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Francesca Giudice, Ernesto E. Ambroggio, Milagro Mottola, Maria Laura Fanani

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The amphiphilic alkyl ester derivatives of L-ascorbic acid induce reorganization of phospholipid vesicles

Francesca Giudice[a], Ernesto E. Ambroggio[a]*, Milagro Mottola[a-1], Maria Laura Fanani[a]*

[a]*Centro de Investigaciones en Química Biológica de Córdoba, CIQUIBIC, CONICET and Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA, Córdoba, Argentina. Haya de la Torre y Medina Allende, Ciudad Universitaria.*

[1] *Present address: Instituto de Investigaciones Biológicas y Tecnológicas (IIByT), CONICET-UNC, Depto. de Química, FCEFyN Córdoba. Argentina.*

**Corresponding authors: ernesto@fcq.unc.edu.ar; lfanani@fcq.unc.edu.ar, +54-351-5353855*

Abstract:

L-ascorbic acid alkyl esters (ASCn) are lipophilic forms of vitamin C, which maintain some of its antioxidant power. Those properties make this drug family attractive to be used in pharmacological preparations protecting other redox-sensible drugs or designed to reduce possible toxic oxidative processes. In this work, we tested the ability of L-ascorbic acid alkyl esters (ASCn) to modulate the structure, permeability and rheological properties of phospholipid bilayers. The ASCn studied here (ASC16, ASC14 and ASC12) alter the structural integrity as well as the rheological properties of phospholipid membranes without showing any evident detergent activity. ASC14 appeared as the most efficient drug in destabilize the membrane structure of nano- and micro-size phospholipid liposomes inducing vesicle content leakage and shape elongation on giant unilamellar vesicles. It also was the most potent enhancer of membrane microviscosity and surface water structuring. Only ASC16 induced the formation of drug-enriched condensed domains after its incorporation into the lipid bilayer, while ASC12 appeared as the less membrane-disturbing compound, likely because of its poor, and more superficial, partition into the membrane. We also found that incorporation of ASCn into the lipid bilayers enhanced the reduction of membrane components, compared with soluble Vitamin C. Our study shows that ASCn compounds, which vary in the length of the acyl chain, show different effects on phospholipid vesicles used as biomembrane models. Those variances may account for subtly differences in the effectiveness on their pharmacological applications.

Keywords: GUVs; LUVs; Laurdan GP; DPH anisotropy; phosphatidylcholine liposomes; confocal microscopy

Abbreviations: ASC_n, L-ascorbic acid alkyl esters; ASC16, ascorbyl palmitate; ASC14, ascorbyl myristate; ASC12, ascorbyl laurate; ASC, L-ascorbic acid; 1-palmitoyl-2-oleoylphosphatidylcholine, POPC; L-α-phosphatidylethanolamine-N-(lissaminerhodamine B sulfonyl), PE-Rho; 1,6- diphenyl-1,35,-hexatriene, DPH; 5(6)-Carboxyfluorescein, CF; laurdan generalized polarization, GP; 6-dodecanoyl-2-dimethyl-aminopnaphthalene, laurdan.

1_ Introduction

L-Ascorbic acid (Vitamin C) has been widely studied especially for being a strong and powerful water-soluble antioxidant that efficiently protects biological molecules against oxidative degradation because of their radical-scavenger activity [1]. However, due to its poor solubility in hydrophobic media, its usefulness is limited to aqueous environments**.** One approach to tackle this issue is the generation of amphiphilic derivatives of Vitamin C like alkanoyl-6-*O*-ascorbic acid esters (ASCn), which are known to maintain the antioxidant character of ascorbic acid and to partition into non-polar milieus [2,3]. These modifications make to this drug family a very attractive tool for being used in pharmacological preparations since there are experimental evidences indicating a strong connection between oxidative damage, cancer, and aging [4,5].

In addition, another advantage of the amphipathic character of ASCn is their ability to form aggregates in aqueous solutions or to be integrated into lipid vesicles, which offer a suitable environment for the storage and transport of hydrophobic substances susceptible to oxidation [6]. In fact, some ASCn such as ascorbyl laurate (ASC12), ascorbyl myristate (ASC14) and ascorbyl palmitate, (ASC16) self-organize as coagels at relative high ASCn/water ratio [1]. All these characteristics make ASCn a promising tool as drug carriers for pharmaceutical dosage formulations.

For example ASC12, which among the drugs studied in this work contains the shorter acyl chain, enhances ophthalmological permeation of other hydrophobic drugs [7]. On the other hand, the ASC16 coagel acts as a good adjuvant in immunization of animal model [8], presumably because some inflammatory-cytotoxic activity. Additionally, it has been recently reported that some members of this drug family show bactericidal and antileishmanial action [9]. All these applications indirectly imply the interaction of ASCn with biological membranes that is the first cell barrier. The length of the acyl chain (acting as the hydrophobic tail) of the ASCn compounds may subtly regulate the effectiveness of those pharmacological applications.

Despite the widespread use of ASCn in pharmacy, there is still lack of information about the physicochemical basis of their interaction with lipid membranes. We recently studied the surface behavior of ASCn with acyl chains of different lengths, showing that these type of drugs have high surface activity where they are able to form stable Gibbs monolayers and were also capable to penetrate into tightly-packed phospholipid monolayers [10]. Subtle chemical differences in these compounds (which contain acyl chains of C16, C14 and C12) have large implications on the surface activity and upon the in-plane organization of the ASCn films as well as in their ability to be integrated into lipid monolayers [10–13]. We also demonstrated that these differences induce differential changes of the physicochemical properties of the targeted lipid membrane and regulate the extent of drug incorporation and the kinetics of the penetration process [12].

The aim of the present work is to delve in the understanding of the consequences of ASCn incorporation into lipid bilayers by exposing phospholipid vesicles, used as a biomembrane model system, to the non-commercial compounds ASC14 and ASC12 (synthesized in our laboratory [10]) and comparing their effect to that observed for the commercial ASC16.

