

Is the amphiphilic carrier structure relevant for α -tocopherol anti-peroxidation efficiency in mitochondrial membranes?

Chiarmoni, N. S.^{1,*}, Duarte, E. L.², Marsanasco, M.¹, Prieto, M. J.¹, Lamy, M. T.², Alonso, S. del V.¹

¹Laboratorio de Biomembranas, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes; Bernal, Buenos Aires
IMBICE-CONICET; Calle 526 y Camino General Belgrano, (B1906APO), La Plata, Argentina

²Instituto de Física, Universidade de São Paulo, CP 66318, CEP 05314-970, São Paulo, Brazil

Email address

nschiara@unq.edu.ar (Chiarmoni, N. S.)

To cite this article

Chiarmoni, N. S., Duarte, E. L., Marsanasco, M., Prieto, M. J., Lamy, M. T., Alonso, S. del V.. Is the Amphiphilic Carrier Structure Relevant for α -Tocopherol Anti-Peroxidation Efficiency in Mitochondrial Membranes. *American Journal of Biological Chemistry*. Vol. 2, No. 1, 2014, pp. 1-7.

Abstract

A study involving liposome- α -tocopherol organization and lipid peroxidation was carried out to contribute to the understanding of the correlation between the structure of the α -tocopherol carrier and the vitamin antioxidant activity in a mitochondrial membrane. Mitochondrial membranes were used as substrates for lipid peroxidation. α -tocopherol was incorporated in liposomes composed by 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at different α -tocopherol concentrations: 1, 5, 10 and 20 mol% relative to DPPC. DPPC membrane packing was studied by Electron Spin Resonance (ESR) of spin labels incorporated into the liposomes and differential scanning calorimetry (DSC). Particle sizes were monitored by light scattering. As expected, ESR and DSC results revealed that α -tocopherol decreases DPPC rigidity when the bilayer is at the gel phase, and the gel-fluid transition is widened. Moreover, the presence of α -tocopherol in DPPC liposomes decreases mitochondrial peroxidation. Surprisingly, in the case of 10 and 20 mol% of α -tocopherol the decrement was found to be lower than with 1 and 5 mol%, with 1 mol% of α -tocopherol producing the best anti-peroxidant activity in mitochondrial membranes. In parallel, ESR and DSC data showed that with 1 mol% of α -tocopherol, at 30 °C, temperature at which the lipid peroxidation assay is performed, the DPPC bilayer is still quite packed, at gel state. At the same temperature, for concentrations of 5 mol% and above, DPPC enters in a broad gel-fluid transition, resulting in a less cooperative process. These findings could be related to the position of α -tocopherol active site nearby DPPC membrane surface. Accordingly, the activity was higher when DPPC membrane was more packed, in the gel phase; hence, the α -tocopherol active site would be more exposed, increasing the probability of α -tocopherol/free radical interaction; thus, decreasing mitochondrial membrane peroxidation. On the other hand, particle size analysis suggests that DPPC dispersions with higher α -tocopherol concentrations (above 5 mol%) are more aggregated than with 1 mol%. That, could also be relevant to the α -tocopherol antioxidant activity, as more α -tocopherol molecules could be exposed to the surface at lower α -tocopherol concentration. Hence, the present work shows that α -tocopherol anti-peroxidation activity in mitochondrial membranes is higher when the molecule is more diluted in DPPC membranes, the latter functioning major as a drug-carrier. That could be either related to the packing of DPPC vesicles and/or to their aggregation in the presence of higher α -tocopherol concentrations.

Keywords

Liposomes, α -Tocopherol, Peroxidation, EPR, DSC, Carrier Structure, Light Scattering, Mitochondrial Membrane

