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Binding and permeabilization of lipid bilayers by natural and synthetic 3-alkylpyridinium polymers

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ABSTRACT

Naturally occurring 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* are membrane-active compounds exerting a selective cytotoxicity towards non small cell lung cancer cells, and stable transfection of nucleated mammalian cells. In view of their possible use as chemotherapeutics and/or transfection tools, three poly-APS based synthetic compounds were tested on their activity using natural and artificial lipid membranes. The tested compounds were found to be very stable over a wide range of temperature, ionic strength, and pH, and to prefer the solid-ordered membrane state. Their membrane-damaging activity increases with the length of their alkyl chains and the degree of polymerization.

1. Introduction

Water-soluble polymeric 3-alkylpyridinium salts (poly-APS), isolated from crude extracts of the Mediterranean marine sponge *Reniera sarai*, belong to the large group of about 80 different biologically active 3-alkylpyridinium and 3-alkylpyridine compounds found in several sponges of the order Haplosclerida.[1–4] Poly-APS are polymers with the molecular weight of 5520 Da, corresponding to 29 *N*-butyl-3-butyl pyridinium units, in which the 3-alkyl chain is bound head-to-tail to the nitrogen of the adjacent subunit.[5] They exert a very broad spectrum of biological activities, which is not surprising given that the activities of 3-alkylpyridinium compounds, as well as their potency, increase with the degree of polymerization. At concentrations above 0.23 mg/mL, poly-APS form large supramolecular aggregates with an average hydrodynamic radius of 23 ± 2 nm.[5] This behavior resembles that of other structurally related cationic detergents.[6]

At concentrations below 0.5 μg/mL, poly-APS have been described as being hemolytic, selectively cytotoxic against non small cell lung cancer cells, and to inhibit the enzyme acetylcholinesterase (AChE), the growth of marine bacteria, and the settlement of marine zoo- and phytoplankton on submerged surfaces (reviewed in[4]). Among the biological effects of poly-APS, their action on biological membranes is intriguing, as it can result in the formation of transient pores and stable transfection of nucleated mammalian cells with heterologous DNA, finding a possible use in gene therapy.[7] At higher concentrations, however (above 1 mg/mL) poly-APS can be toxic and lethal to rodents on intravenous application.[8]

In view of the possible use of poly-APS based synthetic compounds as transfection or chemotherapeutic agents, or as additives to environmental-friendly antifouling paints, several 3-alkylpyridinium oligomers and polymers (3-APS) have recently been synthesized, and their hemolytic, AChE-inhibitory, toxic, antibacterial, antifouling, antifungal, and transfection potential tested.[9–13] Among them APS12 and APS12-2, two large polymeric 1,3-dodecylpyridinium salts of 12.5 and 14.7 kDa molecular mass, were shown to be particularly interesting, as they are more potent and less toxic transfecting agents than the natural poly-APS.[11]

Previous studies have shown that the ability of poly-APS to form lesions in biological membranes can be explained, at least partially, by their surfactant-like characteristics and behavior in aqueous solutions. The hemolytic activity of poly-APS was analyzed and compared to that of the structurally-related monomeric cationic surfactants, cetylpyridinium chloride and cetyltrimethylammonium bromide. It was found that poly-APS induce the formation

Abbreviations: AChE, acetylcholinesterase; 3-APS, 3-alkylpyridinium salt; CH, cholesterol; CMC, critical micelle concentration; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; NSCLC, non small cell lung cancer cells; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPC+, 1-hexadecanoyl-2-(9Z-octadecanoyl)-*sn*-glycero-3-ethylphosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SV, sonicated vesicles.

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of discrete lesions (5.8 nm in diameter) in erythrocyte membranes by a colloid-osmotic type of lysis. Hemolysis was attenuated or prevented by various divalent cations and phosphatidic acid.[6] Recent studies of poly-APS induced transfection showed that this process was, surprisingly, more effective at lower temperatures (7–12 °C).[14] All these results suggest that the structure and physical properties of the membrane could greatly influence the poly-APS membrane activity. In this work, we have further elucidated these interactions, as well as the effects of different conditions on the stability of natural and synthetic 3-APS. The influence of the 3-APS structure (the compounds differ in the length of their alkyl chains and degree of polymerization) on their membrane activity was also studied. The results reported in this study are of interest in view of possible use of these compounds in medicine and/or industry.

