Amino Acids (2007) 33: 459–462
DOI 10.1007/s00726-006-0447-2
Printed in The Netherlands

Amino Acids

Disturbance of erythrocyte lipid bilayer by amino acid-based surfactants

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Received July 14, 2006
Accepted September 20, 2006
Published online November 9, 2006;

Summary. In an attempt to increase our knowledge regarding the mechanisms of surfactant membrane interaction, we studied the action of several anionic and cationic amino acid-based surfactants on membrane fluidity using fluorescence anisotropy. Anisotropy measurements demonstrated that almost all of the surfactants studied disturbed the external region of the erythrocyte membrane without affecting the core of the bilayer. How the physico-chemical properties and structure of these compounds affect dynamics of the lipid bilayer is discussed in detail.

Keywords: Plasma membrane – Fluorescence anisotropy – Surfactants – Erythrocyte – Arginine – Lysine

Introduction

Surfactants, due to their surface and interface properties, are among the most versatile and frequently applied excipients in pharmaceutical, cosmetics, and technology-based industries. They are employed in large quantities every day on a worldwide scale as constituents of many different products (Paulsson and Edsman, 2001).

Since it is well known that surface-active compounds can adversely affect the environment, the biodegradability and biocompatibility of surfactants have become almost as important as their functional performance to the consumer. One interesting strategy to minimize their environmental effects is to synthesize new molecules with analogues structures to such natural compounds as lipo-amino acids.

Amino acid-based surfactants have attracted much interest as environmentally friendly surfactants because of their biodegradability, low aquatic toxicity, low hemolytic activity and their use of renewable sources of raw materials for their synthesis (Infante et al., 1997; Valivety et al., 1998). Our group has considerable experience in the synthesis of surfactants derived from amino acids. Indeed, we have recently developed new families of lysine and arginine-based surfactants (Seguer et al., 1994; Perez et al., 2002).

Both families have been widely studied in recent years in attempts to evaluate their potential risks for eye and skin irritation. Previous reports from our laboratory using in vitro methods revealed low toxicity when compared to conventional surfactants (Sánchez et al., 2004, 2006a; Martínez et al., 2006). Among these methods the red blood cell lysis assay, which quantifies adverse effects of surfactants on the cytoplasmatic membrane, is a specific in vitro tool for evaluating the acute irritant potential induced by surfactants or surfactant-containing preparations (Pape et al., 1999). However, the way surfactants interact with biological membranes is not clearly understood and different research groups have made great efforts to clarify the molecular processes involved in surfactant-induced cell membrane lysis (Hägerstrand and Isomaa, 1991; Vives et al., 1999), which is very closely related to surfactant toxicity. Because the human erythrocyte has no internal organelles and since it is the simplest cellular model obtainable, it is the most popular cell membrane system to study the surfactant-membrane interaction (Svetina et al., 2004).

To increase our knowledge regarding possible mechanisms of surfactant interactions with erythrocyte membranes, we investigated the actions of several amino acid-based surfactants on the alterations on membrane fluidity.

A better understanding of surfactant effect on membrane fluidity may assist in developing surfactants with enhanced selectivity, and in widening their range of applications.

Materials and methods

Materials

L-lysine monohydrochloride, L-arginine monohydrochloride, L-Lysine, caprylic acid, Tris(hydroxymethylmethyl) aminomethane, Sodium dodecyl sulphate (SDS), methanol, NaCl; Na_2HPO_4 and the bases NaOH, LiOH and KOH were purchased from Merck (Darmstadt, Germany). Fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,4-hexatriene p-toluenesulfonate) were purchased from Molecular Probes (Eugene, OR, USA).

Surfactants tested

Two new classes of amino acid based surfactants were investigated in this study:

a) Three different cationic N^{α}-acyl arginine derivatives were tested: N^{α}-lauroyl-L-arginine methyl ester (LAM), N^{α}-myristoyl-L-arginine methyl ester (MAM) and a mixture of different N^{α}-acyl-L-arginine methyl derivatives LAM and MAM were synthesized in our laboratory as previously described (Infante, 1988). The surfactant mixture was synthesized for the first time in our laboratory using a fatty acids mixture from coconut extract (caprylic acid, 5.84%, capric acid, 4.62%, lauric acid, 53.04%, myristic acid, 18.12%, palmitic acid, 8.68%, stearic acid, 9.47%). Our procedure involved the introduction of fatty acid residues as acid chlorides (Sims and Fioriti, 1975; Martínez et al., 2006).
b) Five anionic surfactants, with counterions of different chemical natures from the type N^{α},N^{ε}-dioctanoyl lysine were tested: lysine salt (77KK), tris(hydroxymethyl) amino-methane salt (77KT), sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP). They were synthesized in our laboratory as previously described (Sánchez et al., 2006a, b).

