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Antioxidant Effect of Cardanol in Mixed Nanoformulations with Pluronic

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Abstract. The use of nontoxic, biocompatible and very stable surfactants in the design and preparation of nanoformulations for drug delivery and food industry applications is a quickly expanding area. In this framework, Pluronics are a well explored class of triblokcopolymers presenting hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) in an A-B-A structure (PEO-PPO-PEO) with different PEO/PPO unit ratio. However, polyethers can undergo oxidation with unpredicted concerns. We describe here the design and 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 Pluronics®,[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]

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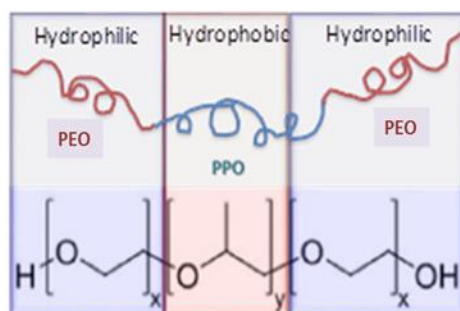
37 **Scheme 1.** Schematic representation of the polymeric class of Pluronics® (Poloxamers).

38

2

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-

82 *tert*-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, D_2O, 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   Na_2HPO_4, NaH_2PO_4 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 MΩ). 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 x 10^{-4} M and 7.5 x 10^{-3} M

114   both above their CMC that are reported to be 7.7 x 10^{-5} and 2.2 x 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 = $(\sigma/\mathrm{Zavg})^2$, where σ is the width of the

120   distribution and Zavg 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   ^{1}H 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 t1, 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 O_2 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, Ri, was determined by the inhibitor method using 2,2,5,7,8-pentamethyl-6-chromanol

147   (PMHC) as a reference antioxidant: $Ri = 2[PMHC]/\tau$, 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 µM, 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% CO_2 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 x 10^{-4} and 7.5 x 10^{-3} M, both, according to

181   literature, above their CMC of 7.7 x 10^{-5} and 2.2 x 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   x 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°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 x

217   10^{-2}, 5 x 10^{-3}, and 2.6 x 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 ^{1}H 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 x 10^{-2} and 5 x 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

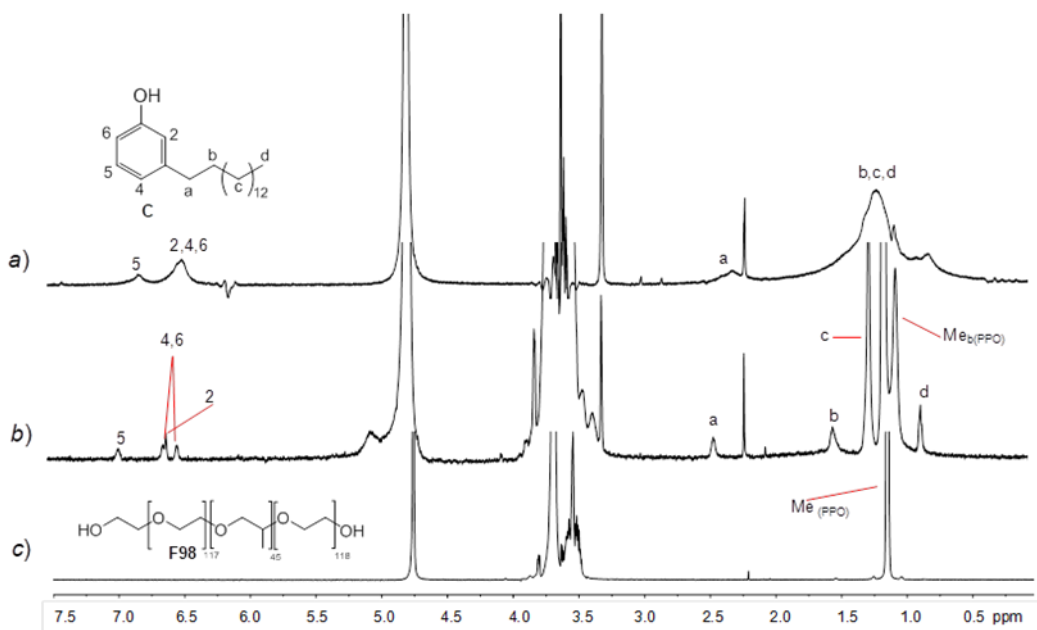242 PPO moiety shifting upfield in the presence of cardanol.[44]