Here, we used PC nano and micro-size liposomes as model lipid membranes and we show that ASCn alter the structural integrity of those vesicles as well as the rheological properties of the phospholipid membrane. We also explored the occurrence of condensed drug-enriched domains laterally segregated from the phospholipid rich phase, as has been shown to occur in analogues monolayer systems [10,11].

2. Materials and Methods

2.1. Materials. Commercial 6-O-Palmitoyl-L-ascorbic acid (ASC16) was supplied by Sigma and exhaustively purified as described in Ref. [10] 6-O-Lauryc-L-ascorbic acid (ASC12) and 6-O-Myristyc-L-ascorbic acid (ASC14) were synthesized and purified as described in Ref. [10]. 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and the lipophilic fluorescent probe L-α-phosphatidylethanolamine-N-(lissaminerhodamine B sulfonyl) ammonium salt (PE-Rho) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). 1,6- dipheniyl-1,35,-hexatriene (DPH) and 6-dodecanoyl-2-dimethyl-aminopnaphthalene (laurdan) were obtained from Invitrogen (Eugene, Oregon, USA). 5(6)-Carboxyfluorescein (CF) and β–casein from bovine milk were purchased from Sigma Aldrich Co. (USA). Chloroform and Methanol were supplied by Merck (Darmstadt, Germany). Deionized water with a resistivity of 18 M Ω cm, was obtained from a Milli-Q Gradient System (Millipore, Bedfore, MA). Indium tin-oxide slides (ITO) where obtained from Nanocs (New York, USA).

2.2. Liposome preparation and analysis. POPC MLVs were prepared by generating a uniform lipid film on the wall of a glass test tube, by solvent evaporation under a N_2 stream, from a chloroformic POPC solution. Final traces of solvent were removed with a high-vacuum chamber. The lipids were hydrated with a buffer solution containing HEPES 10 mM, NaCl 269 mM and EDTA 1 mM (pH ~7.5) applying a vigorous mixing and then subjected to five freezing-thawing cycles (-195 ºC and 40 ºC, respectively). LUVs with an average diameter of 100 nm, were prepared by extrusion (20 times) of MLVs composed by POPC through polycarbonate filters of 100 nm pore size, at room temperature. For fluorescence measurements, appropriate aliquots of the fluorescent probes Laurdan or DPH, dissolved in dimethyl sulfoxide, were added to the chloroformic lipid suspension reaching 0.5 mol %. Laurdan generalized polarization (GP), DPH fluorescence anisotropy and LUVs size distribution were measured before and after LUV incubation with ASCn. In all these experiments the drug was added from

a concentrated ethanolic solution reaching the desired drug:lipid ratio (the added ethanol never exceeded 2% v/v)

Laurdan GP [14] was calculated according to the expression: $GP = \frac{(I_{434}-I_{490})}{(I_{434}+I_{490})}$ eq. (1), where I_{434} and I_{490} are the dye fluorescence emission intensities when Laurdan is mixed with phospholipids adopting a gel phase (434 nm wavelength) or a liquid-crystalline phase (490 nm wavelength). The excitation wavelength was 360 nm. From eq. 1 it can be deduced that high Laurdan GP values correspond to laterally ordered phases (gel-like), whereas low Laurdan GP values correspond to fluid phases.

Fluorescence DPH anisotropy measurements were performed by exciting DPH molecule with polarized light (365 nm wavelength) and detecting the light intensity emitted (wavelength: 428nm) after passing through a polarizer held vertical (v) or horizontal (h) to the direction of the electric vector to the polarized excitation. The anisotropy value, *r*, was calculated according to the following equation [15]:

$$r = \frac{\left(\frac{I_v}{I_h}\right)_v - \left(\frac{I_v}{I_h}\right)_h}{\left(\frac{I_v}{I_h}\right)_v + 2\left(\frac{I_v}{I_h}\right)_h} \quad \text{eq. (2).}$$

r reflects the degree to which the photoselected population of excited molecules becomes randomised during the excited state from the direction of the electric vector to the polarized excitation.

The effect of ASCn on the size-distribution of POPC LUVs (100μM) before and after incubation with the drug was studied with dynamic light scattering (DLS; Submicron Particle Sizer, Nicomp™ 380, Santa Barbara, California,USA). In addition, pure ASCn aqueous suspensions were also analyzed.

2.3. Vesicle content leakage. LUVs encapsulating carboxyfluorescein (CF) were prepared from MLVs formed in a buffer containing CF 50 mM (Tris 10 mM, pH 8). It is worth to mention that this dye at the used concentration is self-quenched. To remove the non-entrapped CF the samples were filtered by Size-Exclusion Chromatography (Sephadex G-25) and size checked by DLS obtaining a size distribution averaging 100 nm of vesicle diameter. The LUVs were incubated with ASCn (1:1 drug:lipid mole ratio) prior to perform fluorescence intensity measurements of CF (ex. 490 nm, em. 513 nm wavelengths [16,17]). The percentage of the released dye was calculated as:

$$CF\% = 100[(I_t - I_0)/(I_\infty - I_0)] \text{ eq. (3)}$$

where I_0 is the fluorescence intensity of the CF-loaded liposome suspension, I_∞ is the fluorescence intensity after maximal release induced by Triton X-100 (final concentration 10 mM). I_t stands for the fluorescence intensity at 45 min after adding ASCn to the liposome suspension. All fluorescence measurements were acquired with a Cary Eclipse spectrofluorometer (Agilent Technologies) equipped with a thermally controlled multi-cuvette holder (21 °C), setting the wavelength slit in 5 nm and using a 2 mm path cuvette.