The physicochemical properties of the surfactants are shown in Table 1.

Table 1. Physico-chemical properties of the surfactants studied

Surfactant	MW[a]	CMC[b] (10^3 µg/ml)	Charge	No. of alkyl chains	Length of alkyl chain
77KK	545.7	1.8	Anionic	2	C8
77KT	519.7	2.3	Anionic	2	C8
77KP	437.6	1.9	Anionic	2	C8
77KS	421.5	3.0	Anionic	2	C8
77KL	405.6	2.9	Anionic	2	C8
LAM	406.6	2.2	Cationic	1	C12
MAM	434.7	0.7	Cationic	1	C14
CCR	418.4	1.7	Cationic	1	Variable (C6–C16)
SDS	288.4	2.3	Anionic	1	C12
HTAB	598.4	0.4	Cationic	1	C16

[a] Molecular weight
[b] Critical micellar concentration

Incubation media

Lysine based surfactants and SDS were dissolved in a PBS buffer. Arginine-based surfactant and HTAB were dissolved in NaCl 0.9% solution as the arginine-based surfactants in PBS solution are not soluble.

Preparation of erythrocyte suspensions

Human blood was obtained from the Blood Bank of the Hospital Clinic (Barcelona, Spain). The erythrocytes were washed three times in a phosphate buffer solution (PBS) containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.6 mM KH_2PO_4 in distilled water (pH 7.4; 300 mOsmol/l). The cells were then suspended at a cell density of 8×10^9 cell/ml.

Fluorescence emission anisotropy measurements

To determine cell membrane fluidity, DPH and TMA-DPH fluorescent probes were selected. To carry out the steady-state fluorescence anisotropy measurements of the probes in treated and untreated red blood cells, the erythrocyte suspensions (hematocrit of 0.01%) in PBS or NaCl were labeled with the fluorescent dyes (final concentration in samples 10^{-6} M) at room temperature for 1 hour. Steady-state anisotropy measurements were carried out with an AB-2 spectrofluorometer SLM-Aminco using polarizers in the L configuration in a quartz cuvette under constant stirring at room temperature. Samples were illuminated with the linearly (vertically or horizontally) polarized monochromatic light ($\lambda_{ex} = 365$ nm) and the emitted fluorescence intensities ($\lambda_{em} = 425$ nm) parallel or perpendicular to the direction of the excitation beam (slit-widths: 8 nm) were recorded. Fluorescence anisotropy (r) was calculated automatically by software provided with the instrument, according to:

$$r = (I_{vv} - I_{vh}G)/(I_{vv} + 2I_{vh}G),$$

where I_{vv} and I_{vh} represented the components of the light intensity emitted, respectively, in parallel and in perpendicular to the direction of the vertically polarized excitation light. A factor $G = I_{hv}/I_{hh}$ was used to correct the inequality of the detection beam to horizontally and vertically polarized emission (Shinitzy and Barenholz, 1978).

Statistical analysis

All anisotropy fluorescence values were expressed as the means ± standard error (SEM) of at least 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) and Student's t-test using the SPSS® software (SPSS Inc. Chicago, IL, USA).

Results and discussion

One of the important parameters relating to the structure and functional state of the cell membrane is membrane fluidity (Shinitzky and Barenholz, 1978). To determine whether membrane fluidity was modified by surfactant treatment, the fluorescent probes DPH and TMA-DPH were incorporated into the membranes of erythrocytes. Knowledge of the probe's location is essential for a consistent interpretation of the observed fluorescence polarization. DPH is a hydrophobic molecule that is incorporated in the region near the center of the bilayer. Differences in the fluorescence polarization of this probe may reflect a direct effect on the motion of the lipid molecules in the core region of the bilayer (Kaiser and London, 1998). The TMA-DPH molecules are believed to accumu-

Table 2. Steady-state fluorescence anisotropy of fluorescence probes DPH and TMA-DPH incorporated into erythrocyte membranes