2. Materials and methods

2.1. Materials

Natural polymeric alkylpyridinium salts (poly-APS) were purified from the marine sponge *Reniera sarai*,[5] and freeze-dried. Before use, they were dissolved in 140 mM NaCl, 20 mM Tris·HCl, pH 7.4 (erythrocyte buffer) or 140 mM NaCl, 20 mM Tris·HCl, 1 mM EDTA, pH 8.0 (vesicle buffer). 3-APS compounds (APS7, APS8 and APS12-2) were synthesized using a microwave-assisted polymerization procedure.[11,13] All the compounds were structurally characterized by NMR, and their molecular weights determined by the use of MALDI-TOF or ESIMS. Deconvoluted data of the high resolution weight spectral data of APS7 indicated a polymerization grade of m = 8 and 24 at a ratio of 2:1 for the monomer $C_{12}H_{18}N^+$.[13] APS8 and APS12-2 were proven to be of 11.9 and 14.7 kDa, respectivelly, which is consistent with 63 and 60 monomer units, respectively.[11] The structures of the used compounds are shown in Fig. 1. Wool grease cholesterol (CH), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) and 1-hexadecanoyl-2-(9Z-octadecanoyl)-*sn*-glycero-3-ethylphosphocholine ($POPC^+$) were from Avanti Polar Lipids (Alabaster, USA). All lipids were dissolved in chloroform or other organic solvents according to the manufacturer's instructions.

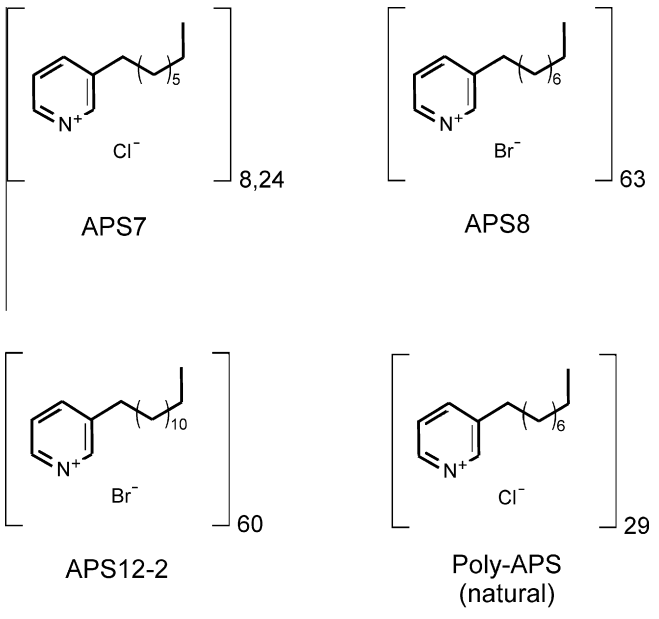

Figure 1. Chemical structures of 3-alkylpyridinium compounds used in this study.

Triton X-100, rhodamine 6G, and calcein were from Sigma. All chemicals were of the highest grade available.

2.2. Methods

2.2.1. Determination of critical micelle concentration

Critical micelle concentrations (CMCs), that is, the aggregation points of different 3-APS were determined using the fluorescent probe rhodamine 6G which was added, to give a final concentration of 10 μM, to vials containing 3-APS ranging from 0 to 1 mg/mL in water. The solutions were mixed and left in the dark for 48 h at, variously, room temperature, 4, 15, 25, or 40 °C. Samples were excited at 480 nm and their fluorescence emission recorded at 550 nm. Light scattering from similar samples was recorded at the excitation and emission wavelengths of 400 nm. In both experiments, a Jasco FP-750 spectrofluorometer (JASCO Ltd., Essex, UK), equipped with a water-thermostated cell holder and a 1 cm path length, magnetically stirred (830 rpm) quartz cuvette, was used. Slit widths with a nominal band-pass of 5 nm were used for both excitation and emission.

2.2.2. Preparation of sonicated vesicles (SV)

Lipid films were formed by removing the organic solvent from a lipid solution by rotary evaporation and vacuum drying. Lipids, at a final concentration of 10 or 5 mg/mL, were swollen in vesicle buffer or in 80 mM calcein, respectively, and vortexed vigorously to give multilamellar vesicles which were further exposed to 8 cycles of freezing and thawing and sonicated using a 750 Watt Ultrasonic Processor (Cole Parmer, USA) as described.[15] After centrifugation (20 min, 16,000*g*, 25 °C), the vesicles were incubated for 45 min at 40 °C. They were kept at 4 °C prior to use for no longer than 5 days. In the case of calcein-containing vesicles, extra-vesicular calcein was removed by gel filtration on a Sephadex G-50 column.