2.4. Confocal Microscopy visualization of ASCn-loaded giant unilamellar vesicles (GUVs). POPC GUVs where prepared by the electroformation method [18]. Briefly, 10 µL of a POPC stock solution (0.5 mg/mL in Chloroform:Methanol 2:1 v/v) doped with 0.5 mol% of the fluorescent probe PE-Rho were spread onto two ITO-coated glass slides. The electrodes were subjected to vacuum in order to remove any remaining trace of organic solvent. Then, the lipids were hydrated with a solution of sucrose (269 mM) and the electrodes were connected to a function generator (UNI-T UTG9002C, Uni-Trend Group Limited, Hong Kong) applying for 5 hours a low-frequency alternating field (sinusoidal function of 10 Hz and an effective tension of 1 V) [17].

A small aliquot of the GUV suspension (20 µL) was transferred to an 8-well observation chamber (Lab-Tek, Thermo Fisher Scientific, Inc. NYSE:TMO) containing an iso-osmotic buffer solution (HEPES-NaCl pH 7.2 containing 1mM EDTA). Before GUV addition into the well, the glass of the observation chamber was treated with a 10 mg/mL β− casein solution, which prevented GUV rupture onto the slide. The giant liposomes where directly observed with a fluorescence confocal microscope (Olympus FV 300, Tokyo, Japan). To monitor over the time the effect of ASCn to the GUV membranes, we acquired time-lapsed images (15 seconds between frames), following the fluorescence of the membrane marker (Rho-PE). In addition, since sucrose and the buffer used have different refractive index, GUVs' content release was monitored by Differential interference contrast (DIC) microscopy. Changes in fluorescence intensity at the GUV membrane and contrast inside the liposome were quantified with the ImageJ 1.43u software (NIH, USA).

3. Results and discussion

3.1. ASCn induce reorganization of POPC vesicles

The chemical structure of the amphiphillic ASCn, with a single hydrophobic tail and a big polar head, mimics the configuration of a detergent. Detergents are able to solubilize/destabilize lipid bilayers damaging membranes and forming mixed lipid/detergent micelles. PCs are zwitterionic lipids abundant in cell membranes and they conform the most studied lipid family [19].

Generally, the most used lipid to build a lipid bilayer or a monolayer as membrane-mimicking systems is POPC (1-palmitoyl-2-oleoylphosphatidylcholine) since it develops, at room temperature, Lα (Liquid-disordered) phase in bilayers, which is proposed to be the major phase state found in cell membranes [19].

In order to test for detergent-like solubilisation of lipid components by ASCn, we incubated POPC multillamelar liposomes (MLVs) with the three different drugs studied, ASC12, ASC14 and ASC16, for 2 hs. Then, the samples were centrifuged (sedimentation of MLVs; detailed experimental procedures are present in the supplementary material file) and the phosphorus content in the supernatant was measured. If ASCn/POPC mixed micelles were formed, they will remain in this soluble fraction. Our study did not detect a significant amount of phosphorus in the supernatant after 2h of MLVs incubation with ASCn (see Figure S1 in Supplementary material file). These results indicate that ASCn did not act as detergents solubilizing membrane components.

We also tested the ability of ASCn to induce aggregation/fusion of lipid vesicles. This can be followed by measuring the change of the size distribution of liposomes when interacting with the drug. We performed these experiments monitoring the size distribution of POPC LUVs after incubation with ASCn by using DLS. Before adding the drugs, LUVs showed a Gaussian diameter distribution with a maximum at ~100 nm (Figure 1). The addition of ASCn, reaching a 1:1 drug:lipid molar ratio, induced restructuration of the particles, changing their size distribution. The drug concentration chosen for this study (100 μM) represents several folds the critical aggregation concentration of ASC12 (33 μM), ASC14 (9 μM) and ASC16 (8 μM) [20]. It is well established that the monomeric form of the amphiphile is the responsible for its interaction with the membrane [21]. Then, a high total drug concentration is required to keep a constant monomer concentration in equilibrium with the membrane, even after depletion due to integration into the membrane phase. Thus, the ASCn aggregates in solution represent drug reservoirs.

The membrane restructuring effect was dependent on the length of the acyl chain of the ASCn compounds. ASC16 induced the occurrence of large structures in the 700 – 4000 nm diameter range only after 45 min of incubation (Figure 1a). It is worth noting that when ASC16 is diluted in an aqueous medium (in the absence of lipids) it results in the formation of large particles corresponding to lamellar structures (Figure 1a and [11]). Large particles were not observed in the presence of lipid vesicles until a considerable long incubation time. This suggests that a fast uptake and slow vesicle restructuration is taking place in the lipid – ASC16 binary system.

On the other hand ASC14, which pure in solution forms large structures, induced little vesicular reorganization, observed as a moderate shoulder of the diameter distribution bell (Figure 1b). Finally, the addition of ASC12 to the vesicle preparation resulted in the occurrence of a second peak in the size distribution (400 – 800 μm diameter range), evidencing the presence of pure ASC12 structures and/or ASC12 loaded LUVs.

Pure ASC12 suspended in an aqueous media showed the occurrence of a low and a high diameter population (see control in Figure 1c). The presence of micelles in ASC12 aqueous suspensions was reported in previous works by the group of Lo Nostro [3]. These authors did not directly check the size of such aggregates, but reported a critical parameter (Pc) for ASC12 of 0.57. According the work of Israelachvili [22], amphiphiles with a $0.33 < Pc < 0.5$ form cylindrical micelles while with $0.5 < Pc < 1$ are expected to form planar lamellas. The Pc reported for ASC12 falls near the boundary limit between cylindrical micelles and lamellas, This geometrical aspect can explain our experimental observation of small structures of 20-40 nm in coexistence with aggregates larger than 100 nm and agrees with the occurrence of cylindrical micelles in dynamic equilibrium with large lamellar structures of the type described for ASC16 by X-ray diffraction [21]. Notably, the low diameter particle population (20 – 40 nm) observed in the pure ASC12 aqueous system was no longer observed after incubation with POPC LUVs (Figure 1c). This may be consequence of a more favorable/faster incorporation into the lipid vesicles of ASC12 from those less compact small structures than from larger particles.

