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1 **Nitrone-Trolox conjugate as an inhibitor of lipid oxidation: Towards synergistic antioxidant effects**

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16

17 **Abstract**

18 Free radical scavengers like α -phenyl-*N-tert*-butylnitron (PBN) and 6-hydroxy-2,5,7,8-
19 tetramethylchroman-2-carboxylic acid (Trolox) have been widely used as protective agents in various
20 biomimetic and biological models. A series of three amphiphilic Trolox and PBN derivatives have been
21 designed by adding to those molecules a perfluorinated chain as well as a sugar group in order to
22 render them amphiphilic. In this work, we have studied the interactions between these derivatives and
23 lipid membranes to understand how they influence their ability to prevent membrane lipid oxidation.
24 We showed the derivatives better inhibited the AAPH-induced oxidation of 1,2-dilinoleoyl-*sn*-glycero-
25 3-phosphocholine (DLiPC) small unilamellar vesicles (SUVs) than the parent compounds. One of the
26 derivatives, bearing both PBN and Trolox moieties on the same fluorinated carrier, exhibited a
27 synergistic antioxidant effect by delaying the oxidation process. We next investigated the ability of the
28 derivatives to interact with DLiPC membranes in order to better understand the differences observed
29 regarding the antioxidant properties. Surface tension and fluorescence spectroscopy experiments
30 revealed the derivatives exhibited the ability to form monolayers at the air/water interface and
31 spontaneously penetrated lipid membranes, underlying pronounced hydrophobic properties in
32 comparison to the parent compounds. We observed a correlation between the hydrophobic
33 properties, the depth of penetration and the antioxidant properties and showed that the location of
34 these derivatives in the membrane is a key parameter to rationalize their antioxidant efficiency.
35 Molecular dynamics (MD) simulations supported the understanding of the mechanism of action,
36 highlighting various key physical-chemical descriptors.

37

38 **Key words:** model membranes; lipid oxidation; amphiphilic nitron; Trolox; antioxidant; synergism

39 1. Introduction

40 Reactive oxygen species (ROS) are produced in living organisms and are involved in several
41 important biological processes such as signaling pathways [1], antimicrobial defense [2] or cell
42 adhesion [3]. However, the deregulation of physiological processes as well as the exposure to
43 environmental factors like pollution, radiations or tobacco smoke may favor an overproduction of ROS
44 [4], which unbalance the redox status of cells and lead to oxidative stress. Because of their ability to
45 damage biomolecules, in particular membrane polyunsaturated lipids which are degraded through a
46 reaction named lipid peroxidation [5]; ROS are involved in the early stages of various diseases [6–8] as
47 well as the deterioration of cosmetic [9], food [10,11] and pharmaceutical supplies [12].

48 In order to limit the deleterious effects of ROS, living organisms use several antioxidant defense
49 systems that are either endogenous – mainly enzymes but also small molecules such as glutathione –
50 or exogenous [13]. Exogenous antioxidants, essentially polyphenols and vitamins, come from food. To
51 improve their properties, bioinspired molecules have been developed based on these natural
52 compounds. In this regard, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water
53 soluble analog of α -tocopherol, has been used to prevent apoptosis [14], *in vitro* or *ex vivo* ischemia
54 [15] and membrane lipid oxidation [16]. Trolox is also used as a reference in the evaluation of
55 antioxidant activities. Namely, the Trolox equivalent antioxidant capacity (TEAC) parameter measures
56 the potency of a given antioxidant. TEAC also allows the comparison of antioxidant efficiency of food
57 matrices and beverages, for which isolation of molecules can rarely be achieved [17,18]. Also,
58 because of their enhanced reactivity against free radicals, spin trapping agents can be used as free
59 radical scavengers [19]. Among them, α -phenyl-*N-tert*-butylnitron (PBN) is one of the most popular.
60 PBN was initially used in electron spin resonance (ESR) experiments for the detection of free radicals
61 under chemical and biological environments, however, it has later been used in biological models as an
62 antioxidant [20,21]. Although PBN has the advantage of being a non-toxic chemically stable antioxidant
63 with a half-life of six hours in animals [22], it presents like Trolox a poor ability to remain inside

64 membranes. To create antioxidants with a greater hydrophobicity and therefore a better affinity for
65 membranes, several molecules deriving from PBN and Trolox have been designed. For instance, Ayuso
66 *et al.* [23], as well as Choteau *et al.* [24], reported the synthesis of PBN derivatives bearing a
67 cholesterol moiety. In both cases, these authors demonstrated that compared to PBN, the derivatives
68 presented an enhanced protective activity against stroke and retina light-induced damages,
69 respectively. Besides the need of increasing the affinity for the membrane, a strategy that has gained
70 attention is to combine two (or more) antioxidant moieties in a single molecule [25]. In this regard,
71 Trolox was combined to lipoic acid [26], silybin [27] as well as a nitric oxide synthase inhibitor [28].
72 Along the same line, Trolox and PBN amphiphilic derivatives [29,30] have recently been synthesized.
73 They bear, on one hand, a perfluorinated chain to increase hydrophobicity without inducing a cytolytic
74 effect [31] and on the other hand, a sugar group to maintain water solubility (Fig. 1).

75 In the present work, we have studied the antioxidant properties of these derivatives related to
76 their capacity to interact with lipid membranes, which in turn has allowed unraveling their antioxidant
77 properties by using biomimetic systems. The antioxidant action at the membrane was evaluated by
78 performing Langmuir monolayers and lipid peroxidation inhibition assays. Surface tension and
79 fluorescence spectroscopy experiments showed the capacity of these derivatives to interact with
80 membrane lipids, with respect to the parent compounds (Trolox and PBN). The nature of the free-
81 radical scavenger moieties and their location in the membranes were analyzed as key parameters to
82 rationalize the antioxidant activity of these derivatives, which was supported by molecular dynamics
83 (MD) simulations.

84

85 2. Materials and methods

86 2.1. Chemicals

87 Vials of 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLiPC) powder were purchased from Avanti
88 Polar Lipids (Alabaster, AL, USA). 2,2'-azo-bis(2-amidinopropane)dihydrochloride (AAPH), 4-(2-
89 hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-
90 carboxylic acid (Trolox) and α -phenyl-*N*-*tert*-butylnitron (PBN) were purchased from Sigma (St. Louis,
91 MO, USA). FAPBN [29], FATx [29] and FATxPBN [30] were synthesized according to the procedure
92 previously published.

93 The water used in all assays was purified using a Millipore filtering system (Bedford, MA, USA),
94 yielding ultrapure water (18.2 M Ω ×cm). Stock solutions of DLiPC were prepared by dissolving the DLiPC
95 powder contained in the vials in chloroform or hexane/ethanol 9:1 (v/v) for the preparation of vesicles
96 and monolayers, respectively. The antioxidants were dissolved in chloroform and ethanol for the
97 experiments involving vesicles and monolayers, respectively. $\log P$ values were calculated with the
98 software ChemSketch version 14.01, Advanced Chemistry Development, Inc. (Toronto, ON, Canada).

99

100 2.2. Preparation of DLiPC vesicles

101 DLiPC alone (control assay) and DLiPC/antioxidant mixtures dissolved in chloroform were dried
102 under a stream of nitrogen and then kept under high vacuum for 2 hours to obtain a solvent-free film.
103 The dried film was then dispersed in Hepes buffer to obtain a solution of DLiPC multilamellar vesicles
104 (MLVs) at a concentration of 0.5 mg/mL. Small unilamellar vesicles (SUVs) were obtained by sonicating
105 MLVs to clarity (3 cycles of 2 min 30 s) with a 500 W titanium probe sonicator at 33 % of the maximal
106 power from Fisher Bioblock Scientific (Illkirch-Graffenstaden, Grand-Est, France). To limit oxidation due
107 to heat and light, sonication was performed in the dark and samples were kept in an ice bath. Then,
108 the SUVs solution was filtered on 0.2 μ m Acrodisc® from Pall Life Sciences (Port Washington, NY, USA)
109 to remove titanium particles. To prepare large unilamellar vesicles (LUVs), the MLVs suspension was

110 extruded 19 times at room temperature through a 200 nm nuclepore polycarbonate membrane filter
111 from Avestin Inc. (Ottawa, Ontario, Canada) with a syringe-type extruder from Avanti Polar lipids Inc.
112 (Alabaster, AL, USA). SUVs and LUVs size distribution were determined by dynamic light scattering with
113 a Zetasizer Nano-S from Malvern Instruments (Malvern, Worcestershire, UK). The sizes were on
114 average 60 nm after sonication and 190 nm after extrusion. The distributions were unimodal.