1. Introduction

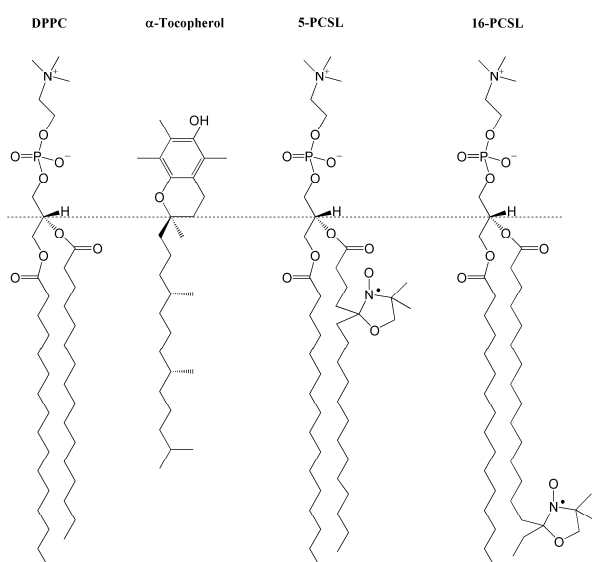
Vitamin E is a generic term including many tocol and

tocotrienol compounds, whose most active form is α -tocopherol (Scheme 1). Several roles of vitamin E have been reported, such as antioxidant, intermediary in arachidonic acid

and prostaglandin metabolism, mitochondrial function, sex hormones production, maintaining the integrity of membranes, protection against hemolytic anemia, impaired erythropoiesis and reducing the risks of heart diseases. Also, preventing cancer, neurological diseases, cataract, retinopathy of premature infants, arthritis, and may be involve in slowing down the aging process in humans or animals [1].

Tocopherol and tocotrienol react with free-radical molecular oxygen and act like antioxidant [2]. They can quench free radicals and act as terminator of lipid peroxidation [3, 4]. The vitamin E radical may be reduced back to vitamin E by ascorbate [5-7] and glutathione [4].

However, there still exist many questions about certain mechanisms involving α -tocopherol. Atkinson *et al.* in 2008 proposed: "Any rationalization of the antioxidant activity of tocopherols must account, by some structurally relevant mechanism, for tocopherol's ability to access both peroxidizing unsaturated lipids in membranes as well as aqueous like ascorbate" [8].



Scheme 1. Chemical structures of DPPC, α -Tocopherol and spin labels 5- and 16-PCSL.

According to Afri *et al.* (2004), for exerting its antioxidant activity, α -tocopherol must have its C-6 hydroxyl hydrogen close to the interface [9]. Other authors also discuss the location of tocopherol in membranes composed by POPC. They reported that α -tocopherol lies perpendicular to the plane of the phospholipid bilayer, with the rigid chromanol ring at the polar headgroup region where the phenoxyl hydrogen group with the possibility of being hydrogen-bonded to either the carbonyl or phosphate oxygen of the phospholipid molecule [10].

Regarding the concentration of tocopherol in a bilayer, some authors have proposed that α -tocopherol can be incorporated in a limited amount only, and that beyond 10 mol% (relative to the lipid), there would be phase separation [11]. It is also interesting to mention that tocopherol exhibits a behavior similar to that of cholesterol [12], broadening the gel-fluid transition of phospholipids, and shifting the

transition to lower values. The broadening of the main transition increases with α -tocopherol content, as lipid-lipid cooperativity decreases [13].

Currently, one of the concerns is to elucidate structure, structure/function and/or mechanisms in the case of liposomes carrying α -tocopherol. The key relevant question that still remains is whether the packing and organization of the α -tocopherol carrier has some relationship to its antioxidant activity in a different system.

Mitochondria are important cell organelles that are ideal for using as substrate to measure lipid peroxidation. They have two membranes: the outer and inner membrane. The inner mitochondrial membrane significantly differs from the outer one due to its high protein content [14]. ATP synthesis is perhaps the most important function of mitochondria. The inner mitochondrial membrane is required for the cellular synthesis of ATP by a pathway involving several proteins, commonly known as oxidative phosphorylation. These proteins need a specific lipid cluster around them. This process is an example of an important cellular function that could not occur without a membrane. The integrity of the membrane is crucial for many vital cell functions.

The present work studies the correlation between the structure of 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes, used as α -tocopherol carrier, and the vitamin antioxidant activity in mitochondrial membranes. DPPC membrane packing was monitored by Electron Spin Resonance (ESR) of spin labels incorporated into the bilayers and differential scanning calorimetry (DSC). Particle size distribution was monitored by light scattering.

2. Materials and Methods

2.1. Materials

The phospholipid 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Scheme 1) and spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, *n* = 5 and 16, Scheme 1) were purchased from Avanti Polar Lipids (Alabama, USA). α -Tocopherol (Scheme 1) was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were used without further purification. Milli-Q water was used throughout.

