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(Article begins on next page)

1

2 **Antioxidant Effect of Cardanol in Mixed Nanoformulations with** 3 **Pluronic**

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11 KEYWORDS: Pluronic F98 and F108; cardanol; polyether oxidation; antioxidant; mixed micelles

12 **Abstract.** The use of nontoxic, biocompatible and very stable surfactants in the design and
13 preparation of nanoformulations for drug delivery and food industry applications is a quickly
14 expanding area. In this framework, Pluronics are a well explored class of triblockcopolymers
15 presenting hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO)
16 in an A-B-A structure (PEO-PPO-PEO) with different PEO/PPO unit ratio. However, polyethers
17 can undergo oxidation with unpredicted concerns. We describe here the design and
18 characterization, in physiological conditions at 37°C, of mixed formulations of Pluronic F98 or

19 F108 with 5 or 10% of cardanol (or tert-butyl cardanol), a natural antioxidant that is able to
20 significantly reduce (up to 80%) the detrimental peroxidation. A systematic study will be necessary
21 to fully address the toxicity of these nanosystems but our preliminary MTT assays on fibroblasts
22 are in favour of their benign nature.

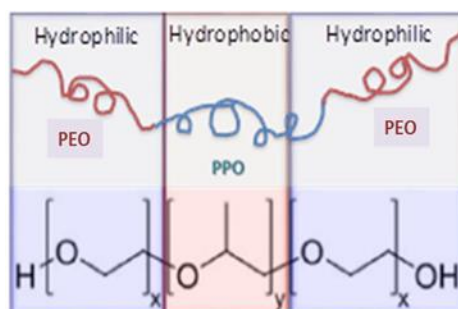
23

24 1. Introduction

25 The impact of nanotechnology on fundamental medical, social and economic fields is constantly
26 increasing, thanks to the wide possibilities that nanosized objects open up to solve problems in
27 new and still unexplored ways.[1][2] The use of nonionic surfactants in the design and preparation
28 of nanoformulations (micelles, nanogels, liposomes, nanosponges etc.),[3] to be used in both
29 pharmaceutical and food industry, has been widely studied in the last decades and it is now a well-
30 established and quickly expanding area. These kinds of surfactants are nontoxic, biocompatible,
31 very stable and able to greatly increase the water solubility of many lipophilic species, including
32 drugs.[4]

33 In these framework, the most exploited class is the one of Pluronic®,[5] amphiphilic triblock
34 copolymers of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide)
35 (PPO), arranged in an A-B-A structure (PEO-PPO-PEO).[6]

36



37 **Scheme 1.** Schematic representation of the polymeric class of Pluronic® (Poloxamers).

38

39 These polymers, interacting with both hydrophobic surfaces and biological membranes [7][8]
40 has been already largely exploited in biomedical applications.[9]

41 In aqueous solution, Pluronic molecules self-assemble into micelles depending on many
42 conditions, not only on the concentration of the copolymers, but also by temperature [10][11] and
43 other environmental conditions such as the presence of other species, in particular electrolytes or
44 polymers, including different mixed Pluronics.[12] This is a critical point since Pluronic exhibits
45 different cell internalization pathways when in the form of single chains (unimers) or in
46 micelles.[13]

47 Micellation takes to nanoobjects with a typical “cargo” architecture that has been exploited for
48 the incorporation of considerable amounts (up to 20–30 wt. %) of water-insoluble drugs,[12][14]
49 but also to efficiently protect molecules with undesirable pharmacokinetics or low stability.[11]

50 The dependence on temperature of phase behavior was exploited in the design of various
51 temperature responsive drug delivery systems.[15]

52 Numerous studies highlight the great versatility of Pluronic micelles in oral delivery of
53 drugs[16][17][18][19] and tumor-specific delivery of antineoplastic agents[20][21] that includes
54 the possibility to be targeted to tumor sites by passive or active mechanisms[22] modifying their
55 surface with specific receptors.

56 The first anticancer micellar formulation to reach clinical evaluation is a mixture of the anticancer
57 drug doxorubicin with co-micelles of Pluronics® L61 and F127 (SP1049C, currently developed
58 by Supratek Pharma Inc.).[23] Pluronic block copolymers, in fact, are one of the very few synthetic
59 polymeric materials approved by the U.S. Food and Drug Administration for use as food additives
60 and pharmaceutical ingredients.

61 It is important to point out, however, that polyethers can undergo oxidation when exposed to
62 air[24]with formation of hydroperoxides at the methylene groups adjacent to the ether bond. The

63 process leads to chain cleavage and to different types of aldehydes as the main scission
64 products.[25] This possible gradual change in physical-chemical properties of solutions of
65 surfactants containing polyoxyethylene chains may cause formulation problems; in particular, the
66 dermatological impact of the oxidative degradation is probably the most severe concern. Even if
67 so many characteristics of these polymeric micelles are known (excellent biocompatibility, low
68 toxicity, enhanced blood circulation time and dissolving of a large number of drugs in their core),
69 problems related to the oxidation of the polyoxyethylene chains are much less discussed and still
70 unsolved.