115

116 2.3. Evaluation of the antioxidant efficiency of the fluorinated derivatives

117 The antioxidant efficiency of the non-modified compounds as well as the derivatives was assessed
118 by evaluating their ability to protect lipid vesicles from oxidation. Because they are frequently used to
119 evaluate the antioxidant properties of various molecules [32,33], SUVs were chosen to perform these
120 experiments. DLiPC SUVs (control assay) and DLiPC SUVs containing fluorinated derivatives, Trolox or
121 PBN at different concentrations were prepared in Hepes buffer as described in section 2.2. The vesicles
122 were diluted to a final DLiPC concentration of 0.1 mg/mL AAPH, an azo compound widely used in
123 biochemical reactions to study lipid oxidation [34], was added at a final concentration of 2 mM and the
124 samples were immediately incubated at 37°C. The heat triggered the degradation of AAPH into peroxy
125 radicals that initiated the oxidation of the vesicles [35]. As they absorb ultraviolet light between 230
126 and 235 nm [36] and since they constitute the primary oxidation products of polyunsaturated lipids
127 [5,37], conjugated dienes were used as an indicator of lipid oxidation. Their appearance was followed
128 by measuring the absorbance at 234 nm with a Specord S300 UV–VIS spectrophotometer (Jena,
129 Thuringia, Germany). The values were normalized to zero by subtracting the initial absorbance (0.8 ±
130 0.2). The results are expressed as percentages of peroxidation, calculated as follows:

$$131 \quad \text{Lipid oxidation (\%)} = \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{maxControl}}} * 100 \text{ (eq. 3)}$$

132 The curves were fitted by applying a dose-response model with variable slope with the software
133 GraphPadPrism version 6.00 for Windows (La Jolla, CA, USA).

134 To further characterize the differences in the antioxidant efficiency of the derivatives, we calculated
135 the lag time of oxidation as follows:

$$136 \quad lag\ time = time_{10/20\%}^{sample} - time_{10/20\%}^{control} \text{ (eq. 4)}$$

137 where $time_{10/20\%}$ corresponds to the time required to reach 10 or 20 % of oxidation for the DLiPC SUVs
138 containing 6,4 μ M of antioxidant (5 %, mol/mol compared to DLiPC) and for the pure DLiPC vesicles
139 (control assay).

140

141 2.4. Surface pressure experiments

142 All the experiments were performed at constant temperature ($21.0 \pm 0.1^\circ\text{C}$). The film balance was
143 built by Nima (Coventry, West Midlands, England) for adsorption experiments on a small Teflon dish.
144 The dish (surface=19.6 cm^2) was equipped with a Wilhelmy-type pressure measuring system. In these
145 experiments, we used a subphase buffer of 62 mL continuously stirred with a magnetic stirrer spinning
146 at a rate of 100 rpm. The buffer contained 10 mM of HEPES, 150 mM of NaCl and was adjusted at a pH
147 of 7.4. To prepare Gibbs monolayers, the compounds – dissolved in ethanol – were injected in the
148 subphase at two ranges of concentrations: from 1 to 40 μ M for PBN and Trolox, from 0.0625 to 10 μ M
149 for the fluorinated derivatives. Surface tension was recorded during 5 min after the injection.

150 In order to study the adsorption of the compounds to DLiPC monolayers, DLiPC was dissolved in
151 hexane/ethanol 9:1 (v/v) and spread at the air-water interface to reach the desired surface pressure.
152 As soon as the initial surface pressure was stabilized (~ 15 min), the compounds were injected into the
153 subphase at a final concentration of 50 nM. Their adsorption at the air-lipid buffer interface was then
154 followed by measuring the variation of surface pressure during 15 minutes. As monolayers of
155 unsaturated lipids tend to oxidize in contact with air [38,39], we made sure the experiments lasted less
156 than 40 minutes to avoid a loss of stability due to lipid oxidation. A control experiment was also
157 performed by injecting ethanol in the subphase, which produced no significant perturbation of the
158 surface pressure. The binding parameters [40,41] were determined with the calculator available on the

159 following webpage: <http://www.crchudequebec.ulaval.ca/BindingParametersCalculator/>. The kinetics
160 of adsorption of the antioxidants in DLiPC monolayers was studied by fitting the data to the following
161 equation [42,43]:

$$162 \quad \ln[(\Pi_{\infty} - \Pi_t)/(\Pi_{\infty} - \Pi_i)] = kt \text{ (eq. 5)}$$

163 where Π_{∞} designates the equilibrium surface pressure after the injection of the antioxidants in the
164 subphase of the monolayer, Π_i the initial surface pressure of the monolayer, Π_t the surface pressure at
165 a time t and k the rate coefficient. $\Pi_{\infty} - \Pi_i$ can also be defined as $\Delta\Pi$, which designates the maximum
166 increase of surface pressure induced by the penetration of the antioxidants in DLiPC monolayers.

167

168 2.5. Effect of the antioxidants on the permeability of the membrane

169 The ability of the fluorinated antioxidants to permeabilize DLiPC vesicles was measured with the
170 calcein release assay. LUVs were chosen instead of SUVs in order to encapsulate larger amounts of
171 calcein and because the extrusion process allows the elimination of the concentric layers of MLVs
172 without releasing the encapsulated calcein. DLiPC LUVs were prepared as described in section 2.2 with
173 HEPES buffer containing 35 mM calcein. Free calcein was separated from encapsulated calcein by gel
174 filtration on a sepharose 4B column equilibrated with HEPES buffer. Lipids were quantified with the
175 method developed by Stewart [44]. The solution containing calcein loaded LUVs was diluted to a final
176 concentration of 10 μ M lipids in HEPES buffer. Fluorinated antioxidants dissolved in ethanol were
177 added after 5 min at a ratio of 5 % (mol/mol) compared to DLiPC. Fluorescence measurements were
178 performed at 21 °C as soon as the LUVs were prepared, immediately after the addition of fluorinated
179 derivatives and after 30, 60 min and 120 min of incubation with a Varian Cary Eclipse fluorescence
180 spectrophotometer (Santa Clara, CA, USA). We used excitation and emission wavelengths of 490 and
181 520 nm respectively. Triton X-100 was added after two hours in order to release the total amount of
182 encapsulated calcein. The intrinsic fluorescence of fluorinated antioxidants at 520 nm was verified and

183 the values we found were not significant. Release percentages were calculated with the following
184 equation:

$$185 \quad \text{Calcein release (\%)} = \frac{(F_1 - F_0)}{(F_{Tot} - F_0)} * 100 \text{ (eq.2)}$$

186 where F_0 designates the fluorescence of the LUVs measured in absence of fluorinated derivatives, F_1
187 the fluorescence of the LUVs measured after addition of fluorinated derivatives and F_{Tot} the
188 fluorescence measured after addition of Triton X100 that triggers the release of the encapsulated
189 calcein.

190

191 2.6. Theoretical methodology

192 Two DLiPC membranes made of 128 and 72 lipids each, were created using the membrane bilayer
193 builder from the CHARMM-GUI server [45]. The membranes were solvated with a hydration number of
194 35 water molecules per lipid. Na^+ and Cl^- ions were added at a 0.154 M concentration, ensuring the
195 neutrality of the system. The DLiPC lipids were described using the lipid 11 force field [46]. The force
196 field parameters for the different compounds (Trolox, anionic Trolox, PBN, FATx, FAPBN, FATxPBN)
197 were derived from the Generalized Amber Force Field version 2 (GAFF2) [47–49] for all moieties but
198 the sugars, which were described by the GLYCAM force field [50], using the antechamber package [51].
199 FATx, FAPBN and FATxPBN were built using a building block approach (*i.e.*, considering the β -D-
200 galactopyranosyl, 2,6-diaminohexanoyl, 1-amino-dodecafluorooctanyl, Trolox and PBN residues).
201 Atomic charges were derived from RESP (Restrained fit of Electro Static Potential) based on the
202 calculations achieved within the Density Functional Theory (DFT) formalism with the IEFPCM-B3LYP/cc-
203 pVDZ method in diethylether [52], accounting for the chemical environment in the building block
204 approach. The DFT calculations and the atomic charge fitting were performed with the Gaussian 09,
205 RevA [53] and R.E.D. III [54] softwares, respectively. The three-point TIP3P water model [55] was used
206 to describe water molecules. The anionic Trolox was used because, regarding the low pKa of the
207 carboxylic moiety, Trolox exists in its deprotonated form in the bulk water, and both (neutral and

208 anionic) forms may co-exist in the bilayer. However, regarding insertion in membrane, no significant
209 differences were observed between the two forms, therefore the results have not been detailed.

210 MD simulations were carried out using CPU-Particle-Mesh Ewald (PME) [56] MD codes available in
211 Amber16 [57,58], and according to the following procedure: minimization of water molecules prior to
212 minimization of the entire system to prevent from steric clash; slow thermalization of the water
213 molecules up to 100 K in the (N,V,T) ensemble for 200 ps; thermalization of the whole system to the
214 final temperature (310 K) for 500 ps in the (N,P,T) ensemble; equilibration of the system for 5 ns
215 (N,P,T) MD simulations. Productions of 600 ns (N,P,T) MD simulations were then achieved. PME MD
216 simulations were carried out using the SHAKE algorithm [59] on H-bonds and a 10 Å non covalent
217 interaction cut-off for both Coulomb and van der Waals interactions. The temperature was maintained
218 using the Langevin dynamics [60] with a collision frequency of 1 ps⁻¹. Semi-isotropic pressure scaling
219 with constant surface tension in the xy-plane perpendicular to the membrane normal (z-axis) was used
220 with Berendsen barostat [61], in which the pressure relaxation time was set to 1 ps.