2.2.3. Hemolytic activity

Hemolytic activity was measured on rat erythrocytes by a turbidimetric method.[15] Rat erythrocytes were obtained by centrifugation of freshly collected citrated blood and washed 3–5 times with excess 0.9% saline and once with 140 mM NaCl, 20 mM Tris·HCl buffer, pH 7.4. Typically, 100 μL of 3-APS solution in erythrocyte buffer was added to 100 μL of rat erythrocyte suspension in the same buffer. The initial turbidity of the mixture at 630 nm was 0.5. The decrease of turbidity was recorded for 30 min using a kinetic microplate reader (Dynex Technologies, USA) to determine the time required for 50% hemolysis, t_{50}. The 3-APS concentration causing 50% lysis (e.g., the drop of the turbidity at 630 nm to 0.25) in 2 min was termed $HC_{0.5}$ (μg/mL). Unless otherwise stated, all the experiments were performed at 25 °C. For pH values ranging from 6.0 to 9.5, the erythrocyte buffer was adjusted to the required pH. Both erythrocytes and 3-APS were dissolved in the same buffer 5 min before the experiment, and the final concentrations of 3-APS in the reaction mixture were calculated to give the $HC_{0.5}$. The same strategy was used for monitoring the effect of ionic strength, where erythrocyte buffers containing different concentrations of NaCl (100, 140, 180, 220, 260, 300, 340, or 380 mM) were used. To assess the effect of sonication on different 3-APS, 2 mL of 3-APS solutions were sonicated using a 750 Watt Ultrasonic Processor (Cole Parmer, USA), output scale 4 and 50% duty cycle (room temperature) for 0, 15, 30, 45, 60, 90 or 120 min, and the hemolytic activity at their $HC_{0.5}$ concentrations assessed immediately and after 24 h. Temperature stability of 3-APS was determined by exposing 2 mL of 3-APS solutions to −196, 25, 50, 75, and 100 °C for 30 min and then the hemolytic activity was assessed. The effect of temperature on hemolytic activity was monitored by following the whole process of measuring hemolytic activity at 4, 10, 25, and 37 °C. In both cases, 3-APS were used at their $HC_{0.5}$ concentrations.

Percent of hemolysis was expressed as 100 − % of inhibition of hemolysis (= $At_{12}/(A_{max} - A_{min})$), where At_{12} is the turbidity at 12 min, A_{max} is maximal turbidity (initial turbidity of the lysing mixture at 630 nm), and A_{min} is the turbidity after total lysis of the erythrocytes resulting from the addition of Triton X-100 to a final concentration of 1 mM.

2.2.4. Inhibition of 3-APS induced hemolysis

Binding of 3-APS to SV with different lipid compositions (DOPC, POPC, DPPC, and POPC:cholesterol, POPC:POPS, and POPC:$POPC^+$ in 1:1 molar ratios) was estimated by measuring the residual hemolytic activity of unbound 3-APS, using a kinetic microplate reader (Dynex Technologies, USA). Typically, 100 μL of SV at various lipid concentrations (0–2.5 mg/mL) in erythrocyte buffer were pipetted onto a multiwell-plate. 50 μL of 3-APS (in final $HC_{0.5}$ concentrations) was added and the plate incubated for 30 min at 25 °C to allow binding of the 3-APS to SV. Hemolysis was then assayed by adding 100 μL of rat erythrocyte suspension in vesicle buffer. The decrease in turbidity at 630 nm was recorded for 30 min to determine the time necessary for 50% hemolysis ($t_{0.5}$). The lysing mixture had an initial turbidity of 0.5 at 630 nm.

