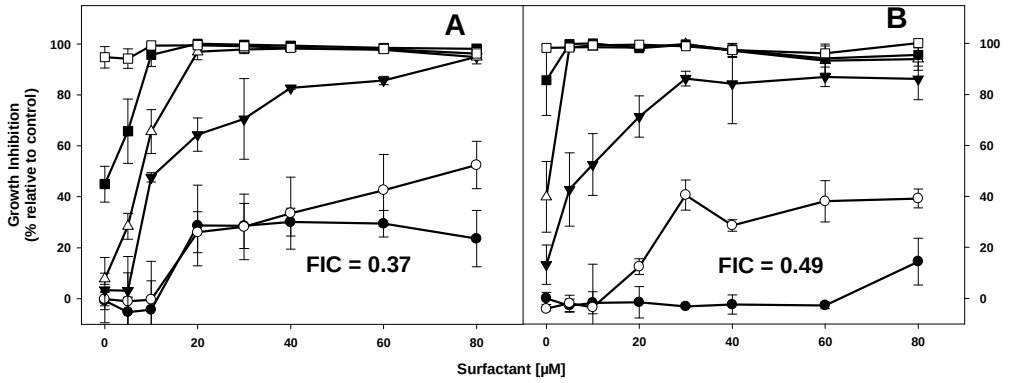

Figure 2. Synergism between surfactant and polymyxin E (PXE) on *Acinetobacter baumannii.* The inhibition of bacterial growth was carried out at different concentrations of both polymyxin E and either 1212RAc (Panel A) or 1414RAc (Panel B) by the chequerboard method, and their respective fractional inhibitory concentration (FIC) values, calculated as stated in Materials and Methods were shown inside the panel. PXE concentrations (μg/ml): 0.0, (closed circles); 0.5 (open circles), 1.0 (closed triangles down), 1.5 (open triangles up), 2.0 (closed squares), 3.0 (open squares).