221 The compounds were inserted in the bulk water of the equilibrated membrane systems, preventing
222 from steric clash with water molecules. MD simulations of 600 ns were then carried out with all the
223 compounds; the total MD simulation time for the six derivatives was 3.6 μs. The analyses were
224 performed along the last 300 ns, which allowed a robust sampling of structural properties, *i.e.*, after
225 the equilibrium was reached. They were carried out using the cpptraj software [62]. The positions of
226 the center of mass (COM) or key moieties of the different compounds in membranes are given with
227 respect to the middle of the bilayer (*i.e.*, z-component of the vector originated at the COM of the lipid
228 bilayer and pointing towards the COM of the compound). The orientation of the compounds in the
229 lipid bilayer was assessed by the α-angle between the z-axis and the normal vector(s) defined for each
230 compound (see supplemental data).

231

232

233 2.7. Statistical analysis

234 Statistical analysis was performed with the software GraphPadPrism version 6.0 for Windows (La
235 Jolla, CA, USA). Since our samples are small ($N < 30$) and because the values do not follow a normal
236 distribution, the Mann-Whitney/Wilcoxon test was carried out to compare the combination indexes of
237 the compounds at low (*i.e.* less than $1 \mu\text{M}$) and high (*i.e.* more than $1 \mu\text{M}$) concentrations. p values <
238 0.05 were considered as statistically significant.

239

3. Results

3.1. Fluorinated derivatives are efficient to limit lipid peroxidation

In order to evaluate the antioxidant efficiency of the compounds, DLiPC SUVs containing the parent compounds or the fluorinated derivatives were prepared and incubated with AAPH. Oxidation percentages were calculated with eq.3 as described in section 2.3 and reported over time. In absence of antioxidant we observed an immediate increase of the percentages of oxidation. The exponential phase was reached after 10 min and the percentages of oxidation kept increasing until reaching a plateau at 60 min (Fig. 2A). This increase is due to the formation of conjugated dienes which are the primary products of lipid oxidation [5,37], and which in turn drive the beginning of the kinetics. Consequently, after 80 min, the percentages of lipid oxidation in absence of antioxidants decreased, as the oxidation process continued beyond the conjugated dienes stage. Namely, conjugated dienes were progressively replaced by secondary peroxidation products that are not detected at 234 nm. With increasing concentrations of FATxPBN, oxidation percentages firstly increased slower than the control, which suggests an ability of FATxPBN to slow down the oxidation process. The exponential phase started after 60 min, at a higher level of oxidation than the control, causing the oxidation percentages to go over 100 % after 100 min. Also, we noticed a gradual decrease of oxidation percentages, showing a dose-dependent antioxidant efficiency of FATxPBN (Fig. 2A). Similar tendencies, although they were less pronounced, were observed with Trolox, FATx and FAPBN (Fig. S1).

To better compare the antioxidant efficiencies, we reported the percentages of oxidation at 80 min (*i.e.*, the time required to reach the plateau in the absence of antioxidant) versus the antioxidant concentration (Fig. 2B-F). For FATxPBN and FATx, concentrations ranging from 1.6 to 6.4 μM appeared sufficient to reach the maximal antioxidant efficiency. FAPBN was significantly less efficient as saturation was reached with a concentration of 38.4 μM . No significant antioxidant efficiency was observed for PBN, even at high concentrations (*i.e.* 25.6-38.4 μM). Trolox, FATxPBN, FATx and FAPBN presented IC_{50} (concentration required to half-reduce the oxidation in absence of antioxidant) values of

265 4.0, 0.6, 0.9, and 29.5 μM , respectively, whereas no IC_{50} could be determined for PBN. The better
266 capacity of Trolox to inhibit lipid oxidation in comparison to PBN agrees with previous work [30],
267 where we showed that Trolox was more efficient than PBN to scavenge $\text{ABTS}^{+\bullet}$ radicals. In the present
268 work, all three fluorinated derivatives exhibited lower IC_{50} than their parent compounds. Indeed,
269 FATxPBN and FATx exhibited IC_{50} values ~ 5 times lower than Trolox (0.6 and 0.9 μM versus 4.0 μM).
270 FAPBN displayed an IC_{50} of 29.5 μM while PBN presented no IC_{50} .

271 To better compare the most efficient derivatives (*i.e.* FATxPBN and FATx), we determined the lag
272 times (Fig. 3), which represent the time required to reach either 10 or 20 % lipid peroxidation for DLiPC
273 SUVs containing 5 % (mol/mol, concentration at which the antioxidant efficiency was optimal) of
274 antioxidant. Except for PBN and FAPBN at 20% oxidation (1.5 ± 2.0 and 6.0 ± 1.0 min, respectively), the
275 lag times of the fluorinated antioxidants did not significantly differ compared to that of the parent
276 compounds. In the case of FATxPBN, the lag time to reach 10 and 20 % of oxidation was significantly
277 higher than the two other derivatives, suggesting that this molecule has a different kinetic inhibition
278 process which efficiently delayed the oxidation process. These data confirmed a significantly improved
279 antioxidant efficiency of the fluorinated antioxidants with respect to the parent compounds.

280

281 3.2. Interactions of the antioxidants with DLiPC membranes

282 3.2.1. Interfacial behavior of the antioxidants

283 To characterize the amphiphilicity of the compounds and gain information on their hydrophobic
284 properties, we measured their ability to adsorb at the air-buffer interface. Increasing concentrations
285 were injected in the subphase and the surface tension was recorded continuously to assess the
286 formation of a Gibbs monolayer at the interface. The injection of 1, 5, 10, 20 and 30 μM of Trolox and
287 PBN exhibited only a poor effect on the surface pressure as the variations of surface pressure ($\Delta\Pi$)
288 recorded were ranging between 0 and 3 mN/m (Fig. S2). Conversely, the fluorinated antioxidants
289 presented a high surface activity since the injection of 0.1 μM was sufficient to observe a significant

290 surface activity (Fig. 4). A maximal surface activity of ~ 40 mN/m was reached at only $1 \mu\text{M}$ for
291 FATxPBN and FATx and at $3 \mu\text{M}$ for FAPBN, indicating that both FATxPBN and FATx are more
292 hydrophobic than FAPBN. Higher concentrations (*i.e.* 5 and $10 \mu\text{M}$) were also tested and the $\Delta\Pi$ values
293 remained close to 40 mN/m, reflecting the formation of stable monolayers at the air-water interface.

294 The micellization was previously studied and results showed that while the parent compounds
295 exhibit no critical micellar concentration (cmc); FATxPBN and FAPBN have cmc values of 10 and $51 \mu\text{M}$,
296 respectively [30]. In accordance with the cmc values, the three fluorinated antioxidants exhibited $\log P$
297 values significantly higher than the parent compounds (Fig. 1). The higher hydrophobicity of Trolox
298 over PBN was clearly observed, with $\log P$ values of 3 and 1.3 , respectively. This was also observed
299 when comparing FATx and FAPBN that present $\log P$ values of 3.8 and 2 , respectively. The same
300 tendency was observed with the experimentally-determined $\log k'_w$ values previously reported [30].
301 FATxPBN and FATx exhibited $\log k'_w$ values ~ 5 times higher than Trolox when that of FAPBN was 3
302 times higher than PBN. This confirms the strong contribution of the perfluorinated chain to the overall
303 hydrophobicity of amphiphilic molecules leading therefore to low cmc values [63]. Altogether, the
304 higher hydrophobic character of the fluorinated antioxidants compared to the parent compounds
305 suggest a better ability to interact with the hydrophobic core of the membrane, which may explain the
306 more pronounced antioxidant efficiency previously observed.

307 3.2.2. Interactions with an unsaturated phospholipid

308 Oxidative stress as well as Gibbs monolayers experiments revealed significant discrepancies
309 regarding the hydrophobic properties of fluorinated antioxidants as well as their ability to prevent
310 membrane lipid oxidation. To evaluate their ability to interact with membranes and understand those
311 differences of efficiency, we performed adsorption assays at lipid monolayers. DLiPC monolayers were
312 prepared at different initial pressures and the derivatives were injected into the subphase. The
313 variation of pressure was recorded to evaluate the ability of the derivatives to modify the surface
314 activity of the monolayer. Due to the strong surface activity of the fluorinated antioxidants and the

315 formation of aggregates above the cmc, the derivatives were injected in the subphase of the
316 monolayers at a final concentration of 0.05 μM ; a concentration significantly lower than their cmc at
317 which they all presented a negligible surface activity (see Fig. 4). The variation of pressure ($\Delta\Pi$) was
318 reported over the initial surface pressure (Π_i) in order to determine the binding parameters of the
319 compounds [40]. The injection of the parent compounds had little effect on the surface pressure, as
320 $\Delta\Pi$ remains below 3 mN/m independently of the initial pressure (Fig. S3). Conversely, the injection of
321 the fluorinated antioxidants (Fig. 5A-C) caused an immediate increase of the surface pressure and
322 $\Delta\Pi$ decreased when the initial pressure was increased. The synergy factor was calculated by adding
323 one to the value of the slope of the regression curve (Table 1). All the compounds presented positive
324 synergy factors, indicating a favorable binding of the compounds to DLiPC membranes. Also, linear
325 regression provided the maximal insertion pressure (MIP), highlighting the highest pressure at which
326 fluorinated antioxidants can penetrate monolayers [40]. As reported in Table 1, FATxPBN, FATx and
327 FAPBN exhibited MIPs of 52.2, 48.6 and 37.6 mN/m, respectively. Because all the MIPs are higher than
328 the average lateral pressure in biological membranes – $\sim 30\text{mN/m}$ [64] –, one can conclude that
329 fluorinated antioxidants can penetrate bilayers under physiological conditions.