	Concentration (μg/ml)	(r) DPH (mean ± SE)	(r) TMA-DPH (mean ± SE)	(r) TMA-DPH reduction (%)
Samples in PBS				
Untreated cells	–	0.2393 ± 0.0076	0.2305 ± 0.0062	–
77KK	300	0.2439 ± 0.0099	0.1778 ± 0.0084**	23
77KT	600	0.2190 ± 0.0017	0.1304 ± 0.0069**	43
77KP	270	0.2368 ± 0.0107	0.1717 ± 0.0094**	26
77KS	180	0.2485 ± 0.0139	0.2215 ± 0.0119	4
77KL	430	0.2444 ± 0.0165	0.2266 ± 0.0113	2
SDS	20	0.2372 ± 0.0077	0.2060 ± 0.0068*	11
Samples in NaCl				
Untreated cells	–	0.2152 ± 0.0096	0.2186 ± 0.0030	–
LAM	30	0.1988 ± 0.0060	0.1747 ± 0.0020**	20
MAM	10	0.1938 ± 0.0073	0.1801 ± 0.0057**	18
CCR	25	0.1972 ± 0.0040	0.1872 ± 0.0074**	14
HTAB	5	0.2363 ± 0.0082	0.2173 ± 0.0061	3

Anisotropy measurements are represented by r values
* Significantly different when compared to values obtained for untreated cells (Student's t-test, $p < 0.05$)
** Significantly different when compared to values obtained for untreated cells (Student's t-test, $p < 0.01$)

late and remain almost exclusively in the outer leaflet of the cell membrane, since their polar heads (trimethylammonium groups) are anchored at the lipid-water interface while hydrocarbon moieties enter the lipid part of the membrane. Therefore, fluidity assessed by steady-state fluorescence with different probes reveals the arrangement and mobility of membrane components at different regions of the bilayer (Mély-Goubert and Freedman, 1980).

Given that fluorescence measurements are very sensitive to medium turbidity, which can result in dispersion, the experiments were carried out in isosmotic mediums to avoid hemolysis. The surfactant concentrations chosen for assessing the membrane fluidity were selected according to their HC_{50} (hemolytic concentration inducing 50% of hemolysis) values determined previously (Sánchez et al., 2004; Martínez et al., 2006). The effects exerted by the surfactants on membrane fluidity, as measured by the fluorescent probes, are shown in Table 2. Low anisotropy values (r) correspond to increased fluidity of cell membrane. None of the surfactants tested altered the core of the membrane as demonstrated by the DPH anisotropy values. However, the arginine derivatives (LAM, MAM and CCR), some lysine derivatives (77KK, 77KT and 77KP) and SDS modified the erythrocyte membrane fluidity on the external region of the membrane as demonstrated by the reductions in anisotropy TMA-DPH values.

From our findings, it is obvious that the fluidity of the erythrocyte membrane was modified by treatments with all of the arginine derivative surfactants, 77KK, 77KT, 77KP ($p < 0.01$) and SDS ($p < 0.05$). Our fluorometric experiments clearly showed that the perturbation caused in membranes by the amino-acid based surfactants incorporated therein was higher in the polar region of erythrocytes membranes and decreased with depth of incorporation, as demonstrated by TMA-DPH and DPH anisotropy values, respectively. A possible explanation for this fact is that the most common phospholipids in the bilayer are 16–18 carbons in length while the alkyl chain length of the tested surfactants was between 8 and 14 carbons. Therefore, these surfactants could not be incorporated more deeply into the membrane bilayer.

It is known that compounds containing counterions interact with biological and model membranes with different efficiencies (Kleszczynska and Sarapuk, 1998). This hypothesis is also supported by our results, specifically in the case of lysine derivative surfactants, which only differ in their counterions. The anisotropy data revealed that 77KK, 77KT and 77KP increased membrane fluidity whereas 77KS and 77KL had no effect. The counterion is also implicated in the differences in the antihemolytic potency and the hemolytic activities of this class of surfactants. Although the mechanisms of action of various surfactants was evaluated in light of their physicochemical properties, the fact that minor changes in surfactant properties may cause dramatic alterations of membrane fluidity supports the idea that specific surfactant-lipid and surfactant-protein interactions should also be considered (Broring et al., 1989).

Acknowledgements

This research was supported by the Project PPQ-2003-01834 from MCE (Spain). Verónica Martínez holds a doctoral grant from the "Universitat de Barcelona" and Lourdes Sanchez holds a doctoral grant from "Unidad Asociada-CSIC" (Spain). The authors are grateful to Robin Rycroft for linguistic assistance.

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