2.2. Liposome Preparation

Liposomes were prepared following the method described before by Bangham *et al* [15]. Firstly, DPPC was dissolved in chloroform and the solvent was dried under a stream of N_2 and left under reduced pressure for a minimum of 2h, to remove traces of the organic solvent. Then the lipid film was hydrated with Milli-Q water in order to form multilamellar vesicles (MLVs). Milli-Q water was used, according to our previous work [16]. Total lipid concentration was 10 mM.

For ESR measurements, spin labels were added to the lipid chloroform solution at 0.2 mol% (16-PCSL) or 0.5 mol% (5-PCSL), relative to the lipid concentration. Those were found to be the maximum spin label concentrations to

display no spin-spin interaction.

2.3. α -Tocopherol Incorporation

Stock solution of α -tocopherol was prepared in ethanol, and its concentration was determined by absorption spectroscopy at $\lambda=295$ nm, considering the α -tocopherol molar extinction coefficient $\epsilon = 3058 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [17]. Due to its hydrophobic character, α -tocopherol was added to the lipid chloroform solution before evaporation, in the following concentrations: 1, 5, 10 and 20 mol% relative to DPPC concentration.

2.4. Purification of Liver Mitochondria

Mitochondria were obtained from hamster's liver. Briefly, 7 grams of liver tissue were suspended in 35 ml of saline solution. Then, they were homogenized using a Potter Elvehjem. In order to extract nucleons, homogenate was centrifuged for 10 minutes at 1,000x g and supernatant was collected and centrifuged for 10 minutes at 11,000x g, the so formed pellet contained mitochondria. It was suspended in 10 ml of saline solution and stored at -20°C until further use [18].

2.5. Efficiency of Peroxidation Protection

Reconstituted mitochondria were used as substrate for lipid peroxidation. They were mixed with fixed concentration of DPPC liposomes and with different concentrations of α -tocopherol. Same mitochondrial batch was used in all lipid peroxidation determinations in order to avoid dispersion due to a different oxidizable lipid concentration. Samples with no α -tocopherol and DPPC were taken as a control of 100% peroxidation. Then, the procedure was completed by determining lipid peroxidation from each sample separately.

2.6. Determination of Lipid Peroxidation

The lipid peroxidation index was determined following the thiobarbituric acid method (TBA) [19]. Briefly, to 0.05 ml of mitochondria, 0.125 ml of Tris-maleate buffer were added (10 mM, pH 7) and 0.025 ml of NaCl 3M. Liposomes and α -tocopherol were prepared as described above, and 0.3 ml of a 5 mM lipid suspension was added to mitochondria. Sample of 100 % peroxidation contained buffer and NaCl. Samples were incubated at 30°C for 20 minutes. Then, a pre-induction was accomplished by adding 10 μl of 1 mM FeSO_4 and 20 μl of 1 mM ascorbic acid and incubated for 20 minutes at 30°C . Then, 10 μl of 1 mM FeSO_4 and 80 μl of 1 mM ascorbic acid were added and samples were incubated for 15 minutes at 30°C . Finally, a 250 μl sample aliquot was combined with 1 ml of TBA reagent solution, and heated for 15 minutes in a boiling water bath. After cooling, the pellet was removed by centrifugation at 12,000 rpm during 6 minutes. Sample absorbance was measured at 535 nm and the concentration of the thiobarbituric acid reactive species (TBARS) was calculated using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [19].

2.7. Electron Spin Resonance Determinations (ESR)

ESR measurements at X band were performed with a Bruker EMX spectrometer. Sample temperature was fixed at 30°C and controlled within 0.1°C by a Bruker BVT-2000 controller-temperature device. ESR data was acquired immediately after sample preparation. A field modulation of 1G and microwave power of 10 mW was used.

All data shown are means of at least two experiments, and the uncertainties are the standard deviations. When not shown, the uncertainties are smaller than the size of the symbols.

2.8. Differential Scanning Calorimetry (DSC)

DSC traces were obtained by heating the samples from 20 to 50°C , with a Microcalorimeter VP-DSC (MicroCal, Northampton, MA, USA), at $10^{\circ}\text{C}/\text{h}$ (identical traces were found at $5^{\circ}\text{C}/\text{h}$). Baseline subtractions and peak integrals were performed using the MicroCal Origin software with the additional module for DSC data analysis provided by MicroCal, as described before [20]. Data shown are representative of at least two different experiments.