330 Since the lower MIP of FAPBN may indicate a less pronounced affinity for DLiPC monolayers [41],
331 another parameter named $\Delta\Pi_0$ can be determined to gain information on the ability of the fluorinated
332 antioxidants to partition into DLiPC membranes [40]. $\Delta\Pi_0$ is determined by performing an
333 extrapolation on Fig. 5 from an initial surface pressure of 0 mN/m and corresponds to the intersection
334 of the linear regression with the y-axis. We performed an extrapolation at the average lateral pressure
335 of membranes – *i.e.* 30 mN/m [64] – to determine $\Delta\Pi_{30}$, which is related to the quantity of compound
336 associated to the monolayer. As reported in Table 1, FATxPBN and FATx exhibited $\Delta\Pi_{30}$ values ~ 4
337 times higher than FAPBN (10.0 and 9.0 versus 2.1 mN/m), indicating that greater amounts of FATxPBN
338 and FATx spontaneously penetrated the membrane in comparison to FAPBN.

339 Fitting the data recorded at 30 mN/m with eq.5 allowed us to determine the rate of penetration (k)
340 of the fluorinated antioxidants in DLiPC monolayers at the average lateral pressure of membranes, as
341 described in [43]. The plot yielded three linear regions (Fig. 6). The first linear region corresponds to
342 the stabilization of the lipid monolayer in absence of antioxidant, the second one to the penetration of
343 the antioxidants in the monolayer and the third one to the rearrangement between the lipids and the
344 antioxidants in the monolayer [42]. The rate of penetration was determined by calculating the slope of
345 the second linear region (Table 1). FATx and FATxPBN present k values twice as important as FAPBN,
346 showing they penetrate the membrane faster than FAPBN. This result, in accordance with the MIP and
347 $\Delta\Pi_{30}$ values, underlines the effect of the fluorinated chain, which has increased the affinity of the
348 parent compounds for DLiPC.

349 Although our results indicate the fluorinated antioxidants interact favorably with DLiPC membranes,
350 we are aware that hydrogenated and fluorinated lipids may not be miscible [65,66]. Nevertheless,
351 there is a general consensus among the community that fluorinated and hydrogenated surfactants are
352 miscible, however whether they do mix completely or only partially is still subject to debate [67].
353 Several reports in the literature suggests various degrees of miscibility between fluorinated and
354 hydrogenated detergents and therefore different scenarios of mixing leading to: (i) the formation of
355 one type of mixed micelles [68]; (ii) the demixing of the two leading to the coexistence of two types of
356 micelles [69] or (iii) both, depending on the mixing ratio [70,71]. In our case, it is worth mentioning
357 that FATx and FATxPBN reached their maximum antioxidant efficiency at concentrations ranging from
358 1.6 to 6.4 μM (*i.e.* molar ratios ranging from 1.25 to 5 % in comparison to DLiPC, Fig. 2D-E). These
359 ratios are sufficiently low to expect only slightly perturbations of the membrane and prevent miscibility
360 issues. Conversely, FAPBN was not efficient at low concentrations and 38.4 μM (*i.e.* 30 % molar ratio)
361 was required to reach a significant antioxidant efficiency (Fig. 2F). The low antioxidant efficiency
362 combined with the poor affinity for membranes as well as the possible impact of a high concentration

363 in membranes highlight the fact that FAPBN presents less interest than the two other fluorinated
364 antioxidants.

365 3.3. The fluorinated antioxidants do not modify the membrane's structure

366 We performed calcein release experiments to evaluate the impact of the fluorinated antioxidants
367 on the membrane integrity. DLiPC LUVs containing calcein were prepared and the fluorinated
368 antioxidants were added to the samples and fluorescence was measured during 2 hours to evaluate
369 the calcein leakage. The fluorinated antioxidants were introduced at a 5 % molar ratio in comparison to
370 DLiPC since at this concentration the antioxidant effect we observed was maximal in the case of
371 FATxPBN and FATx (see Fig. 2). In order to stay consistent, FAPBN was tested with the same
372 concentration although its antioxidant efficiency was limited at low concentrations. Tests of
373 permeability with higher concentrations would have not been relevant from a physiological point of
374 view since an antioxidant is supposed to be efficient at low concentrations [72]. In all cases, the
375 addition of the derivatives caused a weak leakage from the LUVs, with however a percentage of
376 leakage below 5 % during the entire experiment (Fig. S4). The addition of Triton X100 after two hours
377 caused a significant increase of the fluorescence intensity due to the release of the total amount of
378 encapsulated calcein. Because the injection of the derivatives did not significantly modify calcein
379 leakage, we concluded that their insertion has no significant effect on the structure of the membrane.

380 Regarding the interactions between fluorinated surfactants and phospholipids, it is generally
381 admitted that fluorinated surfactants do not solubilize membranes and therefore they have been
382 successfully used to solubilize hydrophobic proteins in the presence of liposomes acting as chaperones
383 [73]. Recently, a phosphocholine based fluorinated derivative (F6OPC) with a perfluorohexyl chain
384 similar to that of FATxPBN was found to partition into POPC bilayers without inducing the solubilization
385 of preformed vesicles [74]. Conversely, Frotscher and co-workers observed the complete solubilization
386 of lipid vesicles after incubation with a fluorinated surfactant bearing the same perfluorohexyl chain
387 and a maltoside polar head group (F6OM) [75]. Our assays did not reveal any significant modification

388 of the permeability of the membrane, indicating that the fluorinated antioxidants tested here did not
389 solubilize the membrane at the concentration they are the most effective. This is very likely due to the
390 very low concentration (physiologically more relevant) used in the present study (5 % mol, *i.e.* 0.5 μ M)
391 compared to the 2 mM used by Frotscher and co-workers.

392

393 3.4. MD simulations to support positioning of the fluorinated antioxidants in DLiPC membranes

394 Each derivative was placed at different positions at the beginning of the MD simulations. It is worth
395 noting that all simulations, each molecule reached similar equilibrium position in the membrane. In
396 other words, when starting from the bulk water all the compounds inserted into the DLiPC lipid
397 bilayers. The preferred (equilibrium) position of the center of mass (COM) of Trolox and PBN were just
398 below the polar head group region in close contact with the phosphates and to a less extent with the
399 choline moieties (14.0 and 13.6 Å for both compounds, respectively from the middle of the bilayer, see
400 Table 2 and Fig. S6 for the radial distribution function). The fluorinated antioxidants also partition just
401 below the polar head group region, the COM being at 14.3, 14.7 and 13.7 Å from the middle of the
402 bilayer for FATx, FAPBN and FATxPBN, respectively (Table 2). The different equilibrium positions
403 obtained from the MD simulations were fully consistent with the chemical structures of these
404 amphiphilic derivatives, as the different moieties drive the preferred location. Namely, from one side,
405 both β -D-Galactose and gluconic acid moieties strongly anchor the derivatives to the choline and to the
406 phosphate groups (*ca.* 16.5 and 20.0 Å for both moieties, respectively, from the middle of the bilayer)
407 by electrostatic and H-bonding interactions (Fig. 6). From another side, the fluorinated chain lines up
408 along the DLiPC lipid chains (COM of the fluorinated chain ranging from 8.2 to 10.4 Å, see Table 2),
409 anchoring the derivatives into the membrane (Fig. 6). In the case of the fluorinated antioxidants, the
410 Trolox and PBN moieties fluctuate below the polar head group region (ranging from 13.8 to 15.7 Å
411 from the middle of the bilayer, see Table 2) with a preferred orientation parallel to the surface (Fig. S7)
412 in close contact with the phosphates, as for the parent Trolox and PBN derivatives.

413 Interestingly, in the case of FAPBN, the MD simulations, which were performed to ensure a
414 sufficient conformational sampling, highlighted the existence of a second (minor) conformer inside the
415 membrane. In this conformer, the PBN moiety was driven deeper in the membrane (9.0 Å from the
416 middle of the bilayer, see Table 2), in close contact with the fluorinated chain (Fig. 6D). This minor
417 conformer corresponds to a 3-dimensional molecular arrangement which has been observed during
418 short-lasting period along the MD simulation time. In this conformer, PBN is lining up along the
419 fluorinated chain (Fig. 6D), whereas in the major conformer (seen during long-lasting period of the MD
420 simulations), PBN is far apart from the fluorinated chain. Conversely, no similar alignment of the Trolox
421 moiety and the fluorinated chain (in FATx and FATxPBN) was observed throughout the whole MD
422 simulations, even though some initial geometries were driven to put these two moieties were in close
423 contact. As a consequence, no intramolecular interactions between the Trolox and PBN moieties were
424 observed in FATxPBN throughout the whole MD simulations.

425

426 **4. Discussion**

427 The antioxidant assays conducted in this work highlight that the three fluorinated antioxidants are
428 much more efficient than the parent compounds PBN and Trolox. This is consistent with various other
429 studies on hybrid antioxidants, which showed how the chemical combination of two antioxidant
430 moieties enhances the activity with respect to both moieties taken separately [26–28]. Along the same
431 line to maximize lipid peroxidation activity, we previously conducted a similar study by combining PBN
432 and cholesterol within amphiphilic derivatives [76]. By using similar experimental setups than in the
433 present study, we showed these cholesteryl-PBN derivatives displayed enhanced hydrophobic and
434 antioxidant properties in comparison to the parent compound PBN, as dynamic light scattering and
435 oxidative stress experiments evidenced a stronger ability of cholesteryl-PBNs to stabilize the structure
436 of the vesicles and to preserve them from the swelling due to the oxidation process. The molecules
437 tested in this work, in particular FATxPBN and FATx, present lower IC₅₀ values than the cholesteryl-

438 PBNs, which underline a stronger ability to prevent lipid oxidation, as expected due to the presence of
439 the Trolox moiety.