71 This challenging point together with our expertise in the study of antioxidant properties of
72 cardanol in micellar systems,[26][27] have taken us to explore mixed Pluronic-cardanol
73 formulations aiming to the preparation oxidation self-preserving carriers suitable for biomedical
74 applications. Cardanol and its derivatives are “green” and “renewable” natural alkylphenols
75 byproducts of cashew nut processing endowed with antioxidant activity that are effective also in a
76 micellar environment.[28][29][30] Initially we proposed the use of sustainable plant-derived
77 cardanol as an additive to commercial surfactants and we demonstrated that its addition, in amount
78 as high as 10% (in moles with respect to the moles of surfactant), to commercial surfactants with
79 different charge does not significantly affect their properties.[26] Moreover, cardanol derivatives
80 [28][31] in dispersed systems of Triton X-100 presents analogous antioxidant activity than
81 commercial synthetic antioxidants BHT (2,6-di-*tert*-butyl-4-methylphenol) and DTBQ (2,5-di-*tert*-
82 butylhydroquinone).[27] The interesting results prompted us to examine in more detail the
83 antioxidant function of cardanol derivatives in nanosystem potentially suitable for biomedical
84 applications both toward the surrounding environment and the oxidative portions of the
85 nanostructure itself.

86 Here we discuss the preparation and careful characterization of mixtures of Pluronic (F98 and
87 F108) and cardanol (C) or *tert*-butyl cardanol (TC) both at 5% and 10% in moles with respect to
88 the surfactant. The antioxidant performance of these natural alkylphenols toward the peroxidation
89 of the polyethylene tail and toward the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical
90 was addressed together with the biocompatibility of these mixed nanoformulations in a MTT assay
91 on fibroblasts. Envisaging potential biomedical applications for these systems, we have
92 investigated their behavior in conditions mimicking as close as possible the physiological ones.

93

94 **2. Materials and Methods**

95 *2.1. Materials*

96 Pluronic F98 and F108, D₂O, diphenylpicrylhydrazyl (DPPH•) radical, 4,4'-Azobis(4-
97 cyanovaleric acid) sodium salt (ABCV), 2,2,5,7,8-pentamethyl-6-chromanol (PMHC), KCl, NaCl,
98 Na₂HPO₄, NaH₂PO₄ and solvents of analytical grade were all purchased from Sigma-Aldrich and
99 used without further purification. A Milli-Q Millipore system was used for the purification of water
100 (resistivity ≥ 18 M Ω). Samples of hydrogenated cardanol (C) and 6-*tert*-butyl hydrogenated
101 cardanol (TC), having a saturated alkyl chain, were kindly provided by Prof. De Crescentini
102 (University of Urbino).

103 Human skin fibroblast (BJ, ATCC® CRL-2522™) were kindly provided by Dott. Lorenzini
104 (DIBINEM, Alma Mater Studiorum – University of Bologna) and grown according to Croco et
105 al.[32]

106 We prepared mixed formulations for both F98 and F108 with C or TC adding 5% or 10% in
107 moles of the antioxidant with respect to the moles of the surfactant in solution ((moles of
108 antioxidant/total moles of surfactant) x 100).

109 2.2. Dynamic Light Scattering (DLS)

110 DLS measurements were carried out using a Malvern Zetasizer Nanoseries equipped with a Laser
111 633 nm. All DLS measurements were performed in PBS solution (pH 7.4), at a total ionic
112 concentration of 0.14 M, at temperature of 25°C or 37°C and at a scattering angle of 173°. The
113 samples were prepared at two different concentrations of surfactant 7.5×10^{-4} M and 7.5×10^{-3} M
114 both above their CMC that are reported to be 7.7×10^{-5} and 2.2×10^{-5} M for F98 and F108
115 respectively, at 37°C at pH 7.4.[33] For both of them (F98 and F108) and for each concentration
116 we explored the effects of the addition of 5% and 10% molar equivalents of C or TC. For the DLS
117 measurements, we used disposable polystyrene cuvettes of 1 cm optical path length.
118 Polydispersion Index (PDI) indicates the width of DLS hydrodynamic diameter distribution and it
119 is calculated by means of cumulant analysis, $PDI = (\sigma/Z_{avg})^2$, where σ is the width of the
120 distribution and Z_{avg} is average diameter of the particle population. Errors on mean effective
121 hydrodynamic diameters have been calculated from the standard deviation (SD) of data obtained
122 from triplicate measurements.

123 2.3. NMR Measurements

124 ^1H and 2D NMR spectra were recorded at 25°C and 37°C on a Varian Inova spectrometer
125 operating at 600 MHz in D_2O solutions using the solvent peak as an internal standard. Chemical
126 shifts are reported in parts per million (δ scale). NOESY data were collected using a 90° pulse
127 width of 5.8 μs and a spectral width of 6000 Hz in each dimension, respectively. The data were
128 recorded in the phase sensitive mode, without spinning the sample. Acquisitions were recorded at
129 mixing times 500 ms. Other instrumental settings were: 256 increments of 2K data points, 8 scans
130 per t_1 , 1.5 s delay time for each scan.

