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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Nelsi Zaccheroni, R.A. (2020). Antioxidant effect of cardanol in mixed nanoformulations with pluronic. JOURNAL OF MOLECULAR LIQUIDS, 316, 113822-113829 [10.1016/j.molliq.2020.113822].

Availability:
This version is available at: https://hdl.handle.net/11585/767499 since: 2023-06-12

Published:
DOI: http://doi.org/10.1016/j.molliq.2020.113822

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05 September 2026

Antioxidant Effect of Cardanol in Mixed Nanoformulations with Pluronic

Nelsi Zaccheroni,*[a] Riccardo Amorati,[a] Elisabetta Mezzina,[a] Andrea Baschieri,[a] Francesco Palomba,[a] Cecilia Prata,[b] Carlotta Facchini,[b] and Susanna Guernelli*[a]

[a]*Dipartimento di Chimica "G. Ciamician", Università degli Studi di Bologna, Via S. Giacomo 11 and Via Selmi 2, 40126 Bologna, Italy*

[b]*Dipartimento di Farmacia e Biotecnologie (FaBiT), Università degli Studi di Bologna, Via Irnerio 48, 40126 Bologna, Italy*

Corresponding Authors: nelsi.zacheroni@unibo.it (NZ) and susanna.guernelli@unibo.it (SG)

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

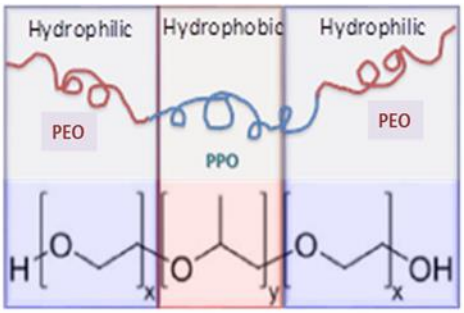
Abstract. The use of nontoxic, biocompatible and very stable surfactants in the design and preparation of nanoformulations for drug delivery and food industry applications is a quickly expanding area. In this framework, Pluronics are a well explored class of triblokcopolymers presenting hydrophilic poly(ethylene 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

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

1. Introduction

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

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



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

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

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

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

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

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

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

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

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

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

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

2. Materials and Methods

2.1. Materials

Pluronic F98 and F108, D_2O, diphenylpicrylhydrazyl (DPPH•) radical, 4,4'-Azobis(4-cyanovaleric acid) sodium salt (ABCV), 2,2,5,7,8-pentamethyl-6-chromanol (PMHC), KCl, NaCl, Na_2HPO_4, NaH_2PO_4 and solvents of analytical grade were all purchased from Sigma-Aldrich and used without further purification. A Milli-Q Millipore system was used for the purification of water (resistivity ≥18 MΩ). Samples of hydrogenated cardanol (C) and 6-*tert*-butyl hydrogenated cardanol (TC), having a saturated alkyl chain, were kindly provided by Prof. De Crescentini (University of Urbino).

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

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

2.2. Dynamic Light Scattering (DLS)

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

2.3. NMR Measurements

^{1}H and 2D NMR spectra were recorded at 25°C and 37°C on a Varian Inova spectrometer operating at 600 MHz in D_2O solutions using the solvent peak as an internal standard. Chemical shifts are reported in parts per million (δ scale). NOESY data were collected using a 90° pulse width of 5.8 μs and a spectral width of 6000 Hz in each dimension, respectively. The data were recorded in the phase sensitive mode, without spinning the sample. Acquisitions were recorded at mixing times 500 ms. Other instrumental settings were: 256 increments of 2K data points, 8 scans per t1, 1.5 s delay time for each scan.

2.4. Determination of DPPH• scavenging

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

2.5. Inhibition of Pluronic peroxidation

The extent of Pluronic peroxidation in the absence and in the presence of the co-micellized antioxidants was evaluated by measuring the O_2 consumption during the reaction by an optical oxygen meter (FirestingO2, Pyroscience GmbH).The reaction was initiated by the hydrosoluble azo-initiator 4,4'-Azobis(4-cyanovaleric acid) sodium salt (ABCV) (50 mM) at 30°C. Initiation rate, Ri, was determined by the inhibitor method using 2,2,5,7,8-pentamethyl-6-chromanol (PMHC) as a reference antioxidant: $R_i = 2[PMHC]/\tau$, where τ is the length of the induction period ($R_i = 1.8 \times 10^{-9}$ M s^{-1}).[36][37]

2.6. Cell viability

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

plates overnight at 37 °C and 5% CO_2 in a humidified atmosphere. The absorbance at 570 nm was measured using a multiwell plate reader (Wallac Victor2, PerkinElmer).