440 We observed significant discrepancies between the fluorinated antioxidants, as both FATxPBN and
441 FATx exhibited IC_{50} values that are 49 and 33 times lower (higher activity) than FAPBN, respectively
442 (see Fig. 2). The differences observed between FAPBN and the other two fluorinated antioxidants can
443 be rationalized by a lower affinity to lipid (slightly lower hydrophobicity, see $\log P$ on Fig. 1) of the
444 former parent compound (*i.e.* PBN) and by the absence of the Trolox moiety in this derivative. The
445 scavenging mechanisms of both Trolox and PBN are different (most probably hydrogen atom
446 abstraction and adduct formation – spin trap –, respectively), which makes the former derivative a
447 much better free radical scavenger. Concomitantly, the amphiphilic feature of the derivatives finely
448 tunes the capacity for lipid peroxidation inhibition, by ensuring a positioning in the membrane below
449 the polar head groups, in close contact with the oxidation process (*i.e.*, as close as possible from
450 unsaturation). At this preferred location, the derivatives are likely to inhibit both the initiation stage or
451 the propagation stage for lipid chains which can adopt a transient snorkel-like shape [77,78] of lipid
452 peroxidation. Also, it is well-known that location of the antioxidants in close contact to the
453 unsaturations of the phospholipid acyl chains favors a better inhibition of lipid peroxidation [79]. The
454 $\log P$ values suggest that the higher the hydrophobicity, the higher the maximal insertion pressure and
455 the deeper the molecules penetrate into the membrane (Fig. S5 A-B). In accordance with our
456 observations, Azouzi and co-workers recently reported that serotonin has the ability to bind and
457 protect DLiPC enriched membranes from oxidation [80], due to frequent interactions between
458 serotonin and phospholipid acyl chains, which might inhibit the propagation stage of lipid
459 peroxidation.

460 Interestingly, PBN exhibited no lipid peroxidation inhibition whereas an IC_{50} was measured with
461 FAPBN. As seen from the MD simulations and highly expected from their chemical nature, both PBN
462 itself and the PBN moiety in the major conformer of FAPBN lie at almost the same preferred position.

463 Therefore, a highly plausible explanation of the better activity of FAPBN with respect to PBN is
464 assigned to an anchoring effect, which is attributed to the fluorinated chain which better constrain
465 (anchor) the positioning of the PBN moiety in the membrane for FAPBN. This can in turn makes the
466 PBN moiety more efficient as a spin trapper. Although hardly directly reachable by experimental
467 techniques, the explanation of the measurable IC_{50} value for FAPBN is likely due to its amphiphilic
468 chemical nature, which allows this anchoring effect in close contact to the region where lipid oxidation
469 occurs. A secondary explanation might be due to the existence of the second (minor) FAPBN
470 conformer (Fig. 7D), in which the PBN moiety may even spend some time deeper in the bilayer to
471 inhibit lipid peroxidation more efficiently than PBN alone, however not as efficient as both FATxPBN
472 and FATx due to a much less efficient free radical scavenging capacity.

473 FATx is 33 times more active than FAPBN, which is mainly attributed to the nature of the
474 antioxidant moiety and not to positioning. Indeed, Trolox is a much more efficient free radical
475 scavenger than PBN, while the MD simulations suggest that both moieties are positioned in the same
476 membrane region, in close contact with the polar head group region due to their relatively hydrophilic
477 and H-bonding donor/acceptor nature. With an IC_{50} of 0.6 μ M, FATxPBN has shown the highest
478 antioxidant activity among all three fluorinated antioxidants. Since FATxPBN presents an IC_{50} lower
479 than the average of FATx and FAPBN, the combination of Trolox and PBN moieties on a same carrier
480 was hypothesized to have created a synergistic antioxidant effect. To confirm or infirm this hypothesis,
481 the activity of FATx and FAPBN was more thoroughly compared to that of FATxPBN to decipher
482 whether the combination result in simple additive, supra-additive (synergistic) or infra-additive
483 (inhibition of one agent by the other) effects. Both FATx and FAPBN are considered here as two
484 separated antioxidant agents engaged in a similar skeleton; *i.e.*, an antioxidant moiety linked to a
485 perfluorinated chain and a sugar moiety. To address this issue, the combination index (CI) of FATx and
486 FAPBN was determined based on the Chou and Talalay method [81]. As one can see in Table 3, at low
487 concentrations (*i.e.*, 0.4 to 0.8 μ M) the combination of FATx and FAPBN creates a synergistic

488 antioxidant effect as CI values are lower than 1. This effect disappears when the concentrations are
489 increased as with concentrations ranging from 1.6 to 38.4 μM , the CI values are higher than 1,
490 suggesting an infra-additive effect (*i.e.* inhibition between both antioxidant moieties). The statistical
491 analysis evidenced significant differences ($p < 0.05$) between the CI values of the low (0.4 to 0.8 μM)
492 and the high concentrations (1.6 to 38.4 μM), which underlines the presence of a synergistic effect at
493 low concentrations. Similar trends (Table. S1) were observed by multiplying the percentages of lipid
494 oxidation of FATx and FAPBN and comparing them to those of FATxPBN according to the Berenbaum
495 method [82,83], leading to similar conclusions.

496 Several researchers have reported on synergistic effects created by combining different antioxidant
497 moieties. For instance, it has been reported that the combination of β -carotene and α -tocopherol
498 results in a better protection of rat liver microsomes from oxidative stress [84]. Recently, Trolox has
499 been mixed with caffeic acid phenethyl ester (CAPE) to create a synergistic antioxidant effect [85],
500 which was attributed to weak interactions (hydrogen bonds) between Trolox and CAPE. Trolox has also
501 been combined to nitrones to create nitrone derivatives of Trolox [86], which revealed more efficient
502 *in vitro* and *in vivo* activities against lipid peroxidation and ischemia with respect to the parent
503 compounds. Synergism between antioxidants can proceed through different mechanisms like sacrificial
504 oxidation [87], regeneration or other mechanisms [88]. In the case of sacrificial oxidation, one
505 antioxidant protects the other by free radical scavenging, while for regeneration, one antioxidant is
506 oxidized to renew the second one. An extensively-described and typical example of regeneration is the
507 interaction between vitamin E and vitamin C [89]. Other researchers attempted to combine, in a single
508 molecule, different types of antioxidants to favor synergistic effects. For instance, lipoic acid has been
509 combined to Trolox [26] as well as a nitric oxide synthase inhibitor [28] with the objective to obtain
510 greater protective effects. Preliminary experiments on biological systems highlighted that these
511 divalent antioxidants have better abilities to prevent neuronal damage caused by glutamate and
512 oxidation of rat microsomal membrane lipids. Recently, Vavrilokova and co-workers, through

513 enzymatic reactions, combined silybin to ascorbic acid, Trolox alcohol or tyrosol to obtain novel
514 divalent antioxidants [27]. Among all the derivatives, the derivative combining silybin to ascorbic acid
515 showed the highest ability to protect rat microsomes from lipid peroxidation. Durand and co-workers
516 previously reported the synthesis of PBNLP, a divalent antioxidant bearing a PBN and a lipoic acid
517 moiety [90]. No synergistic antioxidant effect was observed as PBNLP and its individual constituents
518 showed a similar ability to protect red blood cells from AAPH-induced hemolysis. However, the
519 combination of the two moieties on the same carrier strongly diminished the antagonist effect
520 observed between the individual derivatives.

521 In the case of FATxPBN, no intramolecular interactions between both antioxidant (Trolox and PBN)
522 moieties were observed by MD simulations, which is fully consistent with the amphiphilic nature of the
523 molecule and the chemical nature of the different moieties. This precludes regeneration effect within
524 the same molecule. However, the compounds are likely to accumulate in a narrow region and
525 regarding the similar depth of insertion of both Trolox and PBN moieties, intermolecular interactions
526 are likely to occur. This could favor a regeneration process. This appears possible at relatively low
527 concentration, *i.e.*, below 0.8 μM , as seen by the experimental results. This may correspond to greater
528 local concentration due to partitioning in membrane. In this case small non-covalent clusters can be
529 formed. At higher concentration values this effect disappears (Table 3), most probably due to the
530 formation of higher-order aggregates, in which less antioxidant moieties are available. Also, the ability
531 of FATxPBN to strongly delay lipid oxidation compared to the other antioxidants (see Fig. 3) suggests
532 that this derivative combines the retarding effect of PBN [91] with the free radical scavenging capacity
533 of Trolox [92], thus creating a synergistic effect by retardation. At first, the PBN moiety seems to delay
534 the oxidation during 60 min, as percentages of oxidation of the SUVs are the same, regardless of the
535 concentration of FATxPBN (see Fig. 2A). Then, the dose-dependent antioxidant efficiency observed
536 might be attributed to the Trolox moiety, as FAPBN is only slightly efficient against AAPH-induced
537 oxidation (see Fig. 2F).