2.9. Particle size Distribution (DLS)

Particle size distribution was determined in the range 0.1–1000 μm by laser scattering using a Particle Analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd, UK), as previously described [16]. The liposomal suspensions were diluted in 500 mL Milli-Q water. The dispersion was carried out at 2000 rpm and the degree of obscuration was between 10 and 12%. Sauter mean diameter (D3,2) and De Brouker mean diameter (D4,3) were used as simultaneous parameters. D3,2 and D4,3 are the mean diameters from the surface and volume distributions, respectively. These parameters were used previously in order to characterize the size of liposome population [16] and, more extensively, emulsions [21–24].

3. Results and Discussions

3.1. Membrane Peroxidation

Results corresponding to mitochondrial peroxidation are shown in Table 1, where the control, 100% peroxidation, was obtained in the absence of both α -tocopherol and DPPC liposomes. The incorporation of 1 mol% (relative to DPPC concentration) of α -tocopherol in DPPC liposomes caused a significant decrease in mitochondrial lipid peroxidation: only 8% of the value found in control samples (mitochondria without DPPC and/or α -tocopherol). Interestingly, when α -tocopherol concentration was increased to 5 mol%, instead of increasing the anti-oxidation effect, mitochondrial membrane peroxidation increased to 12% (see Table 1). Accordingly, in the presence of even higher α -tocopherol concentrations, 10 and 20 mol% of α -tocopherol in DPPC liposomes, there

was a decrease in mitochondrial peroxidation with respect to the control, but this decrement was much smaller than the one obtained with 1 mol% or even 5 mol% α -tocopherol: 47 and 30 % of peroxidation in the cases of 10 and 20 mol % in DPPC liposomes, respectively.

Table 1. Mitochondrial Peroxidation

α -tocopherol incorporation(mol%)	Membrane peroxidation(%)
0	78 \pm 3
1	8 \pm 1**
5	12 \pm 1**
10	47 \pm 18*
20	30 \pm 10*

Data is presented as mean \pm SD of four independent measurements.

*: Significant differences with respect to samples with α -tocopherol and without lipids after performing Tukey-Kramer Test($p < 0.01$)

** : Significant differences with respect to samples with α -tocopherol and without lipids after performing Tukey-Kramer Test($p < 0.001$)

It is worth mentioning that DPPC membranes without α -tocopherol also exhibited the ability to avoid peroxidation in mitochondria. Around 22 % remained without being oxidized (see Table 1). This peroxidation protection is probably due to DPPC intercalation into mitochondrial membrane, incrementing the percentage of saturated lipids, hence, increasing the rigidity of the mitochondrial membrane, and affecting the peroxidation efficiency. Other authors reported that in soy phosphatidylcholine membranes, peroxidation was avoided when a saturated lipid such as DPPC was added and this decrement was due to a decrease in the water efflux across lipid membrane [25].

To try to understand the data presented above, which showed that 1 mol% of α -tocopherol in DPPC liposomes is more efficient in preventing mitochondrial peroxidation than higher α -tocopherol concentrations, and knowing that α -tocopherol alters the lipid bilayer structure [8], a structural study of DPPC- α -tocopherol membranes was important to achieve. It was carried out by performing ESR spectroscopy with different spin labels incorporated into the liposomes, combining with light scattering and DSC.

3.2. ESR Data

Figure 1 shows ESR spectra of 5-PCSL and 16-PCSL in pure DPPC bilayers, compared with those yielded by labels in DPPC with 1, 5, 10 and 20 mol % of α -tocopherol, at 30 °C (the same temperature and α -tocopherol/DPPC relative concentrations used in the mitochondrial peroxidation assay). At this temperature, a pure DPPC bilayer is at gel state. Accordingly, the spectra of 5- and 16-PCSL in pure DPPC bilayers are typical of spin labels in a gel phase bilayer. As expected, due to the flexibility gradient towards the bilayer core, the spectrum of 5-PCSL in DPPC membrane is much more anisotropic than that of 16-PCSL (larger A_{max} parameter, indicated in Fig. 1, top spectra),

showing that the movement of the nitroxide moiety in 5-PCSL is more restricted than that in 16-PCSL, as 5-PCSL monitors the membrane hydrocarbon chains closer to the bilayer surface.