2.7. Statistical Analysis

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

3. Results and Discussion

3.1. Morphologic characterization

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

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

in these environments we recorded a bimodal distribution that could be rationalized by the presence of single polymers (or small oligomers) together with micelles, as already reported by other authors. Bahadur and co-authors[38] show that at the concentration $7.7x10^{-3}$ M Pluronic F98 presents a bimodal distribution at 37°C if the ionic concentration in solution is lower than 2 M. For lower amounts of salts, the two peaks coalesce in single one indicating complete micelle formation for Pluronic F98 only above 45°C.

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

3.2 NMR characterization

The NMR analysis was used to provide evidence of the interaction between cardanol and poloxamer F98. The spectra were acquired at different concentrations in deuterium oxide containing 5% of CD_3OD. In particular, we chose three different surfactant concentrations (2 x 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] The spectra were registered in absence and in presence of cardanol both at 25 and 37 °C.

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

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

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

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

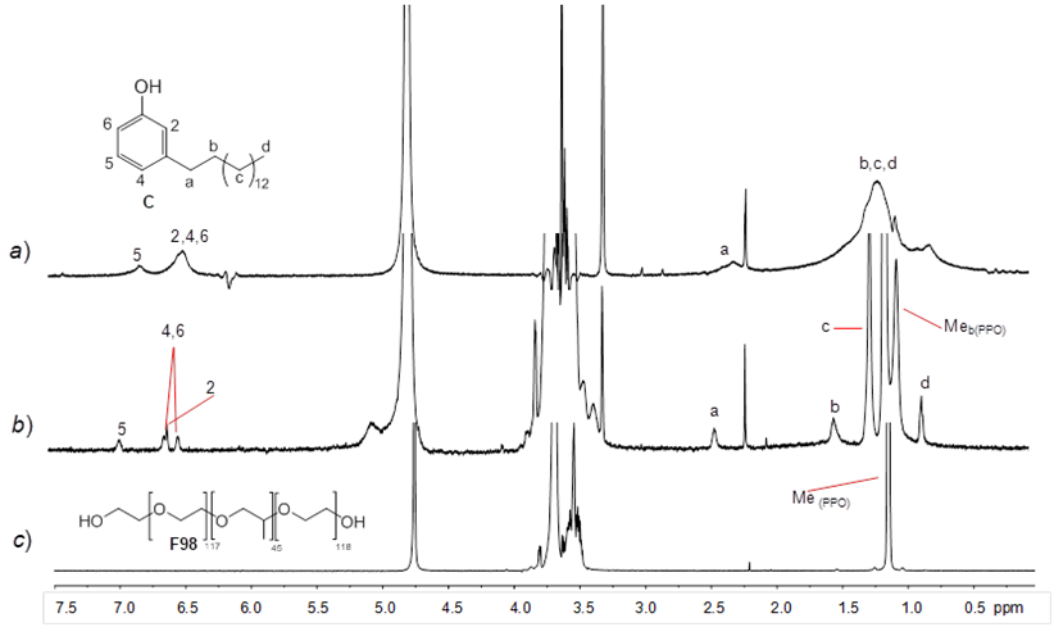


Figure 1. ^{1}H NMR spectra (600 MHz, 95:5 D_2O/CD_3OD, 298 K) of 2.6 x 10^{-4} M of a) cardanol, b) cardanol and F98, and c) Pluronic F98. Signal of spectra are labelled as reported in the structures of C and Pluronic F98.

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.