539 **5. Conclusion**

540 The present work is in line with the strategy aiming at grafting two antioxidant moieties on the
541 same carrier to enhance the global antioxidant action with a nanoscale fine tuning. The key role of the
542 amphiphilic feature of this series of fluorinated antioxidants bearing Trolox and/or PBN moieties was
543 studied in terms of interaction with membranes. The presence of the perfluorinated chain increases
544 hydrophobicity, allowing these derivatives to form monolayers at the air-water interface. Because of
545 higher hydrophobicity, they are anchored to the DLiPC membranes, with a greater antioxidant activity
546 against AAPH-induced lipid oxidation. Interestingly, although PBN is inefficient, its combination with a
547 Trolox moiety on the same carrier creates a synergistic effect that improves the global antioxidant
548 efficiency. Thanks to a dual mode of action, such hybrid antioxidants are very promising molecules for
549 a wide range of applications in cosmetic, food and pharmaceutical industries by lowering the usual
550 concentrations of antioxidant required to efficiently prevent lipid oxidation.

551 **Author's contributions**

552 G.D and S.M conceived this research. M.R synthesized the fluorinated antioxidants. L.S and A.M.G.G
553 performed the experiments with model membranes. B.C and F.D.M designed and performed MD
554 simulations. B.C, F.D.M and P.T analyzed the theoretical data. L.S analyzed the experimental data and
555 wrote the article. P.T, G.D and S.M formatted the manuscript.

556

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567

568 **Conflict of interest**

569 The authors declare no conflict of interest.

570

571 **Supporting material**

572 Supporting material (7 figures and 1 table) can be found at

573

574 **6. References**

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840 **Figure's captions**

841 Fig. 1. Chemical structures and log*P* values of the studied molecules.

842

843 Fig. 2. Effect of the parent compounds and the fluorinated derivatives on the AAPH-induced oxidation
844 of DLiPC SUVs. Kinetics of oxidation in presence of FATxPBN (A). IC₅₀ (μM) of Trolox (B), PBN (C), FATx
845 (D), FAPBN (E) and FATxPBN (F). Results are presented as the mean ± standard deviation of at least
846 three independent experiments.

847

848 Fig. 3. Lag time necessary to reach 10 and 20 % of oxidation. Results are presented as the mean ±
849 standard deviation of at least three independent experiments.

850

851 Fig. 4. Surface activity (Gibbs monolayers) of the fluorinated antioxidants at the air/water interface.
852 FATxPBN (A), FATx (B) and FAPBN (C). Data were fitted with a Langmuir equation with GraphPadPrism
853 software.

854

855 Fig. 5. Interactions of fluorinated antioxidants with DLiPC monolayers. FATxPBN (A), FATx (B), FAPBN
856 (C).

857

858 Fig. 6. Kinetics of penetration of the fluorinated antioxidants in DLiPC monolayers at 30 mN/m, which is
859 the lateral pressure of biological membranes [64]. Evolution of the monolayer pressure after the
860 injection of FAPBN, FATx and FATxPBN in DLiPC monolayers at 30 mN/m (A). Determination of the rate
861 coefficient (*k*) after injection of the fluorinated antioxidants in the subphase of DLiPC monolayers (B).

862

863 Fig. 7. Representative snapshots of the preferred location of FATx (A), FAPBN (B) and FATxPBN (C). Fig.
864 6D depicts the minor conformation of FAPBN, in which the PBN moiety is lined up along the fluorinated
865 chain. The 1LB (β-D-Galactose), GA4 (gluconic acid), DAH (2,6-diaminohexanoic acid) and DFA
866 (3,3,4,4,5,5,6,6,7,7,8,8,8-dodecafluorooctan-1-amine) moieties are depicted in red, blue, brown and
867 yellow, respectively. Trolox and PBN are depicted in grey and pink, respectively. The cholines are
868 depicted in cyan, the phosphates are depicted in magenta, 1st and 2nd unsaturations are depicted in
869 green and dark-green, respectively.

870 **Table's captions**

871 Table 1. Binding parameters and rate of penetration (k) of the fluorinated antioxidants in DLiPC
872 monolayers.

873

874 Table 2. Positions given as distances from the middle of the bilayer (in Å) of key moieties of DLiPC (*i.e.*,
875 choline, phosphates, saturations) and the different derivatives Trolox, Anionic Trolox, PBN, FATx,
876 FAPBN, minor conformer of FAPBN (FAPBN*) and FATxPBN (*i.e.*, Trolox moiety, Trolox OH-phenolic,
877 PBN, PBN amine moiety, 1LB = β -D-Galactose, GA4 = gluconic acid; DAH = 2,6-diaminohexanoic acid,
878 DFA = 3,3,4,4,5,5,6,6,7,7,8,8,8-dodecafluorooctan-1-amine), as well as center-of-mass (COM) of the
879 three fluorinated antioxidants FATx, FAPBN and FATxPBN. The positions were averaged over the
880 second half of the MD simulations.

881

882 Table 3. Combination index (CI) of FATx and FAPBN. CI<1: supra-additive, CI=1: additive, CI>1: infra-
883 additive. Values with different superscripts differ significantly ($p < 0.05$).

884

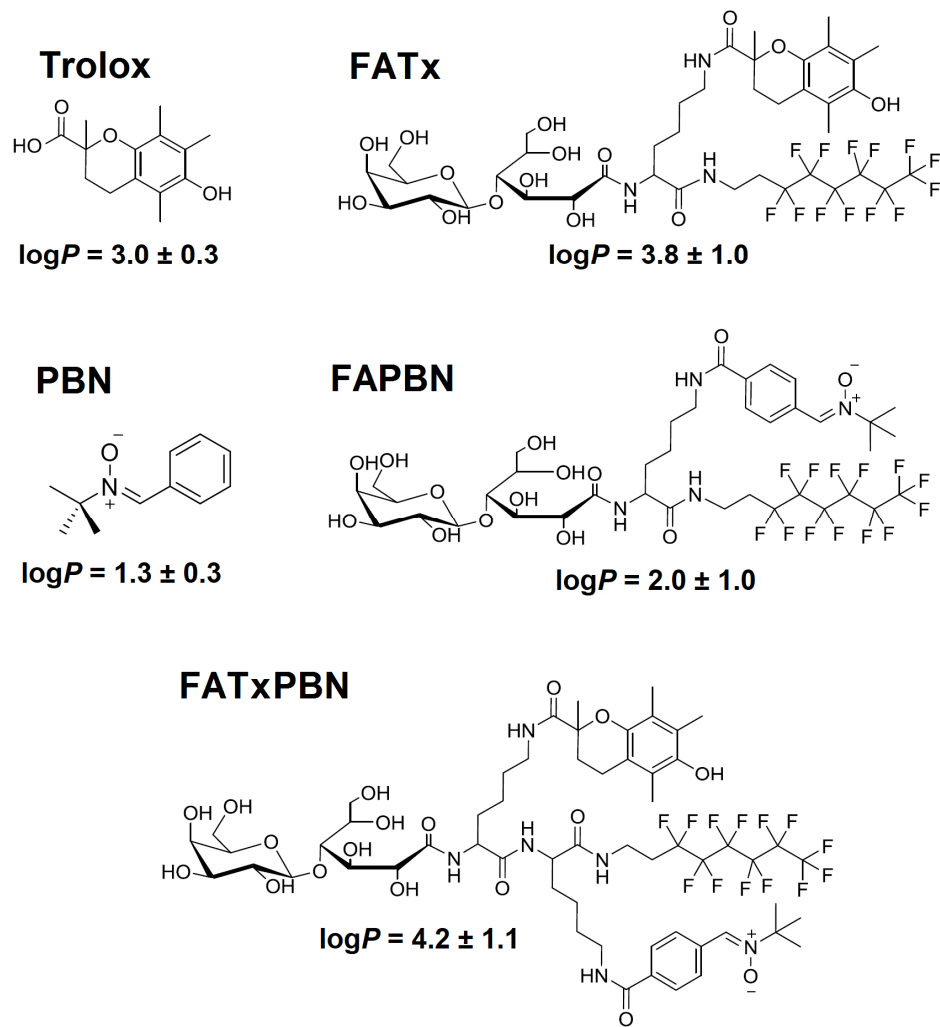


Fig. 1. Chemical structures and $\log P$ values of the studied molecules.