These findings led us to evaluate the membrane integrity and/or pore formation, concomitant to the membrane restructuration induced by ASCn on POPC LUVs. For this reason, we tested the leakage of the soluble fluorophore carboxifluorescein (CF) from the intravesicular compartment (in a self-quenched condition) to the extravesicular medium (where its dilution promotes de-quenching of CF) after ASCn addition. Figure 2 shows that only ASC14 induced a significant content release in conditions above 1:1 drug:lipid mole ratio. This unexpected result reveals that the membrane disrupting/pore formation capacity is not linear with the length of the ASCn, and may be the consequence of concerted and complementary effects. Some of those may be related to the different properties of the ASCn aggregates in equilibrium with the membranes, which may affect their partitioning kinetics and extent. Other factors may be related to changes in the rheology and lateral structure of the ASCn-loaded membranes. Some of those factors are analyzed and discussed below.

3.2. ASCn induce changes in the membrane hydration and microviscosity.

In order to shed light on the effect on the membrane properties mediated by ASCn, we took advantage of the fluorescent properties of Laurdan, a dye sensible to the hydration of the lipid interface [14], and DPH, which allows to monitor changes on the rigidity of membranes by measuring its fluorescence anisotropy.

Laurdan spontaneously partitions into the interfacial region where the phospholipid headgroups locate and its fluorescence is strongly influenced by the dipolar relaxation of the water molecules at this region [14]. The generalized polarization function of Laurdan (GP; eq. 1) allows inferring if the presence of the ASCn derivatives can alter the interfacial properties of the POPC LUVs. We detected that the GP value significantly increases when LUVs were exposed to ASCn (Figure 3), even at the lowest drug concentration tested (1:2 drug:lipid mole ratio). This fact directly evidences ASCn incorporation into the membrane, which leads to a condition where the dipolar relaxation of the surface water is restricted. ASC14 appeard to be the more efficient modulator of the surface hydration of the lipid membrane followed by ASC12 and ASC16. The adjustment of a hyperbolic function to the data revealed not significant differences in the concentration necessary to reach 50% of the GP increase (Figure 3c).

As control, we also tested the surface hydration properties of the ASCn self-assembled structures in the absence of lipid vesicles. Laurdan GP revealed a more structured surface water for the ASC16 aggregates than for the formed by ASC12 and ASC14 (see Figure S2a in the Supplementary material). This result suggests that the poor effect of ASC16 on the vesicle surface hydration (Figure 3) may be a consequence of a scarce presence of the drug in such surface in comparison with ASC14, the most effective drug.

Changes in the microviscosity of the hydrophobic portion of the lipid membrane were assessed by monitoring fluorescence anisotropy of 1,6- dipheniyl-1,35-hexatriene (DPH). DPH is a fluorophore that locates in the hydrophobic core of the bilayer and is oriented predominantly parallel to the fatty acid chains of the phospholipids. Figure 4 shows the increase of DPH anisotropy for LUVs incubated with ASC14 and ASC16. These data evidence an increase in the microviscosity of the hydrophobic core of the bilayer for those cases. On the other hand, small changes were induced by ASC12. Since Figure 3 shows a considerable increase of the Laurdan GP by ASC12, the poor effect of this drug over DPH anisotropy may reflect a superficial integration of this drug into the membrane system. It is worth to point out that the semi-empirical law reported in Ref. [23] allowed the estimation of the average length of an acyl chain when it is in a completed extended conformation resulting in a chain length of 11.3 Å for the C12 and of 15.4 Å for the C16. If we consider that a membrane hemilayer composed of PC in the fluid phase state has a thickness of 15-16 Å [24], therefore, the

shortening in only 4 Å in the acyl chain for ASC12 compared to ASC16 may result in a restriction for ASC12 to reach the hydrophobic center of the membrane.

It is important to note that DPH incorporated into ASCn self-assembled structures showed a highly rigid environment for ASC16 aggregates compared to those of the shorter ASCn (Figure S2b in Supplementary material file). Then, again, the larger increase in microviscosity induced by ASC14, which occurred with an extremely low 50% dose (12 ± 5 μM, see Figure 4c) support a larger incorporation of ASC14 than ASC16 and ASC12.

The more active compounds, ASC14 and ASC16, had very different behavior. Contrary to the observed for ASC14, which reached 50% of the DPH anisotropy increase at very low concentration, ASC16 showed a 22 folds larger $[ASC]_{50}$ value (Figure 4c) and no-saturation in the experimentally accessible drug concentration. This last effect can be related to a tendency of ASC16 to laterally segregate into condensed drug-enriched domains, observed previously in lipid monolayer systems [10,11]. DPH partition into these ASC16-enriched domains may be lower than the partition of the probe into POPC-enriched domains. Then, the ASC16 present in the lipid membrane may be accumulated into drug-enriched domains allowing the maintenance of a low drug concentration in the more fluid phase (where DPH is concentrated). Additionally, previous studies demonstrated that ASC14 has a higher capacity to penetrate into POPC monolayers than ASC16 [12], suggesting that the larger effect found in DPH anisotropy can be also linked to a larger concentration of ASC14 into the probe-enriched phase compared to ASC16.

3.3 Direct observation of ASCn-membrane interaction

We further took advantage of another membrane model system that allows direct visualization of the lipid bilayer using optical microscopy. In this context, we generated POPC GUVs and analyzed the changes in the membrane properties of these vesicles, such as phase separation and vesicle shape/size fluctuations, when were in the presence of the different ASCn.

