

Binary Micellar Solutions of Poly(Ethylene Oxide)-Poly(Styrene Oxide) Copolymers with Pluronic® P123: Drug Solubilisation and Cytotoxicity Studies

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The non-commercial copolymers E₄₅S₈, E₄₅S₁₇ and their mixtures with Pluronic® P123 (E₂₁P₆₇E₂₁) were studied as carriers of the model drug griseofulvin. Critical micelle concentration (cmc) (dye solubilisation method), drug solubilisation capacity (S_{cp} and S_n) determined by ultraviolet-visible (UV-Vis) spectroscopy and ¹H nuclear magnetic resonance (¹H NMR) and cytotoxicity (LDH activity in human neutrophils) were studied. E₄₅S₁₇ 1.0 wt.% dispersions presented colloidal aggregates limiting its S_{cp} in comparison to E₄₅S₈, but in 0.1 wt.% solutions this phenomenon seemed to be absent and E₄₅S₁₇ presented a higher S_{cp}. The mixtures that showed the best S_{cp} results contained 50% of P123 and presented low cmc. An evaluation of literature data suggested a minimum E_m content of 62% in E_mS_n copolymers below which the increase of S_n length does not lead to an increase of S_n. The results suggested no toxicity of the copolymers on human neutrophils, supporting the use of P123 and poly(styrene oxide) containing copolymers as drug carriers.

Keywords: solubilisation capacity, binary micelles, griseofulvin, cytotoxicity

Introduction

The potential of micelles formed from block copolymers for encapsulation and delivery of highly hydrophobic drugs has been widely examined. A major challenge in achieving this objective is the attainment of high drug loading into non-toxic surfactant micelles which remain stable on dilution but dissociate at the target site.¹ Low aqueous solubility is a problem in the formulation of many drugs like griseofulvin (Figure 1), a widely used antifungal antibiotic which has been used for many years as a model drug in studies of solubilisation by polymeric micelles.²⁻⁷

As described in many papers,⁸⁻¹⁴ aqueous micellar solutions of block copolymers based on poly(ethylene oxide) as the hydrophilic component combined with a wide range of hydrophobic blocks have been investigated

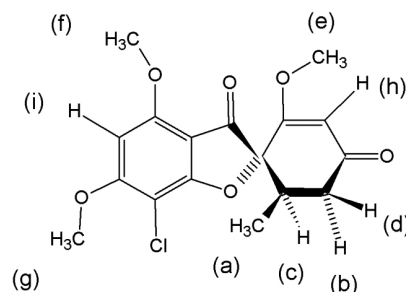


Figure 1. Chemical structure of griseofulvin. The letters assign the different proton peaks observed by ¹H nuclear magnetic resonance (¹H NMR).

as vehicles for drug solubilisation. Nanoparticles with poly(ethylene oxide) surface show the ability to evade scavenging by the mononuclear phagocyte system, so resulting in increased circulation times in the blood, and are among the drug delivery systems most studied for this purpose.¹⁵ Copolymers of E_nP_mE_n type, with hydrophilic

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poly(oxyethylene) and hydrophobic poly(oxypropylene) blocks have been commercially available for several decades and a particular advantage of this family of copolymers is the so-called 'stealth' property of the poly(ethylene oxide) corona of their micelles. Here E denotes oxyethylene (OCH_2CH_2), P denotes oxypropylene ($\text{OCH}_2\text{CH}(\text{CH}_3)$), and subscripts m and n indicate chain length in P or E units. Concentrated solutions of certain $\text{E}_n\text{P}_m\text{E}_n$ copolymers display thermally reversible gelation in the temperature range required for *in situ* gelling in topical use,^{16,17} subcutaneous injection^{11,18} and other vias.¹⁹ Such copolymers attract studies as drug solubilising agents showing, for example, improvements on the bioavailability of the studied drugs.^{20,21} However, they present a relatively low solubilisation capacity.⁴ We have investigated a number of micellar solutions in which the core-forming blocks have been designed to be more hydrophobic and more compatible with the drug to be solubilised.^{4,22,23} Cambón *et al.*²⁴ have also reported the advantage of using copolymers containing poly(styrene oxide) as drug carriers. For example, copolymers $\text{E}_{33}\text{S}_{14}\text{E}_{33}$ and $\text{E}_{38}\text{S}_{10}\text{E}_{38}$ presented potential application as chemotherapeutic carriers, enhancing more than 60 times doxorubicin apparent solubility (loading capacity of 1.8%) and presenting interesting biological properties. In previous works, we have used mixtures of copolymer $\text{E}_{62}\text{P}_{39}\text{E}_{62}$ (commercial notation Pluronic® F87) with copolymer $\text{E}_{137}\text{S}_{18}\text{E}_{137}$ (S denotes styrene oxide: $\text{OCH}_2\text{CH}(\text{C}_6\text{H}_5)$) to obtain a system with useful gelation characteristics (provided by F87) combined with a satisfactory drug-loading capacity (provided by poly(styrene oxide) micelle core) for drugs like griseofulvin.^{3,4} For a given value of S_n (solubilisation capacity in terms of grams of solubilised drug *per* gram of hydrophobic block), the solubilisation capacity measured as S_{cp} will be increased if w_n , the weight fraction of S in the copolymer is increased, e.g., by replacing the triblock copolymer by a comparable diblock copolymer, and may be greatly increased if the chosen diblock copolymer forms cylindrical micelles.^{23,25}