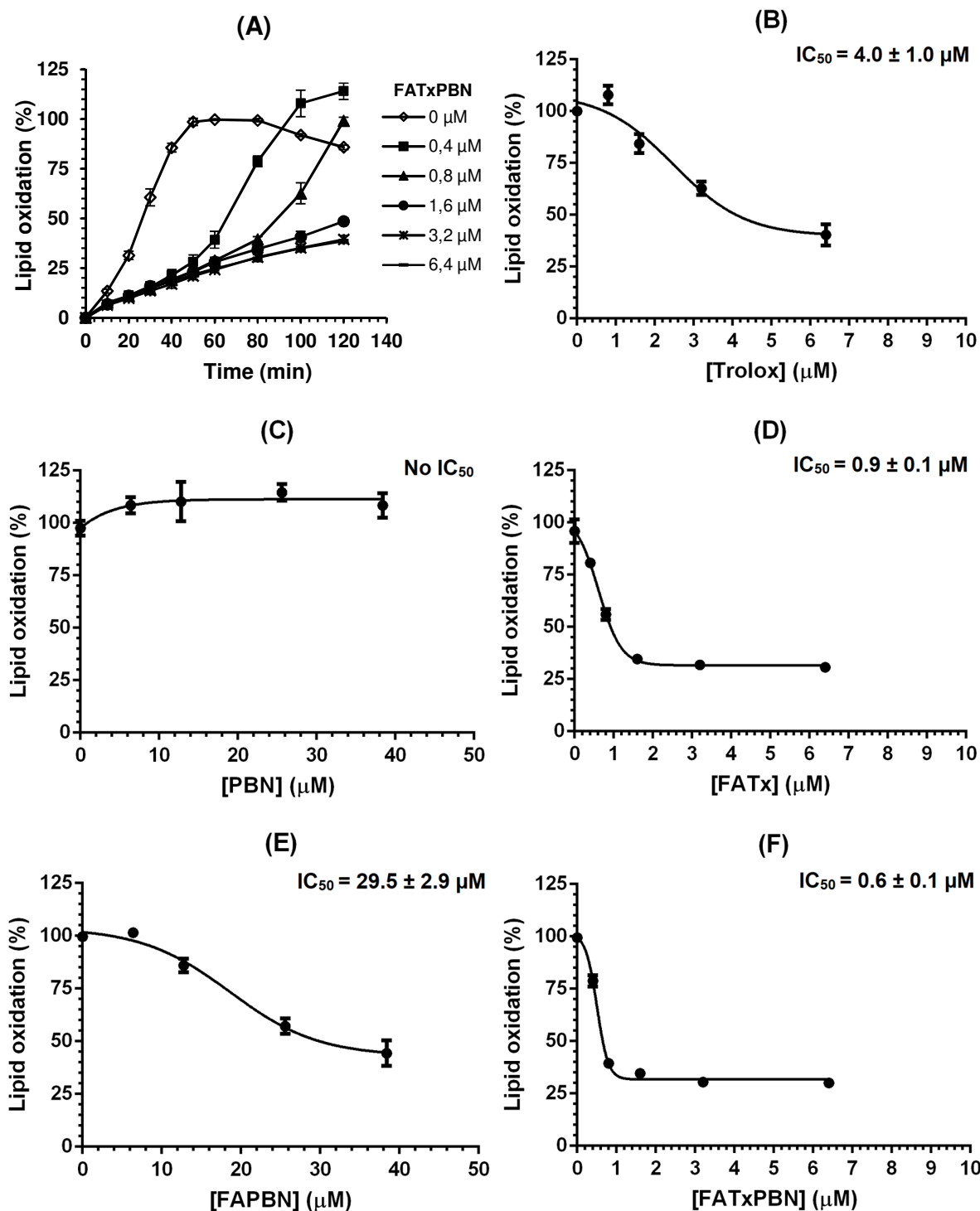


Fig. 2. Effect of the parent compounds and the fluorinated derivatives on the AAPH-induced oxidation of DLPC liposomes. Kinetics of oxidation in presence of FATxPBN (A). IC₅₀ (μM) of Trolox (B), PBN (C), FATx (D), FAPBN (E) and FATxPBN (F). Results are presented as the mean ± standard deviation of at least three independent experiments.

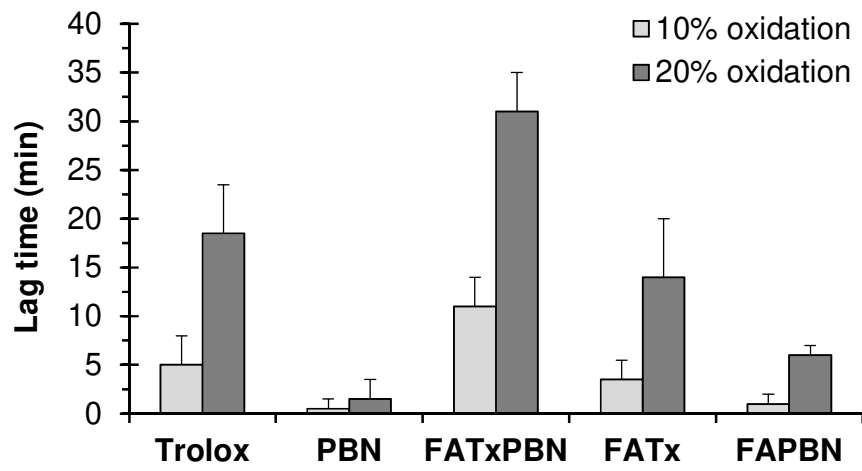


Fig. 3. Lag time necessary to reach 10 and 20 % of oxidation. Results are presented as the mean \pm standard deviation of at least three independent experiments.

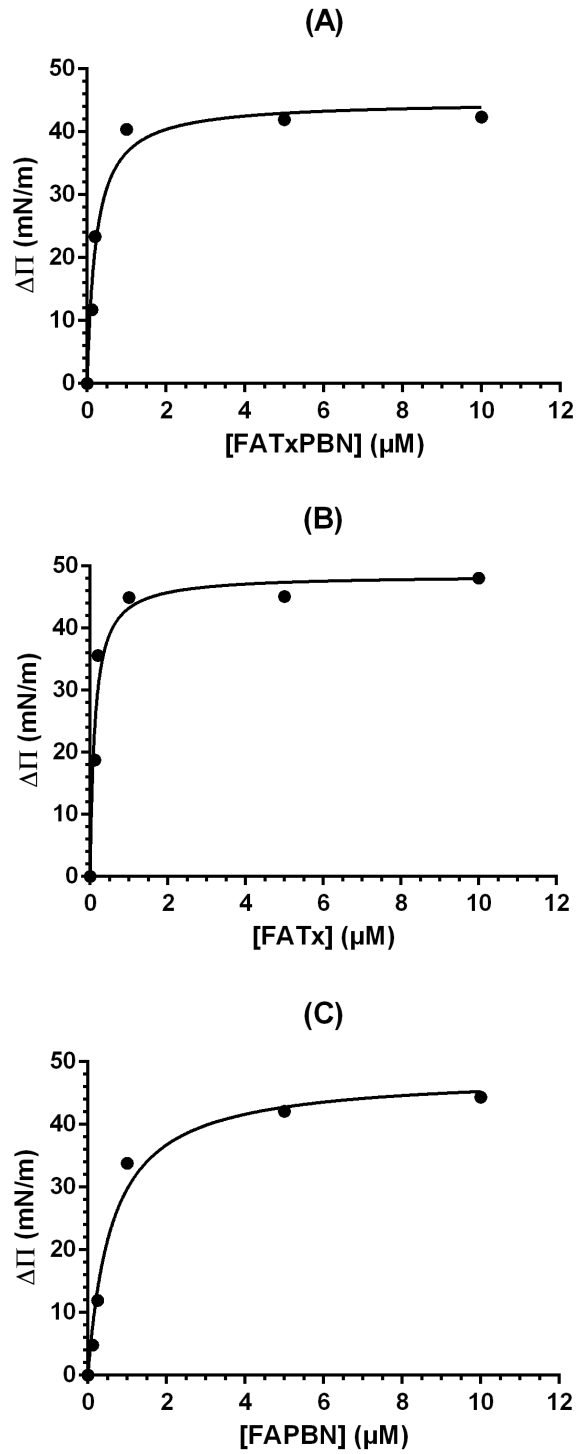


Fig. 4. Surface activity (Gibbs monolayers) of the fluorinated antioxidants at the air/water interface. FATxPBN (A), FATx (B) and FAPBN (C). Each experimental point was performed one time, as described in [40]. Data were fitted with a Langmuir equation with GraphPadPrism software.