131 2.4. Determination of DPPH• scavenging

132 The reactivity of mixed micelles containing antioxidants toward the DPPH• radical was assessed
133 by measuring the disappearance of the DPPH• absorption band at 535nm (an intermediate value
134 between the absorption λ_{\max} of DPPH• in the presence of F98 and F108, that are 533 nm and 537
135 nm, respectively). The proper amount of fresh methanolic DPPH• solution (final concentration
136 1.00×10^{-4} M) was added into a quartz cuvette containing micelles of Pluronic F98 or Pluronic
137 F108 (7.0×10^{-4} M) and antioxidants (C or TC: 7.0×10^{-5} M and 3.5×10^{-5} M) in PBS solution
138 (pH 7.4).[26,27] The spectra were recorded at 37 °C with a Jasco V-550 spectrophotometer. The
139 stoichiometry of the reaction (i.e number of radicals quenched by each antioxidant) was
140 determined by using a slight excess of DPPH•.[34][35]

141 2.5. Inhibition of Pluronic peroxidation

142 The extent of Pluronic peroxidation in the absence and in the presence of the co-micellized
143 antioxidants was evaluated by measuring the O₂ consumption during the reaction by an optical
144 oxygen meter (FirestingO2, Pyroscience GmbH).The reaction was initiated by the hydrosoluble
145 azo-initiator 4,4'-Azobis(4-cyanovaleric acid) sodium salt (ABCV) (50 mM) at 30°C. Initiation
146 rate, R_i , was determined by the inhibitor method using 2,2,5,7,8-pentamethyl-6-chromanol
147 (PMHC) as a reference antioxidant: $R_i = 2[\text{PMHC}]/\tau$, where τ is the length of the induction period
148 ($R_i = 1.8 \times 10^{-9}$ M s⁻¹).[36][37]

149 2.6. Cell viability

150 Cells (fibroblasts, kindly provided by Dott. Antonello Lorenzini – University of Bologna) were
151 treated with increasing concentrations of cardanol or Tert-butyl cardanol (5, 10 or 50 μ M, always
152 10% in moles with respect to Pluronic) in the presence or absence of Pluronic F98 for 48 h in 24-
153 well plates, then incubated with 0.5 mg/mL MTT for 4 h at 37 °C. The blue-violet formazan salt
154 crystals formed were dissolved with a solubilisation solution (10% SDS, 0.01 M HCl) keeping the

155 plates overnight at 37 °C and 5% CO₂ in a humidified atmosphere. The absorbance at 570 nm was
156 measured using a multiwell plate reader (Wallac Victor2, PerkinElmer).

157 2.7. Statistical Analysis

158 Statistical analysis was performed using the Student's t test (GraphPadPrism, GraphPad software
159 Inc., CA, USA), and the level of significance was set at the probabilities of * $p < 0.05$.

160

161 **3. Results and Discussion**

162 *3.1. Morphologic characterization*

163 With the aim of using cardanol to prevent chain cleavage in Pluronic mixed micelles, as a first
164 step, we performed a systematic study to address the influence of C and TC on the micellization
165 properties of Pluronic F98 and F108 in conditions mimicking cell culture media (PBS water
166 solution, pH 7.4, 37°C). Pluronic micelles are known and already exploited in biomedical
167 applications, however, their CMC values are not univocally reported in literature. This is because,
168 for this class of materials, the micelle formation process is significantly influenced not only by the
169 polymer concentration but also by temperature and salt concentrations in solution.[38] In fact, the
170 CMC decreases while the hydrodynamic diameter (D_h) increases with the increment of the last two
171 parameters, and this makes quite tricky to make a direct comparison of CMC and CMT (critical
172 micelle temperature) values in literature.

173 Therefore, in order to have a more complete picture of the self-assembly behaviors in the
174 presence of the cardanol derivatives we measured the D_h and size distributions of Pluronic F98
175 and F108 aggregates and micelles with dynamic light scattering (DLS). Data were obtained in the
176 absence and in the presence of different amounts (5% and 10% in moles/moles with respect to the
177 polymer) of C or TC in solution in defined and comparable conditions. We fixed the total ionic
178 concentration to a value of the same order of magnitude of biological environments or cell cultures,

179 so as the temperature, using 0.14 M and 37°C respectively. We investigated, in this conditions,
180 two different concentrations of the surfactants 7.5×10^{-4} and 7.5×10^{-3} M, both, according to
181 literature, above their CMC of 7.7×10^{-5} and 2.2×10^{-5} M for F98 and F108 respectively, at 37°C
182 at pH 7.4 [33][39][40] to evaluate possible variations of the cardanol influence at two
183 concentration regimes.