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

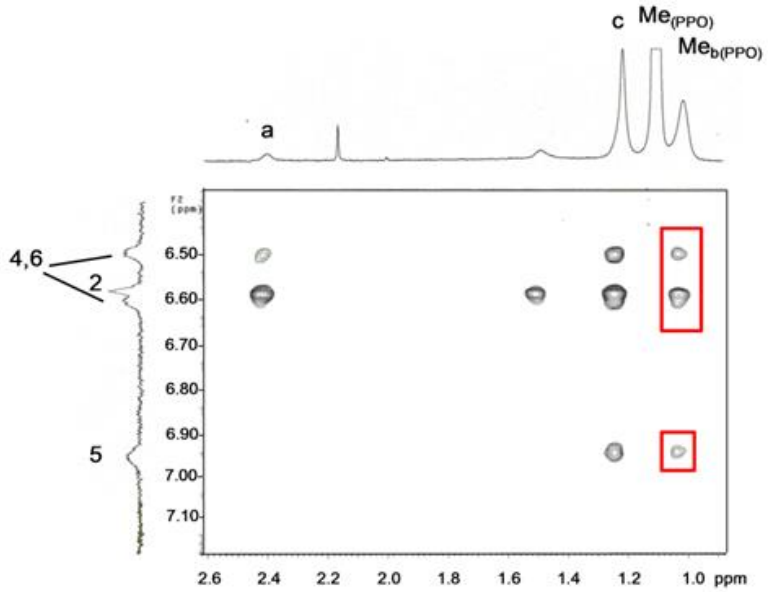


Figure 2. Partial 2D NOESY spectrum (600 MHz, 95:5 D_2O/CD_3OD, 298 K) of 2.6 x 10^{-4} M solution sample of cardanol/Pluronic F98 (1:1). Red-squared cross peaks indicate the intermolecular correlation among phenol protons of C and Me_b(PPO) groups of the polymer. Signals of the 1D spectra are labelled according to the structures of C and Pluronic F98 reported in Figure 1.

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

3.3. Antioxidant activity

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

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

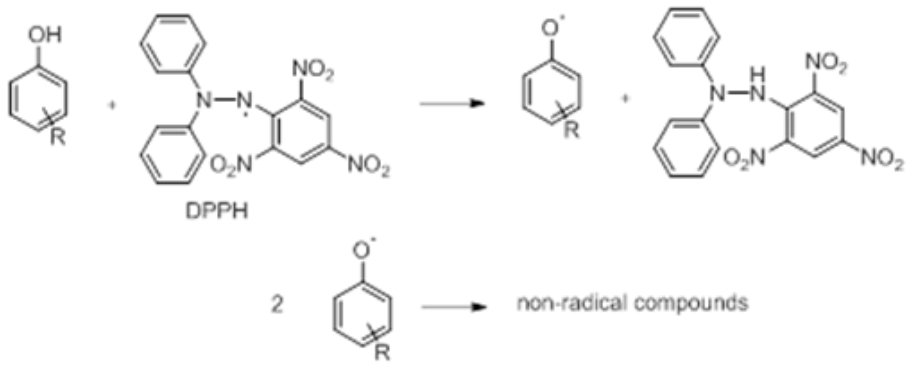
In the presence of Pluronic F98 for both derivatives (C and TC) and concentrations (5% and 10%) the stoichiometry is the same (C 5%: 1.0+/-0.3; C10%: 1.0+/-0.2; TC 5%: 1.0+/-0.6, TC 10%: 1.1+/-0.5), slightly higher values were obtained in the presence of Pluronic F108 (C 5%: 1.3+/-0.2; C10%: 1.1+/-0.2;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

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

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

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

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



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

The antioxidant activity of cardanols was assessed by measuring their ability to retard this peroxidation, which occurs via a radical chain reaction sustained by peroxyl radicals (ROO•) [47][48] under a constant flux of initiating radicals, the water-soluble azoinitiator 4,4'-Azobis(4-cyanovaleric acid) sodium salt (ABCV).[49] The decrease of O_2 concentration in solution directly correlates with the progress of the peroxidation reaction and it was followed by an optical oxygen probe. Oxygen consumption experiments, reported in Figure 3, show that Pluronic F108 oxidizes faster than F98, reasonably because of its larger number of OCH_2 units. It has also to be recalled that the aggregation situation of the polymer is very complex and we do not have only micelles in solution but also unimers and the relative percentage relatively differ for the two polymers having also different CMC values (lower for F108). The presence of cardanol at 5% or 10% ratio, significantly reduces the auto-oxidation of Pluronic in a concentration-dependent fashion.

TC has a larger antioxidant effect than cardanol, in agreement with data reported in literature 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 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.