Thus, the purpose of this work is the determination of the solubilisation capacity of binary micellar solutions of two non-commercial diblock copolymers E_mS_n (E_{45}S_8 and $\text{E}_{45}\text{S}_{17}$) with Pluronic® P123. No studies of $\text{E}_{45}\text{S}_{17}$ have been reported up to date. E_{45}S_8 has already been studied as drug

solubiliser,²³ but to our knowledge, studies of mixed micellar systems containing this copolymer were not reported up to date. The extent of solubilisation was determined by the more usual technique of ultraviolet (UV) spectroscopy and, for comparative purposes, absolutely by ^1H nuclear magnetic resonance (^1H NMR). The dye solubilisation method measured by fluorescence was used to determine the critical micelle concentration (cmc). In addition, to our knowledge, this is the first time that cytotoxicity studies using human neutrophils were performed in copolymers containing poly(ethylene oxide) and poly(styrene oxide) blocks.

Experimental

Materials

Griseofulvin (molecular mass: 352.8 g mol^{-1}) was obtained from Sigma-Aldrich and was used in the form of finely ground (1 mm^2 mesh) powder. Differential scanning calorimetry (DSC) indicated a crystalline form with a melting point of $220.4 \text{ }^\circ\text{C}$ and an enthalpy of fusion of 115.6 J g^{-1} . There was no detectable transition consistent with a glassy component. Copolymer $\text{E}_{21}\text{P}_{67}\text{E}_{21}$ (commercial notation P123) was a gift from ICI Surfactants (now Uniqema), and copolymers E_{45}S_8 and $\text{E}_{45}\text{S}_{17}$ were prepared in the School of Chemistry, Manchester. The molecular characteristics of the copolymers are given in Table 1, i.e., the number-average molar mass (M_n) and weight fraction E (%E) from ^{13}C NMR spectroscopy, and the hydrophilic-lipophilic balance (HLB). Details can be found elsewhere.²³

Critical micelle concentration (cmc)

Stock solutions were prepared by dissolving the copolymers in Milli-Q water and allowing 24 h for complete dissolution before diluting further to concentrations within the range $0.01\text{--}60 \text{ mg dm}^{-3}$. Solubilisation of 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to determine the onset of micellization, as described for triblock copolyethers,²⁶ and before that for ionic surfactants.²⁷ DPH was dissolved in methanol and added to the copolymer solution, so that the final copolymer solution contained 1% (v/v) in methanol and $0.004 \text{ mmol L}^{-1}$ DPH, a mixture shown to

Table 1. Selected properties of the nonionic block copolymers explored in this study

Copolymer	$M_n / (\text{g mol}^{-1})$	E / %	HLB ^a	Reference
E_{45}S_8	2940	67	13	23
$\text{E}_{45}\text{S}_{17}$	4020	49	10	OMIC School of Chemistry (Manchester)
$\text{E}_{21}\text{P}_{67}\text{E}_{21}$ (P123)	5734	32	6.4	28

^aHydrophilic-lipophilic balance (HLB) = E/5.

provide the same values of the cmc as those obtained by other methods for copolymers in water alone. A F-4500 Hitachi fluorescence spectrophotometer was used in the experiments, with solution temperatures maintained at 25 °C and 37 °C (± 0.2 °C).