184 None of our samples, including the ‘blank’ ones containing only the polymer with 0% of C or
185 TC, showed a single peak due to the presence only of the micelles, instead, all the DLS profiles
186 present two main peaks: one below and one above 10 nm of diameter (see fig. S1–S4). This data
187 could be tentatively explained by the coexistence of single polymers (or small oligomers) together
188 with micelles. The different ratio of unimers and micelles depending on the concentration of the
189 polymer is analogously well investigated. The presence of salts help micellization but high
190 polymer concentrations favour modification of the aggregation number and size. Similarly,
191 impurities could influence the micellization features but our results evidence that they don’t change
192 significantly adding C or TC in any of the analyzed amounts with respect to the polymer alone in
193 the same conditions. It is quite interesting to note that this sort of unimer/micelle equilibrium is
194 much more shifted toward the micelle formation for both polymers at the lower concentration (7.5
195 $\times 10^{-4}$ M) in the investigated conditions.

196 In order to evaluate the temperature influence on the system behavior, we repeated all
197 measurements also at 25°C and in all cases, we found a more polydisperse profile: the two peaks
198 diverge even more, and other broad ones are formed due to the presence of large aggregated
199 structures (data not shown).

200 These data, altogether, indicate that F98 and F108 form Pluronic-cardanol co-micelles with 5 and
201 10% of C and TC in PBS water solutions at pH 7.4 and 37°C, conditions mimicking a biological
202 environment and that the presence of C and CT does not significantly affect the system. However,

203 in these environments we recorded a bimodal distribution that could be rationalized by the
204 presence of single polymers (or small oligomers) together with micelles, as already reported by
205 other authors. Bahadur and co-authors[38] show that at the concentration 7.7×10^{-3} M Pluronic F98
206 presents a bimodal distribution at 37°C if the ionic concentration in solution is lower than 2 M.
207 For lower amounts of salts, the two peaks coalesce in single one indicating complete micelle
208 formation for Pluronic F98 only above 45°C.

209 With the aim of addressing a system suitable for biomedical applications (that do not typically
210 envisage $T > 45^\circ\text{C}$) is therefore necessary to carefully characterize the system in conditions where
211 it is a mixed unimers/micelles one and to address the cardanol distribution, its oxidation behavior
212 and the formulation biocompatibility.

213 *3.2 NMR characterization*

214 The NMR analysis was used to provide evidence of the interaction between cardanol and
215 poloxamer F98. The spectra were acquired at different concentrations in deuterium oxide
216 containing 5% of CD_3OD . In particular, we chose three different surfactant concentrations ($2 \times$
217 10^{-2} , 5×10^{-3} , and 2.6×10^{-4} M), where only the third one is under the CMC value at 25°C.[39]
218 The spectra were registered in absence and in presence of cardanol both at 25 and 37 °C.

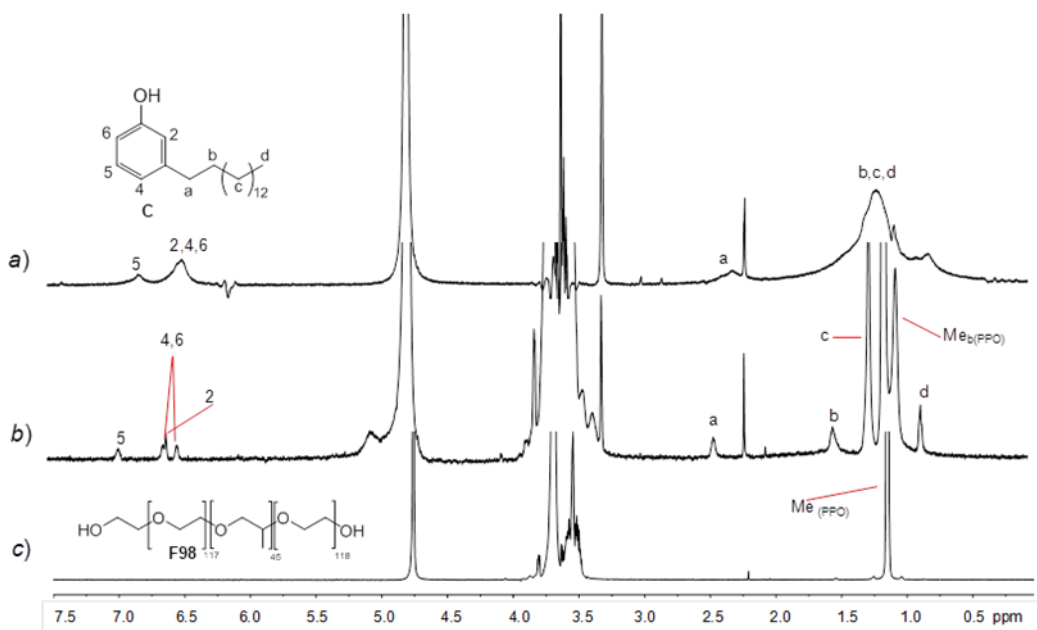
219 The first experimental evidence was that pure Pluronic F98 shows two main sets of signals
220 centered around 1.15 ppm (methyl protons of the PPO polymer block) and in the range 3.45-3.85
221 ppm, due to all the ethereal protons of the PEO and PPO moieties. The ^1H spectra recorded at both
222 temperatures did not present significant differences in the resonance frequencies of the peaks in
223 all the surfactant concentrations investigated.