2.2.5. Permeabilization of SV

Vesicle permeabilization was assayed using a Jasco FP-750 spectrofluorometer (JASCO Ltd., Essex, UK) equipped with a water-thermostated cell holder, and using 1 cm path length, magnetically stirred (830 rpm) quartz cuvette. Slit widths with a nominal band-pass of 5 nm were used for both excitation and emission. The excitation and emission wavelengths were set to 485 and 535 nm. 5–10 μL of calcein-loaded SV with different lipid compositions (DOPC, POPC, DPPC, or POPC:cholesterol, POPC:POPS, and POPC:$POPC^+$ in 1:1 molar ratios) were added to the cuvette containing 1 mL of filtered vesicle buffer, followed by an appropriate amount of 3-APS. The release of calcein was then recorded for 10 min at 25 °C. Maximal (100%) calcein release was obtained by solubilization of SV with Triton X-100, 1 mM final concentration, and the percentage of calcein release then calculated for each well.[16] To assess the influence of different parameters (temperature, ionic strength, pH, sonication) on 3-APS induced membrane permeabilization, POPC vesicles, and 3-APS, in concentrations inducing 50% calcein release from these vesicles under the above-mentioned conditions, were used. For pH values ranging from 6.0 to 9.5, we used the vesicle buffer adjusted to a specific pH. The effect of ionic strength was assessed using vesicle buffers with different final concentrations of NaCl (100, 140, 180, 220, 260, 300, 340, or 380 mM). To assess the effect of sonication on 3-APS, 2 mL of 3-APS solution were sonicated as described in the Section 2.2.3 for 0, 15, 30, 45, 60, 90, and 120 minutes, and their permeabilization potential assessed immediately, and after 24 h. Temperature stability of 3-APS was studied by exposing 2 mL of different 3-APS solutions to −196, 25, 50, 75, and 100 °C for 30 min, while the effect of temperature on vesicle permeabilization was monitored by following calcein release at 4, 25, and 37 °C.

3. Results and discussion

3.1. Determination of critical micelle concentration (CMC)

To assess their critical micelle concentrations (CMCs), 3-APS were tested in the concentration range of 0–1 mg/mL, and their CMCs determined by the use of rhodamine 6G (Fig. 2A), and light scattering (Fig. 2B), as described in the Section 2. The CMC of poly-APS at room temperature was found to be 0.33 mg/mL, slightly higher than that determined previously.[5] It increased with increasing temperature (Fig. 2B). The CMCs of synthetic 3-APS could not be determined (Fig. 2A), which suggests that their CMCs are higher than 1 mg/mL. Thus all the tested compounds exert their biological activities in their monomeric states.

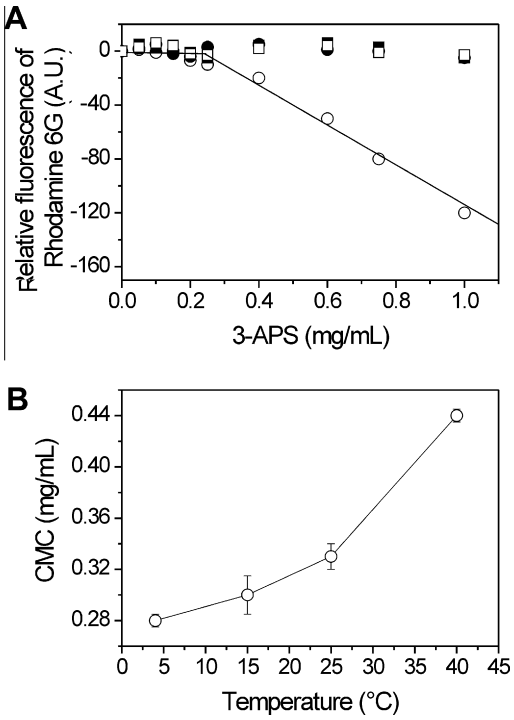

Figure 2. A: Determination of critical micelle concentration (CMC) for poly-APS (○), APS7 (●), APS8 (□) and APS12-2 (■) at 25 °C. 3-APS were tested in the concentration range of 0–1 mg/mL, and CMC was determined by using Rhodamine 6G as described in Section 2.2.1. B: Temperature-dependence of poly-APS CMC. The CMC was determined by recording the light scattering at excitation and emission wavelengths of 400 nm.

3.2. Influence of 3-APS structure on permeabilization of natural and artificial lipid membranes

Three synthetic 3-APS, differing in the length of their alkyl chains (7–12 C-atoms), degree of polymerization (8 to 63 monomeric subunits), and the nature of their counterions (chloride or bromide), were assayed for their ability to induce the formation of lesions in natural and artificial lipid membranes. Their activities were compared to that of the natural polymeric alkylpyridinium compound, poly-APS (Fig. 3A). Hemolysis rates of 1 s^{-1} for APS12-2, APS8, and APS7 were measured at 0.5, 1.2, and 7 μg/mL, respectively, indicating that the hemolytic activity increases with the increasing alkyl chain length. The membrane activity of these synthetic 3-APS also correlates with their degree of polymerization. However, all the three tested compounds were far more active than the natural compound, poly-APS.