Figure 5 shows that the addition of ASCn induced a reduction of membrane tension in all the systems studied but in different extension evidenced by massive membrane deformations and/or increase in the bilayer fluctuation. In this sense, ASC14 appeared as the more active compound, inducing enlargement of the vesicles in the direction of the media flow. ASC12 showed a similar behavior but in a moderate manner. On the other hand, ASC16 only induced an increase in the roughness of the membrane (decrease of circularity). In all cases, these observations were correlated with a loss of contrast (detected by DIC microscopy) inside the liposome (Figure 5a-c). This last effect can be associated to a destabilization of the membrane integrity and content release (exchange between sucrose and buffer from both compartments).

LUVs content leakage experiments (Figure 2) showed only a significant effect for ASC14-lipid systems. This difference may be due to the excess of drug in the GUV/ASCn experiments since the lipid content used is much lower (high drug:lipid mole ratio).

When GUVs were incubated with ASC16, the occurrence of domains was observed. Those domains appeared as dark, flower-like shapes, depleted of the fluorescent probe Rho-PE (Figure 5d). Since Rho-PE is known to be excluded from gel phases, this supports a gel character for these domains. Furthermore, the non-circular flower-like shapes of the gel domains close resemble the ASC16-enriched liquid-condensed domains observed in binary ASC16-phospholipid monolayers [10,11,13].

Interestingly, early infrared spectroscopy studies reported by Casal and co-workers [25] evidenced that ASC16 induces an increase in the gel to Lα transition temperature of dipalmitoyl-PC. The transition process occurs in a single step, meaning mixing of the components, but the stabilization of the gel phase strongly suggests a preferential partitioning of ASC16 into this phase (and also a poor solubility of ASC16 into the fluid Lα phase). These data resembles our experimental finding were ASC16 induced the formation of an ASC16-enriched gel phase in coexistence to a fluid phase, probably depleted of ASC16.

Confocal microscopy experiments also showed a noticeable decrease in the fluorescence intensity of Rho-PE after ASCn addition (Figure 6). This decrease was not caused by photobleaching due to light exposure (see control curve in Figure 6) but likely by chemical reduction of the Rhodamine group of the amphiphilic fluorophore used. ASCn were reported to keep an important part of the antioxidant (reducer) power of ascorbic acid (ASC) [1,10]. We further explored if this effect could be induced by the addition of soluble ASC. Figure 6 shows that there was no effect of soluble ASC on the fluorescence of Rho-PE contrasting to the effect of ASCn during the working time. Quantification of fluorescence intensity over time after drug addition showed an increasing effect for ASC12 $<$ ASC14 $\sim$ ASC16. This result suggests that the ASC-containing compound should be integrated into the lipid membrane in order to be effectively able to reduce membrane components. Since the reduction power of the ASCn studied are similar [9,26] the differences in fluorescence intensity decay may be a direct measurement of the ability for membrane incorporation of the compounds. Then, ASC12 appears as the compound with the poorest membrane incorporation capacity as well as the less effective on enhancing the membrane core microviscosity (Figures 4 and 6) and, in general, behaving with moderate effect on vesicles restructuring.

At this point is important to note that the previously shown fluorescent measurements may also undergo such global fluorescence intensity decrease by ASCn reduction, but the reported

parameters such as Laurdan GP and DPH anisotropy are calculated form relative intensity measurements, balancing this effect.

4. Conclusions

The ASCn, used in different pharmacological preparations due to its amphiphilic nature and anti-oxidative power, have shown subtly differences on therapeutic effectiveness [7–9,27]. Those differences may be related to differential interaction of ASCn with cell membranes. Our study demonstrated that ASCn compounds, which vary in the length of the acyl chain from 12 to 16 carbon atoms, develop different effects on phospholipid vesicles used as biomembrane models (see Table 1). A similar chain-length dependent effect was described in early studies of free fatty acid interaction with cell membranes. For instance, C12-C14 (medium chain-length) show a concentration-dependent biphasic behavior, with protection against hypo-osmotic hemolysis at a low concentration range (10-100 μM) and inducing subsequent hemolysis at high concentrations (0.2 to 1 mM range) [28]. On the other hand, long-chain saturated fatty acids only show the stabilization effect in the micromolar range [29]. The overall effect evidenced a favorable interaction of fatty acids with membranes and some membrane perturbation capacity in the same concentration range that was found for ASCn. Furthermore, similar to the present work, for free fatty acids the differences in the effects observed were explained as a consequence of different partition extent of the amphiphiles into the membranes [28] .Among the compounds studied in the present work, ASC12 is the only that forms small structures as well as large lamellar particles in aqueous solution (see Figure 1c). The presence of those loose small structures may be of importance in the loading of other hydrophobic drugs [7] and also responsible of a rapid molecular exchange with biomembranes. ASC12 appeared able to partition into the liposome surface, but not deeply penetrate it. In addition, it shows considerable effects on membrane tension decrease and release of vesicular content, a capacity that may enhance hydrophobic drug delivery [7,9]. ASC14 appeared as the most efficient membrane-perturbing drug (Table 1), with high incorporation extent and being able to laterally mix with the lipid bilayer (no phase separation is observed). As far as we know, no pharmacological approach has been reported using this compound, but from our results we can expect that there will be a compromise between the anti-oxidant effect localized at the membrane level and the considerable destabilization of the lipid bilayer.

On the other hand the commercially-available ASC16, the ASCn family member mostly used in pharmacy, showed important membrane restructuring properties with the additional feature of being able to form drug-enriched condensed domains once incorporated into the lipid

membrane. This, together with an aqueous organization as large and highly compacted structures (Figure 1 and [11]), may account for its good adjuvant capacity recently reported for ASC16 coagels [8]. In addition, the effect of these coagels, when used as adjuvants, suggests a local inflammatory response induced by ASC16 as a main feature responsible for the obtained immunization. Evidences of local necrosis in the injection area supported this hypothesis. This picture may be better understood on the light of our results. ASC16 can be slowly released from coagel structures and integrated into cell membranes, where it can also accumulate, and laterally separate into rigid domains acting as reservoirs of the drug. The moderate membrane destabilization and enhanced permeation may attempt against cell viability at high drug concentrations (near the injection site). In a similar place, the shorter ASCn compound ASC14, with an enhanced membrane perturbation capacity, may go further and threaten animal survival.