224 Looking at the cardanol, instead, the presence of 2×10^{-2} and 5×10^{-3} M of polymer causes a
225 drastic change in the signal pattern of the alkylphenol of a solution of 5% cardanol, if compared
226 with the spectrum of pure cardanol in the same solvent mixture. In particular, (see Figure S5 of

227 SI) the spectral regions of the aromatic ring protons of C present well resolved peaks, especially
228 at 37°C, with respect to the broad signals of free cardanol.[41][42][43] In these experiments it is
229 reasonable the instauration of intermolecular interactions among cardanol and the polymer chains
230 that cause a significative improvement of the spectral lines of the phenol in the micelles, unimers
231 and in other possible self-organized aggregates (5×10^{-3} M).

232 To get insight on the specific molecular interactions between C and Pluronic F98, we decided to
233 investigate the behavior of the alkylphenol below the CMC of the polymer at 25°C (and at 37°C,
234 data not shown). Figure 1 reports NMR proton spectra of 2.6×10^{-4} M of cardanol, Pluronic F98,
235 and equimolar amounts of both the alkylphenol and the polyether. As previously described, also
236 the spectrum of 1:1 mixture of F98 and C (trace b, Figure 1) shows resolved signals for all the
237 resonances of cardanol in both the aromatic and the aliphatic regions. In addition, a new signal
238 appears in the spectrum, falling in the aliphatic region at 1.05 ppm close to the methyl peaks of the
239 PPO fragment [Me(PPO)] of Pluronic. Integration of peak areas of both these signals, i.e. the
240 Me(PPO) and the new one, corresponds to the sum of the methyl protons of the whole PPO
241 polymer block, and this suggests that the new signal belongs to the surfactant methyl groups of the
242 PPO moiety shifting upfield in the presence of cardanol.[44]

243



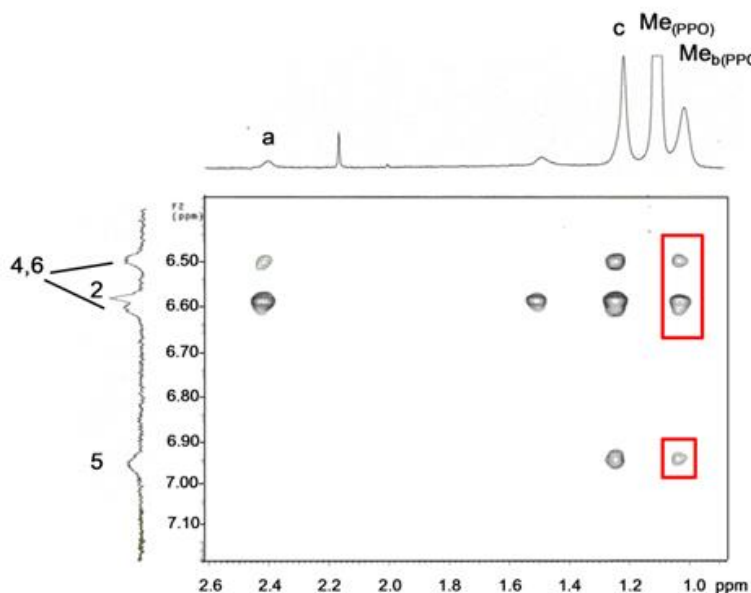
244
 245 **Figure 1.** ^1H NMR spectra (600 MHz, 95:5 $\text{D}_2\text{O}/\text{CD}_3\text{OD}$, 298 K) of 2.6×10^{-4} M of a) cardanol,
 246 b) cardanol and F98, and c) Pluronic F98. Signal of spectra are labelled as reported in the structures
 247 of C and Pluronic F98.

248
 249 These two well-resolved signals on the NMR time scale due to the lower chemical exchange rate
 250 between the free $[\text{Me}(\text{PPO})]$ and complexed $[\text{Me}_b(\text{PPO})]$ protons at 25°C , are visible also at 37°C ,
 251 indicating the robustness of the complex.

252 If we suppose that all cardanol interact with the PPO core of pluronic, the integration area value
 253 of the new signal $\text{Me}_b(\text{PPO})$, should coincide with the number of methyl groups involved in the
 254 complexation with C. Actually, this value corresponds to about six methyl protons that undergo a
 255 chemical shift variation due to the presence of the phenol guest.