A similar trend was observed when monitoring the membrane-permeabilizing activity on artificial lipid vesicles composed of POPC (Fig. 3B). All the compounds showed membrane-permeabilizing activities in a concentration range similar to that in the case of erythrocyte membranes, however the sequence of the compounds according to their permeabilization potential was slightly different. 50% calcein release was achieved by 0.35, 1, 1.2, and 10 μg/mL of APS8, APS12-2, APS7, and poly-APS, respectively. APS8 also proved to be the most potent membrane-permeabilizing agent in the case of artificial vesicles made of other pure phospholipids (DOPC, DPPC, Fig. 5) or POPC:POPS equimolar mixtures (not shown). However, when phospholipids (POPC, DPPC) were combined with an equimolar content of cholesterol, the permeabilizing

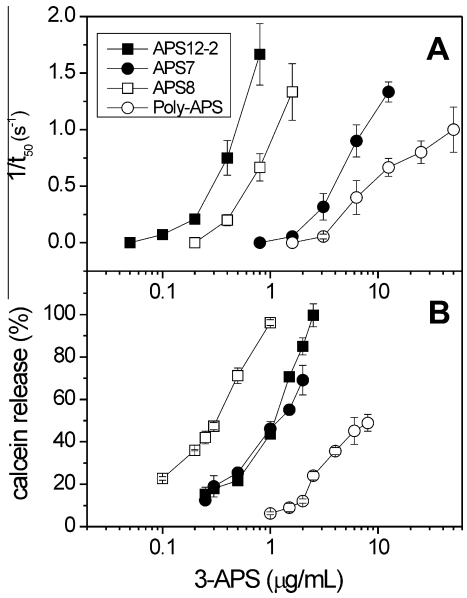

Figure 3. Hemolytic activity on rat erythrocytes (A) and permeabilization of calcein-loaded POPC vesicles (B) induced by different concentrations of APS12-2 (■), APS8 (□), APS7 (●), and poly-APS (○) at 25 °C. The experiments were performed as described in the Sections 2.2.3 and 2.2.5. $1/t_{50}$ = reciprocal half-time of hemolysis, i.e., time necessary to induce the lysis of 50% of the erythrocytes. Each point is the mean of three independent experiments ± SE.

ability of all the compounds was considerably lower, and APS12-2 was always the most active (Fig. 5). Overall, these results corroborate those obtained by other authors, showing that the membrane-disrupting potential of amphiphilic compounds correlates with their alkyl chain length and the number of positive charges.[17–19] The higher hemolytic potential of APS12-2 and APS8 could also be explained by the nature of their counterions, since the pyridinium salts containing Br^- were found to be more membrane-damaging than those containing chloride or other counterions.[20,21] Hydrated bromide ions, having the greater mobility and the smaller radii, could be more effective in modifying the membrane surface potential, thus enhancing the interaction between the pyridinium compound and the membrane.[20,21]

3.3. Influence of a lipid membrane phase on 3-APS activity

The lysis of red blood cells induced by poly-APS (Fig. 4) is temperature-dependent, with the lowest value observed at 4 °C, the maximum at 10 °C, and slightly decreasing hemolysis at 25 and 37 °C. Similar results, with an even more pronounced maximum at 10 °C, were obtained with bovine erythrocytes (not shown).

The same temperature trend was observed with all the three tested synthetic 3-APS. These results are in accordance with those of McLaggan et al.,[14] which showed more effective transfection of poly-APS at lower temperatures (7–12 °C), and also with the results obtained by other authors[21–24] on temperature-dependence of hemolytic activity of ionic detergents. The phase transition in erythrocyte membranes occurs between 18 °C and 25 °C,[25] indicating that at lower temperatures the erythrocyte membranes exist in a solid-ordered state that obviously facilitates 3-APS membrane activity. On the other hand, at higher temperatures (>37 °C), local rearrangements deriving from the formation of a multibilayer state, and subsequent exposition of lipid-free areas of the erythrocyte surface, facilitate a spontaneous membrane lysis.[26] The requirement of a solid ordered membrane phase for 3-APS activity is further confirmed by the results obtained with lipid vesicles, where all the tested 3-APS showed the highest activity with DPPC vesicles, followed by those composed from POPC and DOPC (Fig. 5). The fact that these lipids show phase transitions at 41 °C,[27] −3 °C,[28] and −19°C[29] respectively, indicates that only the DPPC ones exist in the solid ordered phase at room temperature. The 3-APS lytic potential is further reduced by the introduction of 50 mol % of cholesterol (Fig. 5). The addition of this lipid broadens the phase transition and leads to the formation of the so-called liquid ordered phase, in which membrane lipids are tightly packed, but still exhibit rapid lateral mobility.[30] At 25 °C, the POPC:cholesterol (1:1, mol:mol) vesicles exist in this state,[31] and are clearly the most resistant to the action of all studied 3-APS (Fig. 5). The liquid ordered phase is believed to be the physical state of membrane rafts—transient, dynamic and unstable cell membrane entities involved in several important biological functions, such as exocytosis and endocytosis, signal transduction, pathogen entry, and attachment of various ligands.[32–34] Our combined results suggest that 3-APS are excluded from lipid rafts when interacting with cell membranes.