243

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

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

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

To confirm the location of hydrophobic Cardanol into the PPO block copolymer, we recorded a 2D NOESY spectrum of 1:1 solution of 2.6 x 10^{-4} M of F98 and C at 25°C to detect proton-proton interactions occurring in the two species. Figure 2 reports the partial 2D contour plot showing 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
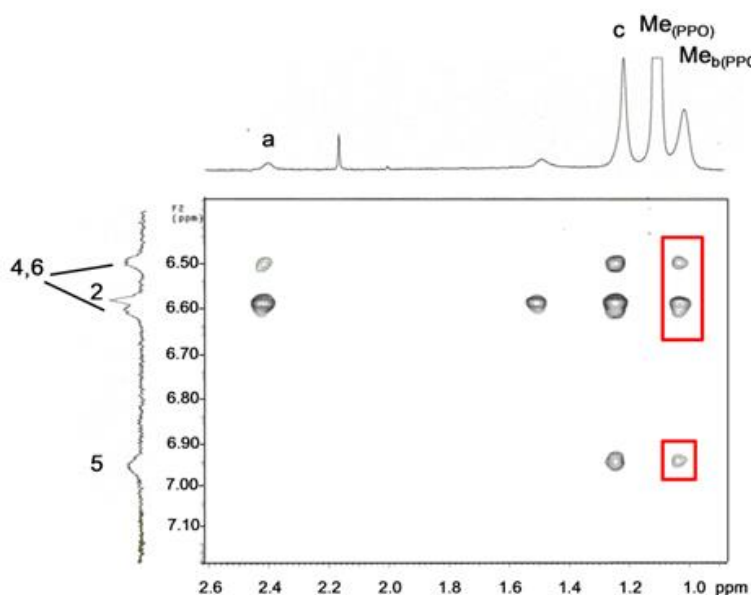
261   1.05 ppm [Me$_b$(PPO)] are clearly visible.

262

263   **Figure 2.** Partial 2D NOESY spectrum (600 MHz, 95:5 D$_2$O/CD$_3$OD, 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 Pluronics). 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.

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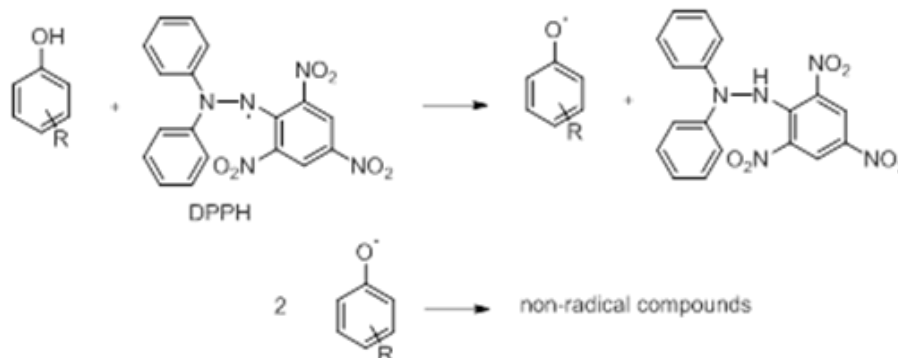
302   **Scheme 2.** Schematic representation of the reaction of cardanols with the DPPH• radical.