256 To confirm the location of hydrophobic Cardanol into the PPO block copolymer, we recorded a
 257 2D NOESY spectrum of 1:1 solution of 2.6×10^{-4} M of F98 and C at 25°C to detect proton-proton
 258 interactions occurring in the two species. Figure 2 reports the partial 2D contour plot showing
 259 cross peaks correlating phenol protons and aliphatic signals of Pluronic and cardanol itself.

260 In this region, the cross peaks (in the red boxes) connecting protons 2, 4, 5, and 6 with those at
261 1.05 ppm [Me_b(PPO)] are clearly visible.



262
263 **Figure 2.** Partial 2D NOESY spectrum (600 MHz, 95:5 D₂O/CD₃OD, 298 K) of 2.6 x 10⁻⁴ M
264 solution sample of cardanol/Pluronic F98 (1:1). Red-squared cross peaks indicate the
265 intermolecular correlation among phenol protons of C and Me_b(PPO) groups of the polymer.
266 Signals of the 1D spectra are labelled according to the structures of C and Pluronic F98 reported
267 in Figure 1.

268
269 This supports the previous hypothesis of an intermolecular interaction between C and the
270 hydrophobic part of Pluronic. Also, the NOE experiment detects other intermolecular interactions
271 (see Fig. S6) connecting the aliphatic chain of the phenol guest (i.e. protons a, b and c) with the
272 Me_b(PPO) resonance, again confirming the hosting of cardanol in the PPO copolymer block
273 region.

274 *3.3. Antioxidant activity*

275 The radical trapping ability of Cardanol and *tert*-butyl Cardanol in F98 and F108 micelles was
276 assessed by studying their reaction with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•). This is
277 an experiment commonly used as preliminary test for the estimation of antioxidant activity.[27] In
278 the presence of reducing molecules, the purple DPPH• radical is reduced to the yellow hydrazine
279 through a formal H atom transfer reaction.[25]

280 We monitored the reaction by UV-Vis spectroscopy, measuring the decrease of the absorption
281 maximum of DPPH• (λ_{\max} 535 nm) as a function of time in a water solution of polymeric mixed
282 micelles (cardanol derivatives and Pluronic). To mimic the cellular conditions the polymer
283 micelles were prepared in PBS solution at pH 7.4 and measurements were done at 37 °C. The
284 results show that cardanols are able to quench one molecule of DPPH•, accordingly with the
285 mechanism reported in Scheme 2.

286 In the presence of Pluronic F98 for both derivatives (C and TC) and concentrations (5% and
287 10%) the stoichiometry is the same (C 5%: 1.0+/-0.3; C10%: 1.0+/-0.2; TC 5%: 1.0+/-0.6, TC
288 10%: 1.1+/-0.5), slightly higher values were obtained in the presence of Pluronic F108 (C 5%:
289 1.3+/-0.2; C10%: 1.1+/-0.2; TC 5%: 1.2+/-0.2, TC 10%: 0.9+/-0.5).

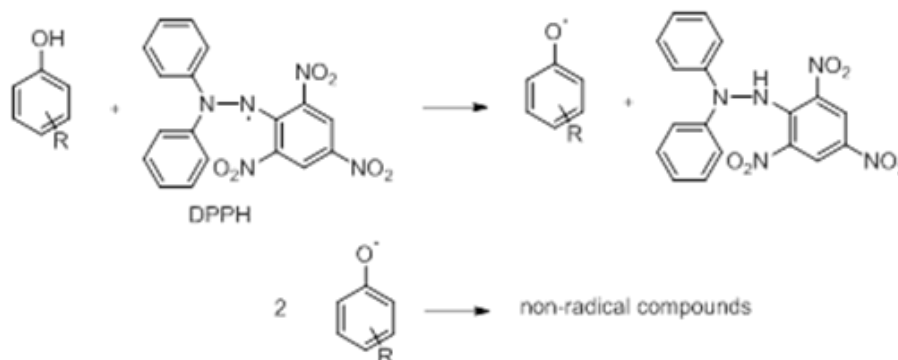
290 The experiments confirmed that the stoichiometry of aggregates is that expected for a
291 monophenolic antioxidant,[45] this supports the evidence that cardanol derivatives as co-
292 surfactants maintain their native properties.

293 As shown in Figure S7, the absorption wavelength maximum of DPPH• shifts from 511 nm in
294 methanol to 533 and 537 nm in the presence of the mixed micelles of Pluronic F108 or F98
295 respectively.

296 The absorbance shift of DPPH• toward lower energies with the increasing water fraction in the
297 solvent is a well-known effect that has been explained with the rise of aggregation processes.[46]

298 We then investigated the ability of cardanol and *tert*-butyl cardanol to prevent the peroxidation
299 of the oxyethylene or oxypropylene units of Pluronic.

300



302

302 **Scheme 2.** Schematic representation of the reaction of cardanols with the DPPH• radical.