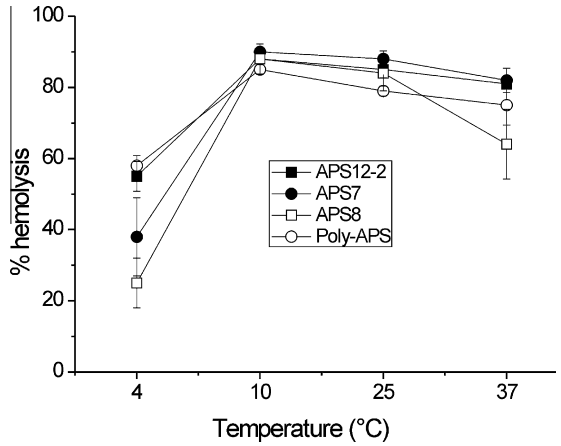

Figure 4. Temperature-dependence of 3-APS induced hemolysis. A suspension of rat erythrocytes was incubated for 2 h at each temperature, then APS12-2 (■), APS8 (□), APS7 (●), and poly-APS (○) at their final $HC_{0.5}$ concentration were added and hemolysis measured as described in the Section 2.2.3. Each point is the mean of three independent experiments ± SE.

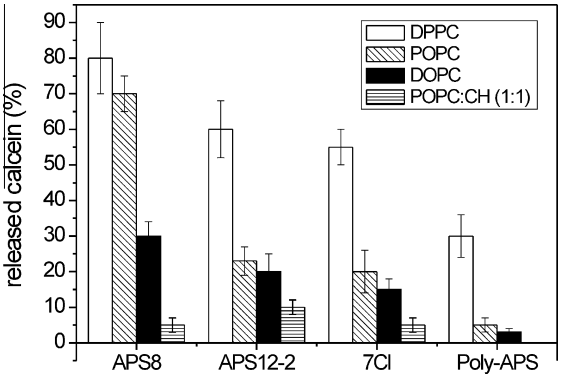

Figure 5. Effect of lipid composition on 3-APS induced permeabilization of calcein-loaded vesicles composed of phosphatidylcholines with different degrees of saturation of fatty acid chains (DOPC, POPC, DPPC), and equimolar mixture of POPC and cholesterol at 25 °C. The 3-APS concentration in all the experiments was 0.5 μg/mL. Each bar is the mean of three independent experiments ± SE. DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; CH, cholesterol.

3.4. Influence of membrane surface charge, ionic strength, and pH on 3-APS activity

The effect of the membrane surface charge on 3-APS binding was determined using the hemolysis-inhibition assay, by pre-incubating 3-APS with different concentrations of lipid vesicles. The results clearly indicate the importance of the charge for the initial membrane—3-APS interaction (Fig. 6A). The highest degree of poly-APS induced inhibition was exhibited by vesicles composed of POPC in combination with negatively charged phosphatidylserine (50% inhibition at 3 μg/mL SV), followed by those of pure POPC (50% inhibition at 30 μg/mL SV), and finally vesicles composed of POPC supplemented with a positively charged phosphatidylcholine, where no inhibition of hemolysis was observed up to 3 mg/mL of lipid vesicles. As discussed previously, addition of cholesterol significantly decreased the 3-APS binding step, and 50% inhibition of poly-APS induced hemolysis could be observed only at 350 μg/mL of POPC:cholesterol (1:1, mol:mol) SV. The above-described trends were similar with all other tested 3-APS (not shown). The hemolytic activity of poly-APS is abolished in the presence of negatively charged phosphatidic acid.[6] These combined results indicate that the preference of poly-APS for negatively charged membrane lipids results from their polycationic structure. This preference was clearly observed also in the further steps of 3-APS-membrane interactions, that is, in the process of membrane permeabilization, as shown for poly-APS in Fig. 6B.