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 peroxyl 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 O_2 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 OCH_2 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
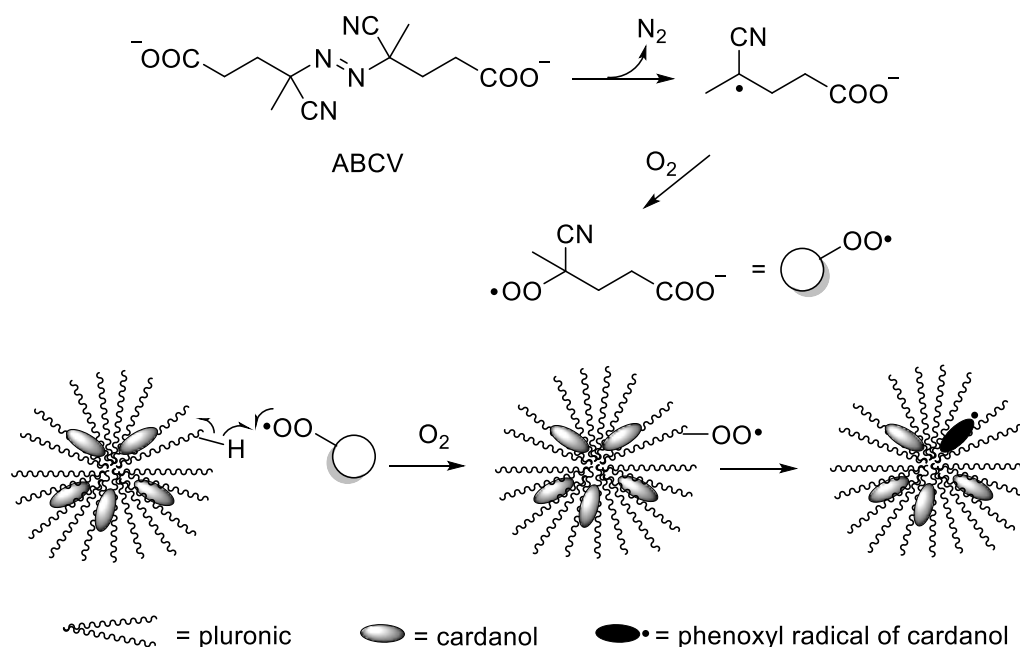
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317 substituents in ortho and para positions to the reactive phenol group lower the bond dissociation

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 O_2 consumption reaches a lowest limit of about 4 nMs^{-1} for F108, that is approximately twice

322 the rate of radical generation by ABCV (Ri = 1.8 nMs^{-1}). This observation provides some

323 mechanistic insight, since measured O_2 consumption corresponds to the sum of the O_2 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 O_2 consumption, reached on increasing the

327 cardanol concentration, should have been equal to R_i. Instead, if cardanols can quench only

328 Pluronic-derived peroxyl radicals, the O_2 uptake limit would be expected to be twice the value of

329 Ri, as experimentally observed. In conclusion, cardanols can trap peroxyl radicals formed on the

330 alkyl chain of Pluronic, but are relatively less effective at quenching the charged ABCV-derived

331 peroxyl 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 x 10^{-3} 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 O_2 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

= pluronic   = cardanol   •= phenoxyl radical of cardanol

341

342

343   **Scheme 3.** Reaction of the water soluble ABCV-derived peroxyl radical with the outer portion of

344   Pluronic (here we represent only micelles), and subsequent trapping of the peroxyl 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 peroxyl radicals formed on the alkyl chain of Pluronic, but are unable

350   to quench the charged ABCV-derived peroxyl 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.

17

352

3.4. Cell activity

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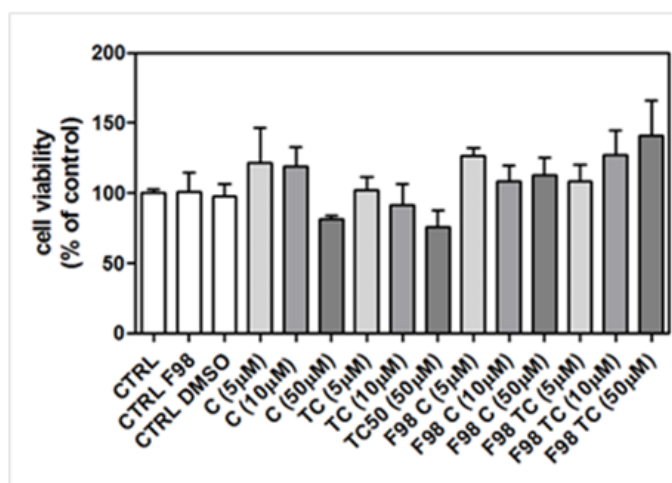

359

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

365

4. Conclusions

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

18

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 access 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

391

392   **Author contributions**

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

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