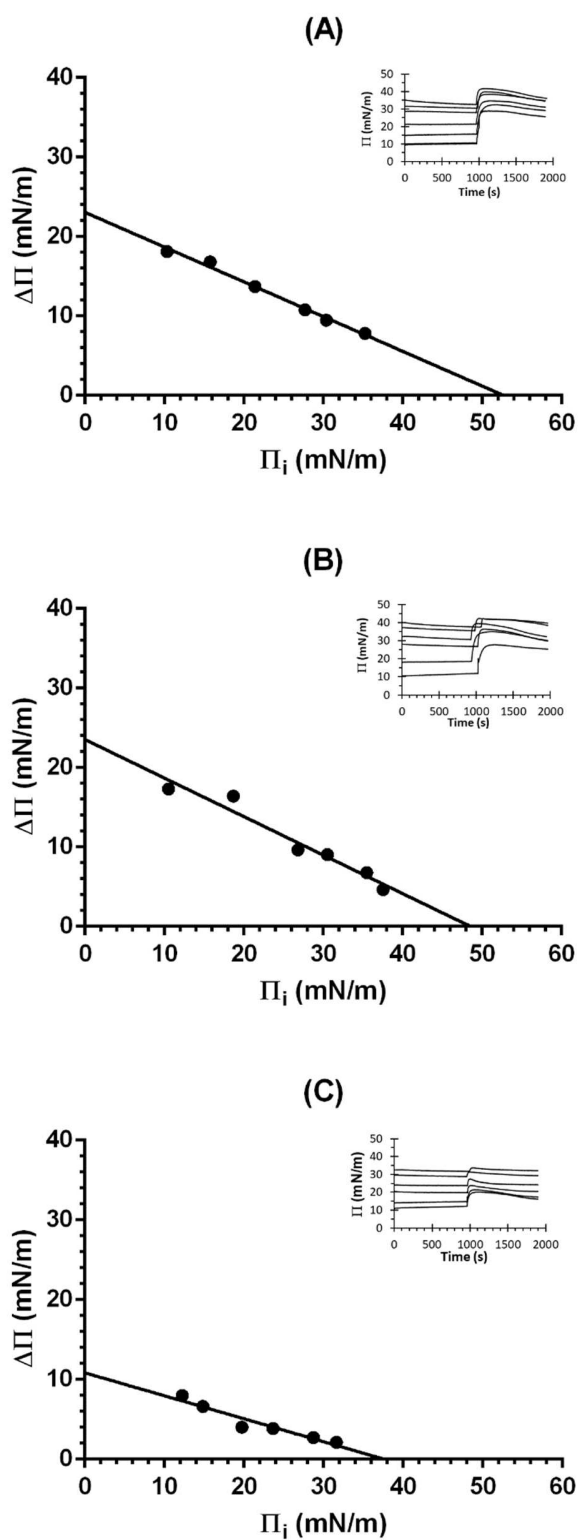


Fig. 5. Interactions of fluorinated antioxidants with DLPC monolayers. FATxPBN (A), FATx (B), FAPBN (C). Upper right boxes: Kinetics of insertion of antioxidants at different surface pressures. Each experimental point was performed one time, as described in [40].

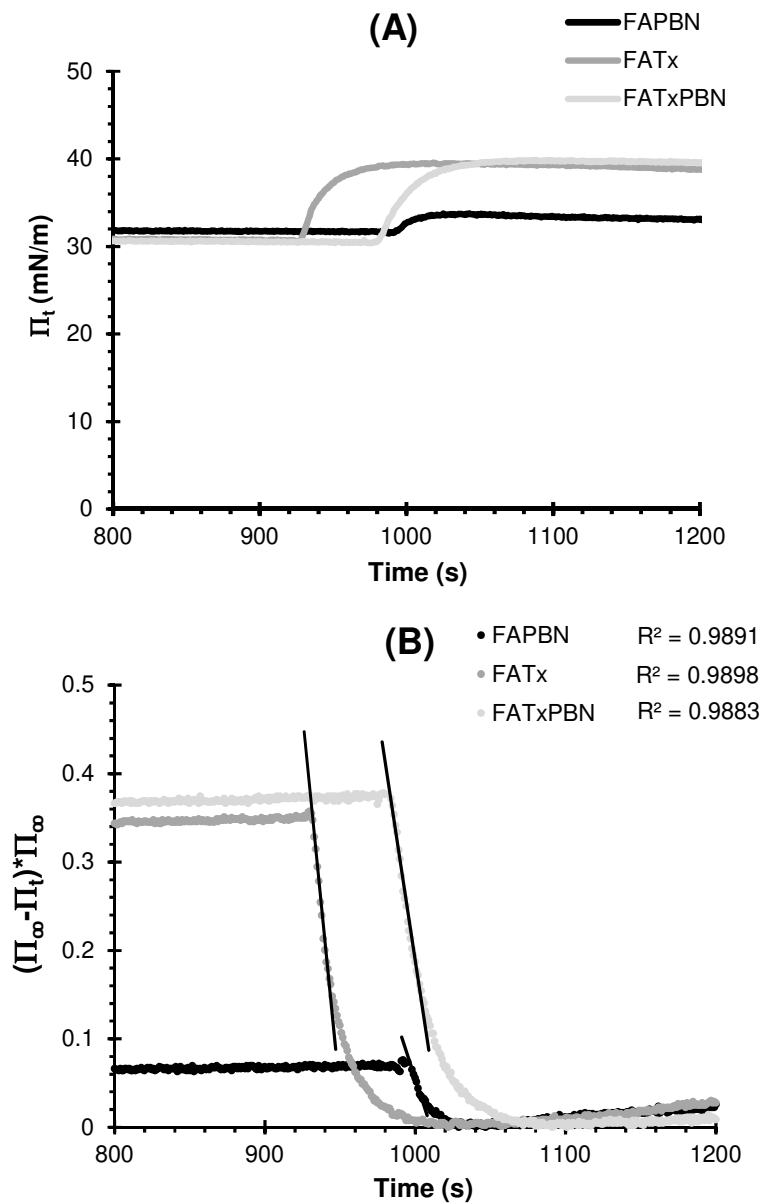


Fig. 6 Kinetics of penetration of the fluorinated antioxidants in DLiPC monolayers at 30 mN/m, which is the lateral pressure of biological membranes [64]. Evolution of the monolayer pressure after the injection of FAPBN, FATx and FATxPBN in DLiPC monolayers at 30 mN/m (A). Determination of the rate coefficient (k) after injection of the fluorinated antioxidants in the subphase of DLiPC monolayers (B).

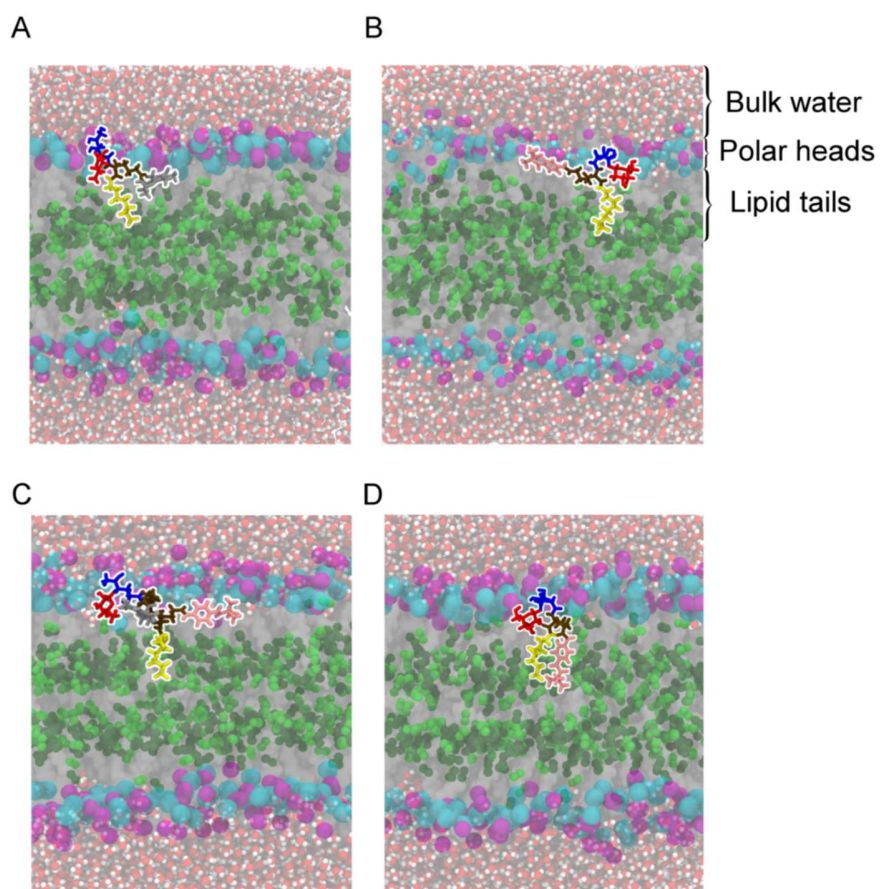


Fig. 7. Representative snapshots of the preferred location of FATx (A), FAPBN (B) and FATxPBN (C). Fig. 6D depicts the minor conformation of FAPBN, in which the PBN moiety is lined up along the fluorinated chain. The 1LB (β -D-Galactose), GA4 (gluconic acid), DAH (2,6-diaminohexanoic acid) and DFA (3,3,4,4,5,5,6,6,7,7,8,8,8-dodecafluorooctan-1-amine) moieties are depicted in red, blue, brown and yellow, respectively. Trolox and PBN are depicted in grey and pink, respectively. The cholines are depicted in cyan, the phosphates are depicted in magenta, 1st and 2nd unsaturations are depicted in green and dark-green, respectively.

Table 1. Binding parameters and rate of penetration (k) of the fluorinated antioxidants in DLiPC monolayers.

	FATxPBN	FATx	FAPBN
MIP (mN/m)	52.2 ± 2.3	48.6 ± 5.3	37.6 ± 5.3
$\Delta\Pi_{30}$ (mN/m)	10.0 ± 0.4	9.0 ± 1.5	2.1 ± 1.1
Synergy factor	0.55 ± 0.02	0.52 ± 0.06	0.71 ± 0.05
k (s ⁻¹)	1.11*10 ⁻²	1.17*10 ⁻²	0.52*10 ⁻²

Table 3. Combination index (CI) of FATx and FAPBN. CI<1: supra-additive, CI=1: additive, CI>1: infra-additive. Values with different superscripts differ significantly ($p < 0.05$).

Concentration (μM)	Combination index	Effect
0,4	0,46 ^a	Supra-additive
0,6	0,69 ^a	Supra-additive
0,8	0,92 ^a	Supra-additive
1,6	1,83 ^b	Infra-additive
3,2	3,66 ^b	Infra-additive
6,4	7,33 ^b	Infra-additive
12,8	14,66 ^b	Infra-additive
25,6	29,31 ^b	Infra-additive
38,4	43,97 ^b	Infra-additive