303

304 The antioxidant activity of cardanols was assessed by measuring their ability to retard this
305 peroxidation, which occurs via a radical chain reaction sustained by peroxy radicals (ROO•)
306 [47][48] under a constant flux of initiating radicals, the water-soluble azoinitiator 4,4'-Azobis(4-
307 cyanovaleric acid) sodium salt (ABCV).[49] The decrease of O₂ concentration in solution directly
308 correlates with the progress of the peroxidation reaction and it was followed by an optical oxygen
309 probe. Oxygen consumption experiments, reported in Figure 3, show that Pluronic F108 oxidizes
310 faster than F98, reasonably because of its larger number of OCH₂ units. It has also to be recalled
311 that the aggregation situation of the polymer is very complex and we do not have only micelles in
312 solution but also unimers and the relative percentage relatively differ for the two polymers having
313 also different CMC values (lower for F108). The presence of cardanol at 5% or 10% ratio,
314 significantly reduces the auto-oxidation of Pluronic in a concentration-dependent fashion.

315 TC has a larger antioxidant effect than cardanol, in agreement with data reported in literature
316 performed in homogeneous organic solutions.[50][31] The reason is that electron donating

317 substituents in ortho and para positions to the reactive phenol group lower the bond dissociation
318 enthalpy of the O-H bond, making it more reactive toward H-atom abstraction from ROO•
319 radicals.[26][51]

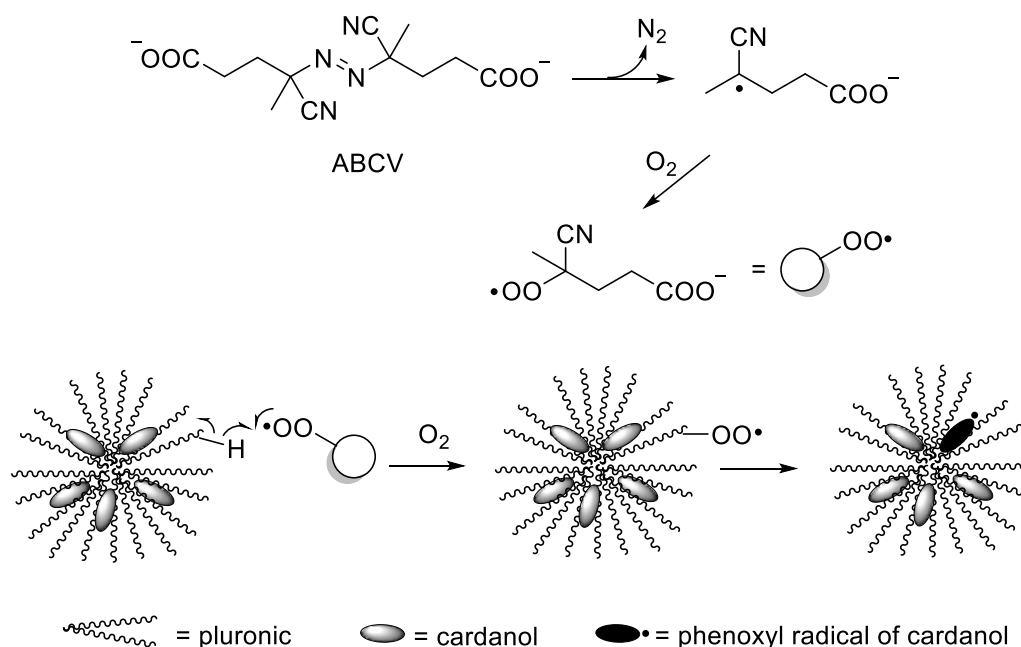
320 The data reported in Table 1 show that in presence of the maximum amount of cardanols, the rate
321 of O₂ consumption reaches a lowest limit of about 4 nMs⁻¹ for F108, that is approximately twice
322 the rate of radical generation by ABCV ($R_i = 1.8 \text{ nMs}^{-1}$). This observation provides some
323 mechanistic insight, since measured O₂ consumption corresponds to the sum of the O₂ uptake from
324 the alkyl radicals formed from the azoinitiator (that is equal to R_i), and from the secondary alkyl
325 radicals formed by Pluronic (Scheme 3). If cardanols were able to quench the initiating radicals
326 produced by ABCV decomposition, the lowest limit of O₂ consumption, reached on increasing the
327 cardanol concentration, should have been equal to R_i . Instead, if cardanols can quench only
328 Pluronic-derived peroxy radicals, the O₂ uptake limit would be expected to be twice the value of
329 R_i , as experimentally observed. In conclusion, cardanols can trap peroxy radicals formed on the
330 alkyl chain of Pluronic, but are relatively less effective at quenching the charged ABCV-derived
331 peroxy radicals, that react with the outer moieties of the polymers that are exposed toward the
332 solvent and that are not protected by the antioxidant.

333
334 **Figure 3.** Oxygen consumption during the autoxidation of Pluronic ($7 \times 10^{-3} \text{ M}$) initiated by ABCV
335 at 30 °C in the absence of antioxidants (black line) and in the presence of: 5% (a) or 10% (b) of
336 cardanol; 5% (c) or 10% (d) of tert-butyl cardanol. Panel (A): Pluronic F98; panel (B): Pluronic
337 F108.