Both 3-APS induced hemolysis (Fig. 7) and permeabilization of POPC vesicles (not shown) are slightly inhibited on increasing the ionic strength (in this case the NaCl concentration) of the buffer. This phenomenon strongly suggests charge shielding of the negatively charged groups at the membrane by Na^+, preventing the access of 3-APS to binding sites on the membrane, as already described for the inhibitory action by some divalent cations.[6,35]

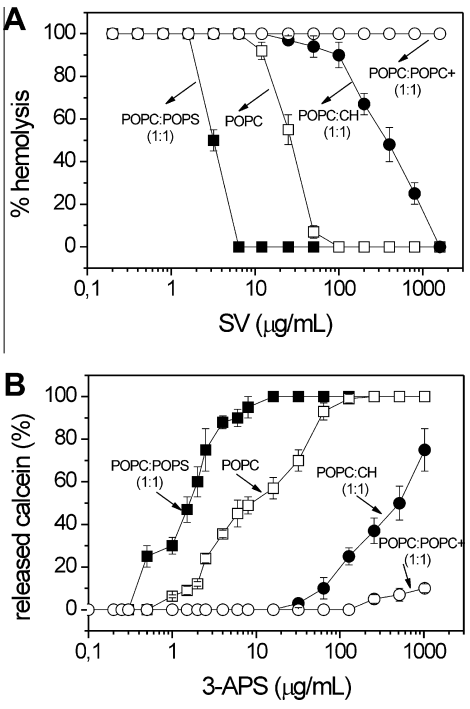

Figure 6. Inhibition of poly-APS induced hemolysis of rat erythrocytes by sonicated lipid vesicles (A), and poly-APS induced permeabilization of calcein-loaded sonicated lipid vesicles (B). The lipid vesicles were composed of POPC:POPS (1:1, mol:mol, ■), POPC (□), POPC : cholesterol (1:1, mol:mol, ●), and POPC:$POPC^+$ (1:1, mol:mol, ○). The experiments were performed at 25 °C, as described in the Sections 2.2.4 and 2.2.5. Each point is the mean of three independent experiments ± SE. POPS = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine, $POPC^+$ = 1-hexadecanoyl-2-(9Z-octadecanoyl)-*sn*-glycero-3-ethylphosphocholine. Other abbreviations are as given in Fig. 5.

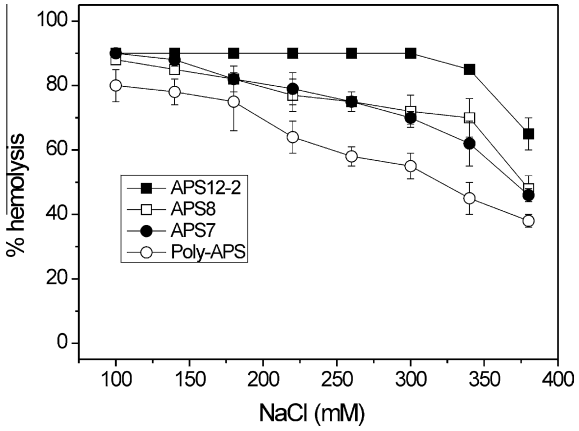

Figure 7. Effect of NaCl concentration on 3-APS induced hemolysis of rat erythrocytes. Erythrocyte buffer with different NaCl concentrations was used in the test. The concentrations of 3-APS used were 2 μg/mL (APS12-2, ■), 3.5 μg/mL (APS8, □), 8 μg/mL (APS7, ●), and 10 μg/mL (poly-APS, ○). Each point is the mean of three independent experiments ± SE.

On the other hand, the rates of hemolysis and vesicle permeabilization showed a steady, slow increase with increasing pH, as shown in Fig. 8 for hemolysis in the pH-range from 6 to 9.5. This could reflect stronger interactions of 3-APS with negatively charged groups at the membrane surface, exposed after their deprotonation at higher pH.