Finally, our results suggest that ASCn accumulation into the biomembrane enhances its capacity as reducer (or antioxidant) because of the site-specific drug localization. This supports previous reports that evidence an enhanced bactericidal capacity for the ASCn compounds in comparison with the addition of soluble ASC together with the corresponding fatty acid [9].

In summary, the ASCn family shows biomembrane perturbation activity in different extent depending on the length of its acyl chain. The differential activity for each ASCn found in the present work may account for a characteristic pharmacological potential and also may contribute for the design of new applications that have biological interfaces as a main target for these types of drugs.

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Contributors. F.G. developed the experimental work, M.M. synthetize and purified the non-commercial ASCn. E.E.A and M.L.F. designed the project, and performed or guided the experimental process. All the authors helped in the writing and discussion of the work and approved the final version of the manuscript.

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Legends to figures

Figure 1. Restructuration of POPC LUVs by ASCn incorporation. Size distribution over time of POPC LUVs (100μM) analyzed by dynamic light scattering (DLS) before (t=0) and after being exposed to ASC16 (a), ASC14 (b) or ASC12 (c) (1:1 drug:lipid molar ratio). Gray dashed lines and symbol represent the size distribution of the corresponding aqueous ASCn suspension. The Frequency axes indicate the relative distribution ranging 0 to 1 for all cases. Error bars correspond to the Standard Error of the Mean (SEM) of six independent experiments.

Figure 2. Effect of ASCn on membrane permeability: Percent of carboxyfluorescein release (CF%; see eq. 3) from POPC LUVs, 45 min after ASCn (Red: ASC12; Green: ASC14; Blue: ASC16) addition. The final lipid concentration was 100 μM. Error bars represent the SEM of two independent experiments.

Figure 3. Changes in Laurdan generalized polarization (GP) induced by ASCn. POPC LUVs (100 μM) containing laurdan (0.5 mol%) were incubated with the ASC12 (red), ASC14 (green) or ASC16 (blue) for 45 minutes. GP (eq. 1) was plotted as a function of ASCn final concentration (panel a). Panel (b) shows the GP maximal increase after ASCn incorporation and panel (c) the drug concentration needed to reach 50% of the GP increase, obtained by fitting a hyperbolic function of the type: $y=maxGP.x/([ASCn]_{50}+x)$ to the experimental data in (a) . Error bars represent the SEM of two independent experiments.

Figure 4. DPH anisotropy changes induced by ASCn. POPC LUVs containing DPH (0.5 mol%) were treated with ASC12 (red), ASC14 (green) or ASC16 (blue) and incubated for 45 minutes. DPH anisotropy was calculated (eq. 2) and plotted as a function of ASCn final concentration (panel a). Panel (b) show the maximal anisotropy increase after ASCn incorporation and panel (c) the drug concentration

needed to reach 50% of the anisotropy increase, obtained by fitting a hyperbolic function of the type: $y=max.Anisotropy.x/([ASCn]_{50}+x)$ to the experimental data in (a) . Error bars represent SEM of two independent experiments.

Figure 5. Confocal Microscopy of POPC GUVs doped with 0.5 mol% of Rho-PE and treated with ASC12 (a), ASC14 (b) or ASC16 (c-d). (a-c) show fluorescence confocal views (upper panels) and DIC microscopy (lower panel) of the equatorial section of GUVs at the indicated time after ASCn addition. Fluorescence images at long incubation times (right panels in a,b and c) were bright and contrast enhanced in order of counteract the chemical bleaching developed by ASCn, as shown in Figure 6. Panel (d) shows apical views of the GUVs treated with ASC16. Scale bars are 5 μM.

Figure 6. Rho-PE fluorescence decay at the GUV membrane after ASCn incorporation. Panel (a) shows representative time curves of normalized GUV membrane fluorescence intensity after incorporation of ASC12 (red), ASC14 (green) or ASC16 (blue) and without ASCn (black). Control experiments with soluble ascorbic acid (ASC) are also shown (grey line). Panel (b) show the statistical data of the fluorescence decrease at 5 min after ASCn addition. Error bars represent SEM of 4 - 6 vesicles from two independent experiments.