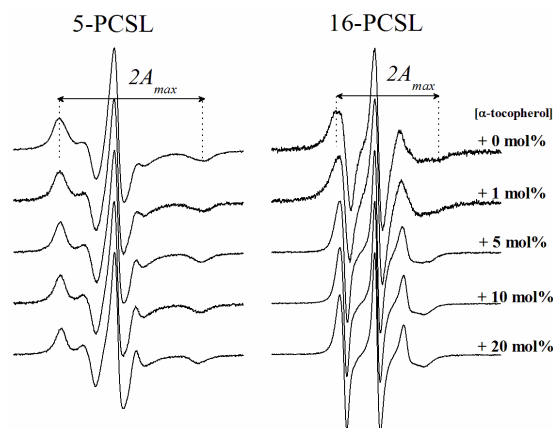


Figure 1. X-band ESR spectra of 5- and 16-PCSL incorporated in 10 mM DPPC in the absence and presence of different α -tocopherol concentrations (mol% relative to DPPC), at 30 °C. Total spectra width 100 G.

Though some qualitative conclusions can be drawn from the spectra, the best parameter to comparatively analyze the ESR spectra of DPPC, with and without α -tocopherol, is the maximum hyperfine splitting, A_{max} . This parameter increases with the label microenvironment viscosity or packing [26], and can be directly measured in the ESR spectra of both labels at 30 °C, as indicated in Fig. 1.

Both labels report a decrease in DPPC bilayer packing as the α -tocopherol concentration increases (decrease in A_{max} in Fig. 2). At the bilayer core, monitored by 16-PCSL, there is no distinction between the presence of 10 or 20 mol% of α -tocopherol, though 5-PCSL indicates a further increase in bilayer fluidity from 10 to 20 mol%.

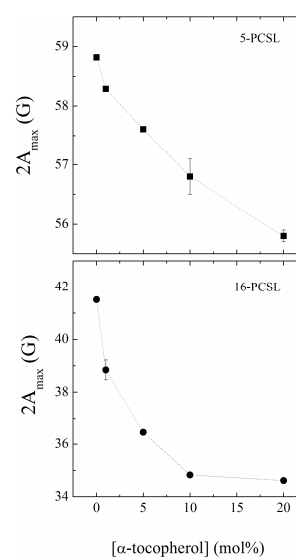


Figure 2. Outer hyperfine splitting ($2A_{max}$), measured on ESR spectra of 5- and 16-PCSL incorporated in DPPC, as a function of the α -tocopherol relative concentration (mol% relative to DPPC).

Hence, analyzing the above ESR data, which give an average value of the DPPC bilayer packing with different α -tocopherol concentrations, one could infer that an effective anti-peroxidation action of α -tocopherol on mitochondrial membranes requires a particular DPPC bilayer packing achieved around 1 mol% of α -tocopherol: in a more fluid DPPC membrane, α -tocopherol is less active.

Even though packing has been analyzed by ESR, we confirmed the membrane state by an independent methodology (DSC).

3.3. DSC Data

For a better understanding of the alterations caused by α -tocopherol on DPPC membranes, DSC scans were performed with DPPC membranes containing the same α -tocopherol relative concentrations used for the peroxidation and ESR studies (Fig. 3).

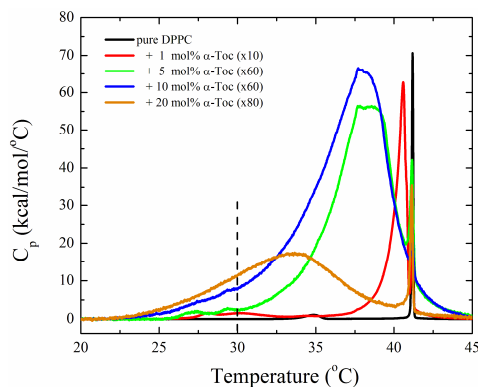


Figure 3. DSC heating scans of 10 mM DPPC in the absence and presence of different concentrations of α -tocopherol. The traces for DPPC in the presence of α -tocopherol were amplified, as indicated; hence the C_p scale on the left is related to pure DPPC only. The dashed line indicates the temperature at which the peroxidation assay was performed (30 °C).