3.5. Influence of temperature and physical stress on 3-APS stability

Aqueous solutions of poly-APS are stable for months.[36] Synthetic 3-APS are also stable; their membrane-damaging activity was unaltered even after 1-hour exposure to 100 °C, or after five cycles of freezing–thawing (not shown). In contrast, sonication had a pronounced negative effect on their hemolytic and vesicle-permeabilizing activities, as shown in Fig. 9 for permeabilization of calcein-loaded POPC lipid vesicles. This effect was especially pronounced on poly-APS, which lost about 78% of its membrane activity after a 15-min sonication. However, this is unlikely to be due to sonication-induced disruption of the supramolecular

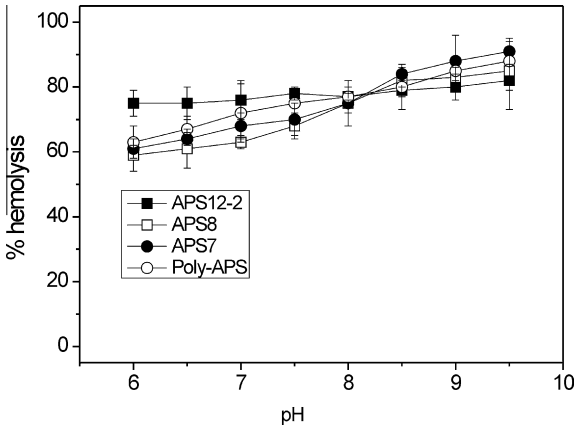

Figure 8. Effect of pH on 3-APS induced hemolysis of rat erythrocytes. Erythrocyte buffer with different pH values was used in the experiment. The concentrations of 3-APS used were 1.5 μg/mL (APS12-2, ■), 2.25 μg/mL (APS8, □), 6.3 μg/mL (APS7, ●), and 7 μg/mL (poly-APS, ○). Each point is the mean of two independent experiments ± SE.

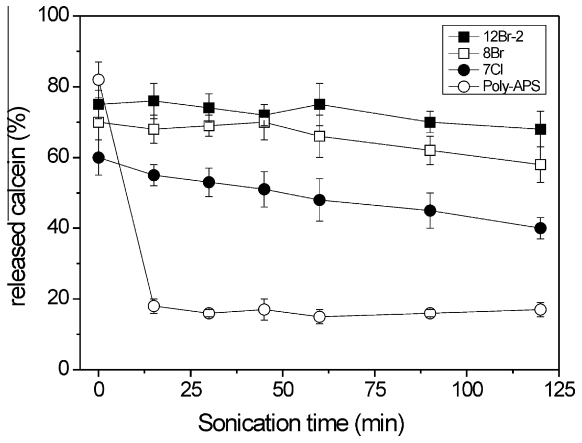

Figure 9. Effect of time of sonication on 3-APS induced permeabilization of sonicated vesicles composed of POPC. The concentrations of different 3-APS used in the test were 0.7 μg/mL (APS12-2, ■), 0.2 μg/mL (APS8, □), 0.7 μg/mL (APS7, ●), and 3 μg/mL (poly-APS, ○). Each point is the mean of three independent experiments ± SE.

structures that can be formed by these natural alkylpyridinium salts, since (i) the concentration of poly-APS used was far below their CMC (Fig. 2,[5]), and (ii) the loss of membrane-damaging potential was irreversible, that is, it did not recover in the next 24 h (not shown). Synthetic 3-APS were far more resistant, even to longer sonication times (Fig. 9).

4. Conclusions

The three tested synthetic 3-APS analogues are shown to be very stable over a wide range of temperature, ionic strength, and pH. Their membrane-damaging activity is greatly influenced by their structure, and increases with the length of their alkyl chains and the degree of polymerization. When interacting with artificial and natural lipid membranes, these compounds appear to prefer the solid-ordered membrane state. This could be the reason for their relatively low cytotoxicity to non-target cell membranes that, according to the current model of cell membrane structure, consist of co-existing liquid-disordered and liquid-ordered domains.[37] Recently, it was found that poly-APS exert a selective cytotoxicity towards non small cell lung cancer (NSCLC) cells, which are the most common form of lung cancer and are also linked to tobacco use.[38] These cells express molecules belonging to the cholinergic system, such as cholinoacetyltransferase, vesicular acetylcholine transporter and acetylcholinesterase, so the selective cytotoxicity of poly-APS probably derives from the interruption of the NSCLC cells' cholinergic system through acetylcholinesterase inhibition, resulting in their apoptosis. The cytotoxic concentrations of poly-APS against NSLC cells are hence significantly lower than those inducing lysis of other cell types. The results obtained in this study, combined with preliminary encouraging results on NSCLC cytotoxicity and the antifouling activity of synthetic 3-APS (to be published elsewhere), support these compounds as promising candidates for use in non-toxic antifouling paints, and/or in medicine as selective chemotherapeutics.

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