Figure 1

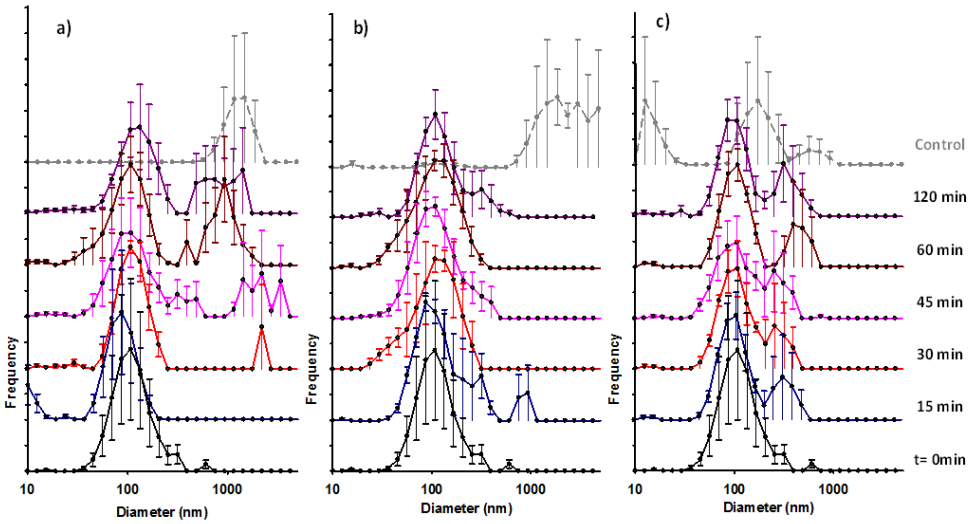


Figure 2

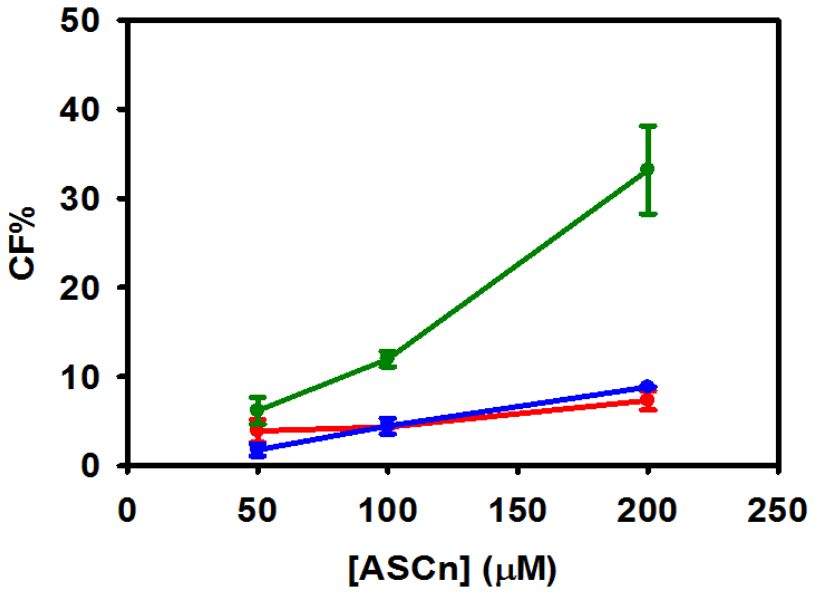


Figure 3

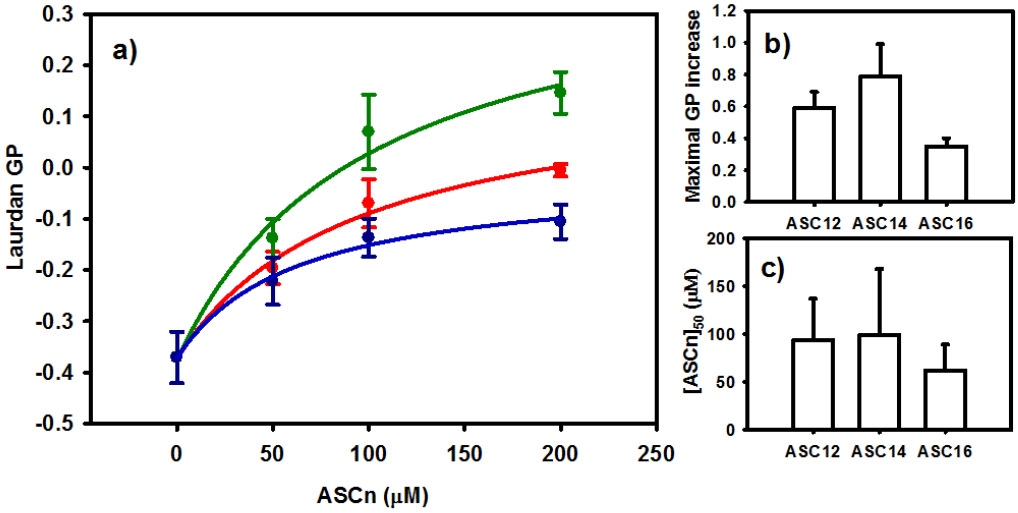


Figure 4

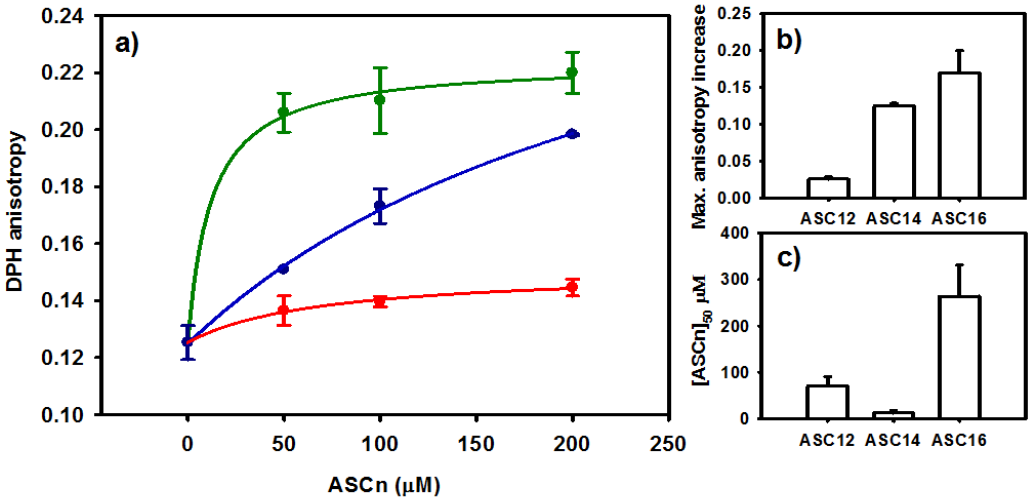


Figure 5

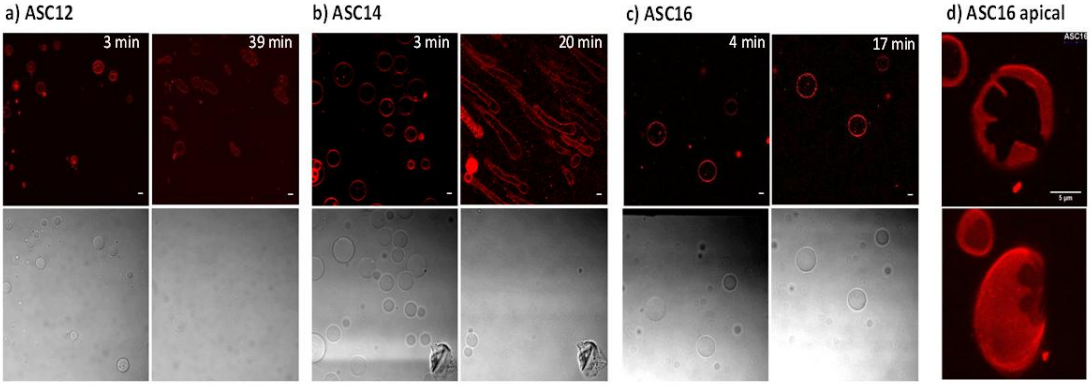


Figure 6

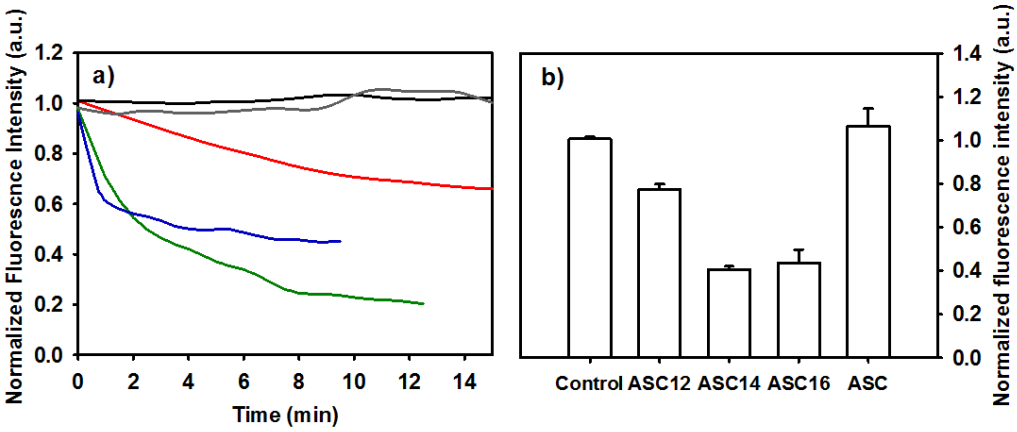
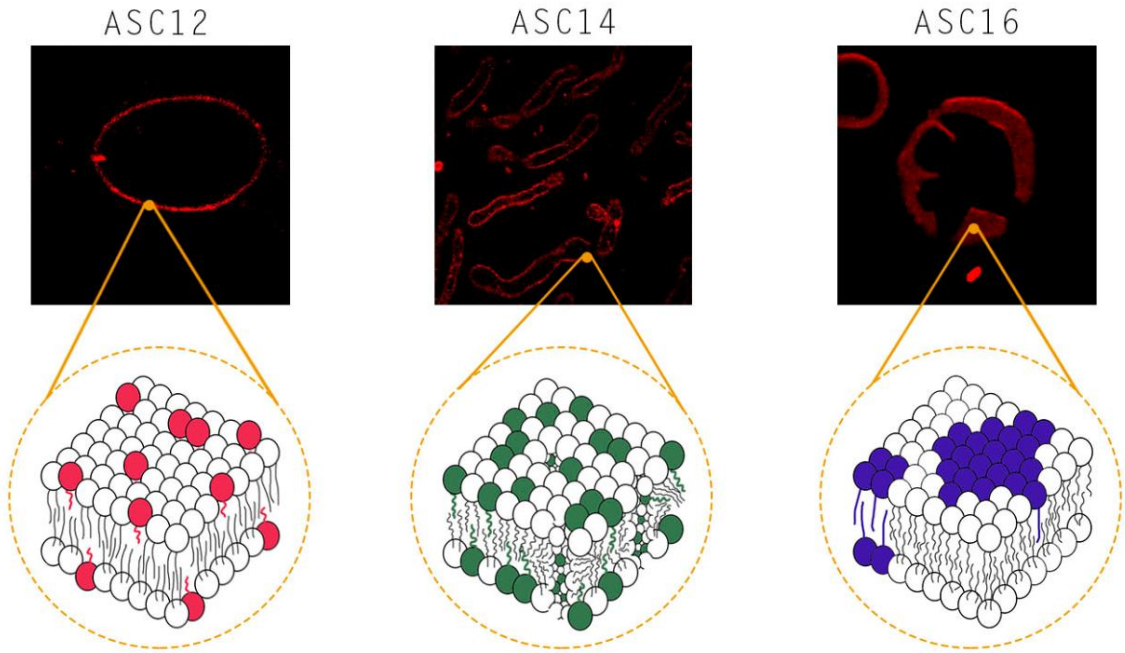


Table 1. Summary of the activity of ASCn family at the membrane level.

Membrane activity	**ASC12**	**ASC14**	**ASC16**
Detergent effect on POPC MLVs (soluble phosphorus determination)	-	-	-
Changes in the size distribution of LUVs (DLS)	-	++	+++
LUVs content leakage (CF release)	-	++	-
Increased LUVs surface water structuring (Laurdan GP)	++	+++	+
Increased microviscosity of the hydrophobic bilayer core (DPH anisotropy)	-	+++	++
Changes in membrane tension and GUV shape elongation (confocal microscopy)	++	+++	+
GUVs content leakage (DIC microscopy)	++	+++	+
Condensed domains occurrence in GUVs (confocal mic.)	-	-	+++
Reduction of fluorescence intensity of GUVs (confocal mic.)	+	+++	+++



Graphical abstract

Highlights

L-ascorbic acid alkyl esters (ASCn)develop different effects on POPC vesicles

ASC12 partitions scarcely and superficially into the bilayer affecting its properties

ASC14 strongly modulates the membrane tension, permeability and rheology

ASC16 forms drug-enriched condensed domains in the lipid membrane

ASCn accumulation into the biomembrane enhances its local capacity as antioxidant