The present work focuses on DPPC membranes at 30 °C: the temperature at which the peroxidation assay was carried out. As mentioned above (ESR section), and can be seen in Fig. 3, at this temperature pure DPPC is at the gel phase. DPPC main gel-fluid transition is at 41.2 °C. The addition of 1 mol% of α -tocopherol broadens the DPPC main transition, and slightly decreases its transition temperature (Fig. 3). Still, at 30 °C, the bilayer is at the gel phase. For DPPC with 5 mol% of α -tocopherol, the temperature of the main transition decreases even further (down to 38.9 °C), together with a huge broadening: apparently, at 30 °C the bilayer gel-fluid transition has already started. Due to the even larger broadening caused by 10 and 20 mol% of α -tocopherol, DPPC- α -tocopherol bilayers are certainly much more fluid at 30 °C than those with lower α -tocopherol concentrations.

3.4. Particle Size Distribution

Particle size distributions of DPPC with different concentrations of α -tocopherol are shown in figure 4.

Distribution is expressed as percentage of area or D3,2 (Figure 4 A) and percentage of volume or D4,3 (Figure 4B). Mean values of each distribution are depicted in the graphs as well. The surface particle size distribution was bimodal and maintained nearby the same range values (1-10 μ m) for all concentrations, but volume percentage did vary with no definite trend in the range 1-100 μ m. It is important to mention that D4,3 is more sensitive to larger particles size than D3,2, thus an increase in aggregation is manifested by an increase in D4,3 values, while an increase of the population of smaller particles is evidenced by a decrement of the D3,2 values. If D3,2 remains constant and D4,3 shifts its maxima to higher values, that can be interpreted as an increment in the population of particles of larger size. In the case of liposomes, this can be conceived as an increase in aggregation [16].

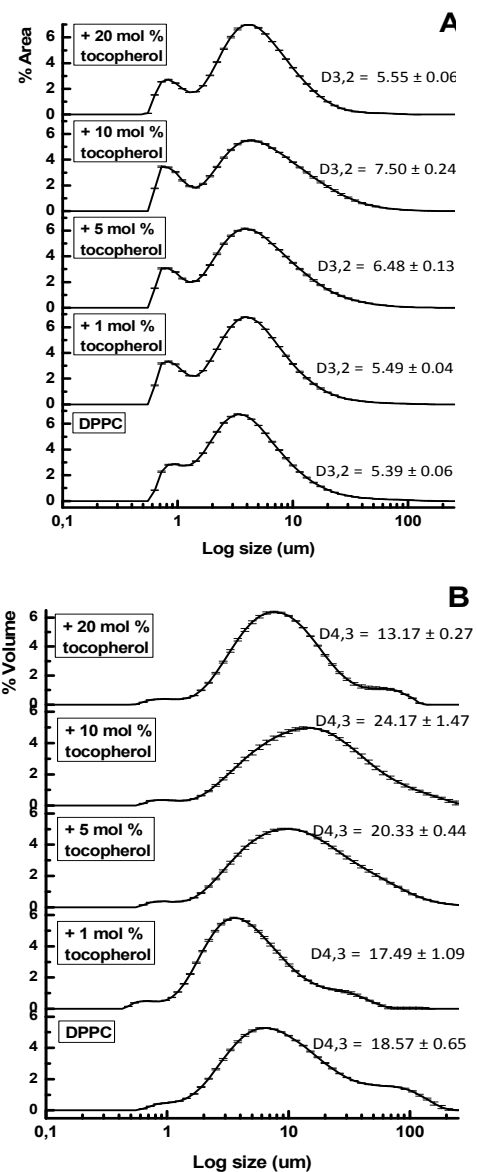


Figure 4. Particle size distribution of DPPC liposomes with different α -tocopherol concentrations, expressed as area (A) and volume (B) percentage. Data is presented as mean \pm SD of three measurements.