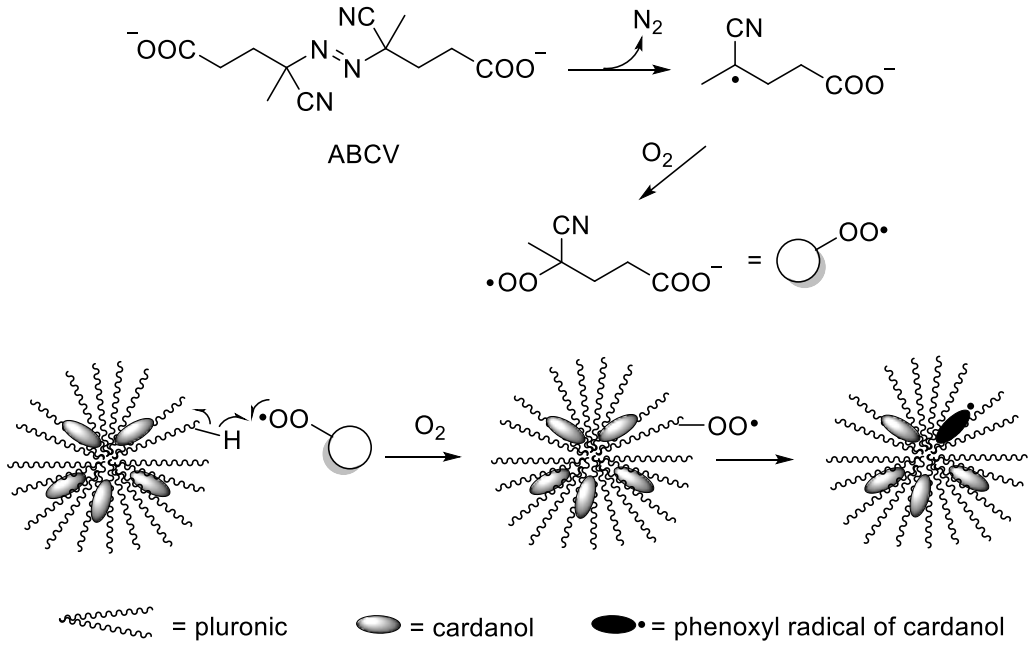
333

334 **Figure 3**. Oxygen consumption during the autoxidation of Pluronic (7×10^{-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



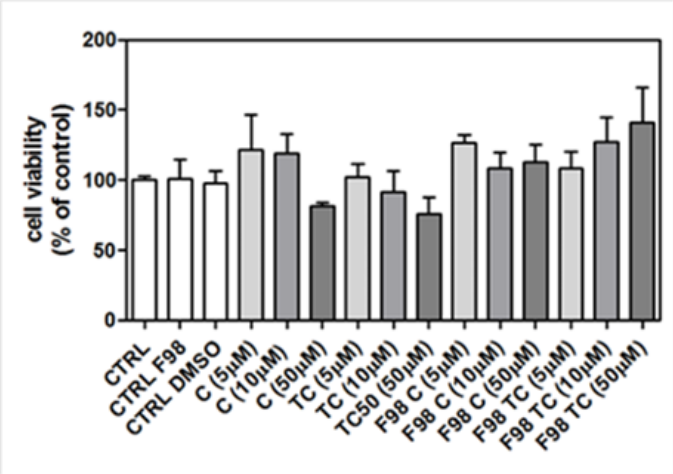
Scheme 3. Reaction of the water soluble ABCV-derived peroxyl radical with the outer portion of Pluronic (here we represent only micelles), and subsequent trapping of the peroxyl radical by cardanol.

Actually, if cardanols were able to quench all the initiating radicals produced by ABCV decomposition, the rate of O_2 consumption should have been equal to R_i. A reasonable explanation is that cardanols can trap all peroxyl radicals formed on the alkyl chain of Pluronic, but are unable to quench the charged ABCV-derived peroxyl radicals, that react with the outer portion of the 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 μM 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 μM always 10% in moles with respect to Pluronic) and cell
363 viability was assessed by means of MTT assay. Values are expressed as means ± 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

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

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

Conflict of interest

There are no conflicts to declare.

Supplementary Material

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

Acknowledgement

The authors would like to thank Prof. L. De Crescentini (University of Urbino) for providing cardanol derivatives. The research was founded by the grant Fondo di Finanziamento Ordinario (FFO) of the Italian Ministry of University and Research (MIUR).

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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