338
339 **Table 1.** Rates of O₂ consumption in nM s⁻¹ during ABCV-initiated Pluronic autoxidation.

Pluronic	-	5% C	10% C	5% TC	10% TC
F98	11±1	8.7±0.5	6.5±0.5	5.9±0.5	5.7±0.5
F108	15±1	6.7±0.5	4.1±0.5	3.9±0.5	4.0±0.5

340



341

342

343 **Scheme 3.** Reaction of the water soluble ABCV-derived peroxy radical with the outer portion of
 344 Pluronic (here we represent only micelles), and subsequent trapping of the peroxy radical by
 345 cardanol.

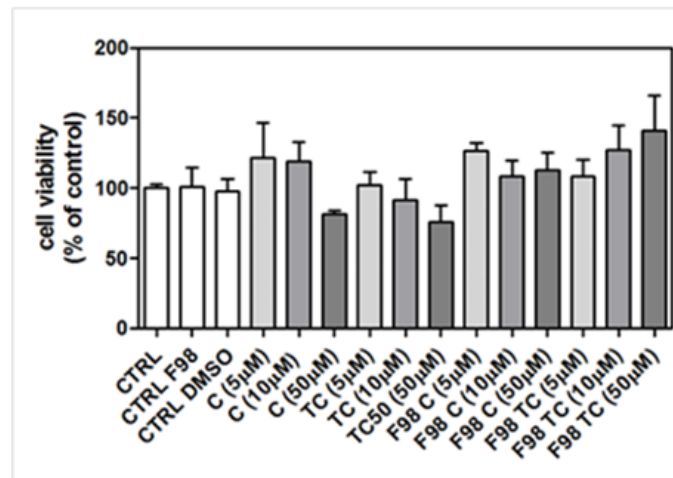
346

347 Actually, if cardanols were able to quench all the initiating radicals produced by ABCV
 348 decomposition, the rate of O_2 consumption should have been equal to R_i . A reasonable explanation
 349 is that cardanols can trap all peroxy radicals formed on the alkyl chain of Pluronic, but are unable
 350 to quench the charged ABCV-derived peroxy radicals, that react with the outer portion of the
 351 polymers that is exposed toward the solvent and that is not protected by the antioxidant.

352

353 3.4. Cell activity

354 In order to investigate the potential cytotoxicity exerted by cardanol or *tert*-butyl cardanol
355 included in Pluronic (F98), a MTT assay was performed in fibroblasts after 48 h incubation. In all
356 the tested experimental conditions (5, 10 or 50 μ M of cardanol or *tert*-butyl cardanol, always 10%
357 in moles with respect to Pluronic) we did not detect any significant difference from control on cell
358 viability as shown in Figure 4.



359

360 Figure 4. Effect on cell viability of nanoformulations based on Pluronic F98-cardanol
361 Fibroblast (BJ) were incubated for 48 h with Pluronic F98 micelles including cardanol (C) or *tert*-
362 butyl cardanol (TC) (5, 10 or 50 μ M always 10% in moles with respect to Pluronic) and cell
363 viability was assessed by means of MTT assay. Values are expressed as means \pm SD (n = 4).
364 Statistical analysis showed no significant differences between treated and control cells (p>0.05)

365

366 4. Conclusions

367 We have described here the design and characterization of mixed systems of Pluronic F98 or
368 F108 with 5 or 10% moles/moles of the natural antioxidant cardanol. Our data show how, in the
369 mixed formulations, cardanol reduces of a high percentage (up to 80%) the detrimental

370 peroxidation of Pluronics. This is a very interesting result due to the quite stable hosting of
371 cardanol in the polymer (see NMR data) also in the coiled unimers. This is important since DLS
372 measurements, in physiological conditions, at 37°C showed a bimodal distribution indicating the
373 simultaneous presence of single polymers (or small oligomers) and micelles

374 In conclusion, the insertion of cardanol, a green and renewal species, in a FDA approved material
375 can yield oxidative self-preserving nanoformulation, efficiently reducing unwanted oxidative
376 instability of the Pluronic chains. The biomedical application of these nanosystems requires a
377 systematic study to fully assess their toxicity but preliminary MTT assays on fibroblasts are in
378 favour of their benign nature.

379

380 **Conflict of interest**

381 There are no conflicts to declare.

382

383 **Supplementary Material**

384 Supplementary Material: DLS data, NMR data, determination of DPPH• scavenging: solvent
385 effect. This material is available free.

386

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391

392 **Author contributions**

393 SG and NZ: Conceptualization and original draft preparation; EM, RA and CP: contributed in
394 writing their part of competence and in reviewing the paper; FP and NZ: data collecting and formal
395 analysis for the DLS measurements; EM: data collecting and formal analysis for the NMR
396 measurements; RA, SG and AB: data collecting and formal analysis for the antioxidant activity
397 investigation; CP: data collecting and formal analysis for the cell activity investigation.

398

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