Addition of α -tocopherol induces no significant changes in maxima distribution and overall shape of D3, 2, for 1 to 20 mol%, though small increments in D3,2 mean distribution for 5 and 10 mol% was observed (Figure 4A), as compared to control. Regarding D4,3, a small decrement in liposome size was observed for 1 mol% of tocopherol, in comparison to control (Figure 4B): distribution maxima shifts to higher sizes. Besides, a more homogenous population is observed by a narrowing of the main distribution peak. With 5 and 10 mol%, an increment of both, mean distribution and a shift of peak maxima value were observed. Liposome populations became more heterogeneous, reflected in the increase of the width of the distribution peak. With 20 mol% of tocopherol, the maximum distribution peak and the same shape as the control were observed, although a small population of larger size was also observed.

In the case of 5 and 10 mol %, differences in liposome size could be due to aggregation, evidenced by an increase in D4,3, while D3,2 value is kept constant (figure 4). In this way, the active site of α -tocopherol could be blocked, and hence, less active than when there is 1 mol% present. On the other hand, in the case of 20 mol%, differences in liposome size could be due to the formation of enriched α -tocopherol domains. These domains would be due to α -tocopherol aggregates, and belong to a larger size population reflected in volume distribution values. D4,3 distribution, that correspond to 20 mol % of α -tocopherol, has slightly larger size than DPPC without vitamin and for both the D3,2 remain constant. However, liposome aggregation, in the case of 20 mol %, was lower in comparison with 5 and 10 mol %. α -tocopherol domains were also reported by Wang and Quinn, who discuss the presence of domains enriched with α -tocopherol in POPC membranes with vitamin concentrations higher than 10 mol% [11], in accordance with our light scattering results. In the so obtained population, the α -tocopherol active site would be blocked due to particle aggregation, not allowing the chromanol ring active site available to free radicals.

4. Conclusions

Obtained results here strongly suggest that DPPC membrane organization has an important role in the activity of α -tocopherol in mitochondrial membranes. The best anti-oxidation effect was obtained with 1 mol% of α -tocopherol in DPPC liposomes, and not with higher vitamin concentrations. Moreover, ESR of spin labels incorporated into the membranes and DSC clearly shows that DPPC membranes, at 30 °C, are significantly more packed with 1 mol% of α -tocopherol, than with higher vitamin concentrations. Studies of particle size distribution revealed that DPPC vesicles with 1 mol % of α -tocopherol are less aggregated in comparison with the other α -tocopherol concentrations analyzed.

Results conveyed in suggesting that for higher α -tocopherol concentrations, in a DPPC more fluid

environment, some of the vitamin molecules could be localized deeper in the bilayer, and some gathered forming enriched α -tocopherol aggregates, such that α -tocopherol active sites would not be available, lowering anti-oxidation activity. Moreover, liposome aggregation, observed with higher α -tocopherol concentrations, may also prevent α -tocopherol exposition to the medium.

In all, the present study contributes to the general understanding of the correlation between the α -tocopherol/carrier organization and the antioxidant activity of the vitamin in mitochondrial membranes. Certainly, other α -tocopherol carriers should be tested for a more comprehensive analysis.

References

- [1] M.L. Thakur, U.S. Srivastava, Vitamin-E metabolism and its application, *Nutrition Research*, 16 (1996) 1767-1809.
- [2] H. Sies, W. Stahl, A.R. Sundquist, Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids, *Annals of the New York Academy of Sciences*, 669 (1992) 7-20.
- [3] G.W. Burton, A. Joyce, K.U. Ingold, Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes?, *Archives of biochemistry and biophysics*, 221 (1983) 281-290.
- [4] C.K. Chow, Vitamin E and blood, *World review of nutrition and dietetics*, 45 (1985) 133-166.
- [5] D.C. Liebler, J.A. Burr, Oxidation of vitamin E during iron-catalyzed lipid peroxidation: evidence for electron-transfer reactions of the tocopheroxyl radical, *Biochemistry*, 31 (1992) 8278-8284.
- [6] P.B. McCay, Vitamin E: interactions with free radicals and ascorbate, *Annual review of nutrition*, 5 (1985) 323-340.
- [7] J.M. May, S. Mendiratta, Z.-C. Qu, E. Loggins, Vitamin C recycling and function in human monocytic U-937 cells, *Free Radical Biology and Medicine*, 26 (1999) 1513-1523.
- [8] J. Atkinson, R.F. Epand, R.M. Epand, Tocopherols and tocotrienols in membranes: a critical review, *Free radical biology & medicine*, 44 (2008) 739-764.
- [9] M. Afri, B. Ehrenberg, Y. Talmon, J. Schmidt, Y. Cohen, A.A. Frimer, Active oxygen chemistry within the liposomal bilayer. Part III: Locating Vitamin E, ubiquinol and ubiquinone and their derivatives in the lipid bilayer, *Chemistry and physics of lipids*, 131 (2004) 107-121.
- [10] J.B. Massey, Interfacial properties of phosphatidylcholine bilayers containing vitamin E derivatives, *Chemistry and physics of lipids*, 109 (2001) 157-174.
- [11] X. Wang, P.J. Quinn, The distribution of alpha-tocopherol in mixed aqueous dispersions of phosphatidylcholine and phosphatidylethanolamine, *Biochimica et biophysica acta*, 1509 (2000) 361-372.
- [12] T.P. McMullen, R.N. McElhaney, New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry,

- Biochimica et biophysica acta, 1234 (1995) 90-98.
- [13] A. Ortiz, F.J. Aranda, J.C. Gómez-Fernández, A differential scanning calorimetry study of the interaction of α -tocopherol with mixtures of phospholipids, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 898 (1987) 214-222.
- [14] P.L. Yeagle, *The membranes of cells*, Second edition ed.(Academic Press, USA, 1993).
- [15] A.D. Bangham, *Model membranes*, *Chemistry and physics of lipids*, 8 (1972) 386-392.
- [16] M. Marsanasco, A.L. Márquez, J.R. Wagner, S. del V. Alonso, N.S. Chiaramoni, Liposomes as vehicles for vitamins E and C: An alternative to fortify orange juice and offer vitamin C protection after heat treatment, *Food Research International*, 44 (2011) 3039-3046.
- [17] J.A. Mendiola, D. García-Martínez, F.J. Rupérez, P.J. Martín-Álvarez, G. Reglero, A. Cifuentes, C. Barbas, E. Ibañez, F.J. Señoráns, Enrichment of vitamin E from *Spirulina platensis* microalga by SFE, *The Journal of Supercritical Fluids*, 43 (2008) 484-489.
- [18] N. Lambert, R.B. Freedman, The latency of rat liver microsomal protein disulphide-isomerase, *The Biochemical journal*, 228 (1985) 635-645.
- [19] J.A. Buege, Aust, S.D., Microsomal lipid peroxidation, in: L. Packer, Fleisher, S. (Ed.) *Biomembranes*, Academic Press, USA, 1997, pp. 413-421.
- [20] K.A. Riske, R.P. Barroso, C.C. Vequi-Suplicy, R. Germano, V.B. Henriques, M.T. Lamy, Lipid bilayer pre-transition as the beginning of the melting process, *Biochimica et biophysica acta*, 1788 (2009) 954-963.
- [21] D.I. Comas, J.R. Wagner, M.C. Tomás, Creaming stability of oil in water (O/W) emulsions: Influence of pH on soybean protein-*lecithin* interaction, *Food Hydrocolloids*, 20 (2006) 990-996.
- [22] A.L. Márquez, A. Medrano, L.A. Panizzolo, J.R. Wagner, Effect of calcium salts and surfactant concentration on the stability of water-in-oil (w/o) emulsions prepared with polyglycerol polyricinoleate, *Journal of colloid and interface science*, 341 (2010) 101-108.
- [23] G.G. Palazolo, P.A. Sobral, J.R. Wagner, Freeze-thaw stability of oil-in-water emulsions prepared with native and thermally-denatured soybean isolates, *Food Hydrocolloids*, 25 (2011) 398-409.
- [24] G.G. Palazolo, D.A. Sorgentini, J.R. Wagner, Coalescence and flocculation in o/w emulsions of native and denatured whey soy proteins in comparison with soy protein isolates, *Food Hydrocolloids*, 19 (2005) 595-604.
- [25] M.A. Soto-Arriaza, C.P. Sotomayor, E.A. Lissi, Relationship between lipid peroxidation and rigidity in L- α -phosphatidylcholine-DPPC vesicles, *Journal of colloid and interface science*, 323 (2008) 70-74.
- [26] J.H. Freed, *Spin Labeling: Theory and Applications*, (Academic Press, New York, 1976).