Micellar size

The hydrodynamic diameter (D_h) of the pure diblock copolymers $E_{45}S_{17}$ and the mixtures P123/ $E_{45}S_{17}$ 50/50 and P123/ $E_{45}S_8$ 50/50 mixtures in aqueous solutions were determined at 25 °C, using the equipment Malvern Zetasizer Nano ZS model ZEN 3500. The systems were submitted to 30 scans with an acquisition time of 30 s for each scan. All the measurements were made in triplicate.

Solubilisation of griseofulvin

UV spectroscopy is commonly used to determine the extent of drug solubilisation, but involves an initial calibration procedure. In this study we used the UV spectroscopy and compared the solubilisation capacity determined by this method with that from the absolute method based on 1H NMR spectroscopy.

UV spectroscopy quantification

A UV-Vis spectrometer (Hitachi U-2000) was calibrated by recording the absorbance (wavelength range 200-350 nm) of methanol solutions of griseofulvin (2-20 mg dm^{-3}) against a solvent blank. The strong absorbance at 292 nm gave a satisfactory Beer's law plot. In a solubilisation experiment, a 10 cm^3 portion of a stock 1 wt.% copolymer solution was added to finely-ground (1 mm^2 mesh) griseofulvin powder (0.02 g). The mixture was stirred at 25 °C for 72 h before being filtered (0.45 μm from Millipore) to remove any unsolubilised drug. The sample was then diluted with methanol to enable analysis by UV spectroscopy. The water content after dilution was low enough to allow the calibration for methanol solutions to be used without correction. After dilution the samples were held at 37 °C for 72 h before analysis. Measurements were carried out in triplicate and the results were averaged.

1H NMR spectroscopy quantification

Solutions with solubilised drug were prepared and filtered as described for the UV method, but were then freeze dried (24 h, 10^{-3} mm Hg) to remove water. The entire sample was dissolved in $CDCl_3$ and its 1H NMR spectrum recorded at ambient temperature (22 °C) using a

Bruker DRX 500 11.7 T (499.80 MHz for 1H) spectrometer in a 5 mm inverse detection z-gradient probe at 298 K. The spectra were obtained using 90° rf pulse (9.20 μs), a spectral width of 12019 Hz, 256 transients with 64000 data points, an acquisition time of 2.73 s, and a relaxation delay of 10 s before being converted to 32000 data points and the phase and baseline corrected manually using the Bruker software. The amount of griseofulvin solubilised *per* gram of polymer was determined using appropriate peak integrals. All measurements were carried out in triplicate and the results averaged.

Cytotoxicity study: determination of lactate dehydrogenase (LDH) assay

Human neutrophils were isolated by Lucisano and Mantovani's method^{29,30} with slight modifications. Cells pellets were suspended in Hank's balanced salt solution (HBSS) containing 80-90% neutrophils with viability of $90 \pm 2.0\%$ established by Trypan Blue exclusion test. Human neutrophils (2.5×10^6 cells mL^{-1}) were incubated (15 min at 37 °C) with HBSS (non-treated cells), Dimethyl sulfoxide (DMSO) (0.4% (v/v), vehicle-control), Triton X-100 (0.2% (v/v), standard cytotoxic drug) and following test drugs (1, 10, 50 and 100 $\mu g mL^{-1}$): diblock copolymers E_mS_n ($E_{45}S_8$ and $E_{45}S_{17}$), Pluronic® P123, the binary mixtures of copolymers (P123/ $E_{45}S_8$ 50/50 and P123/ $E_{45}S_{17}$ 50/50, named as P50/S8 and P50/S17, respectively) or their versions loaded with griseofulvin (P123/ $E_{45}S_8G$ and P123/ $E_{45}S_{17}G$, named as P50/S8G and P50/S17G, respectively).

The LDH activity was determined by a commercially available method (LDH liquiform of Labtest Diagnosis).

Statistical analysis

The results are expressed as mean \pm standard deviation (SD). The statistical significance of differences between groups was determined by one-way ANOVA, followed by Tukey for multiple comparisons as a *post hoc* test. The significance level was set at $p < 0.05$.

Results and Discussion

Regarding binary mixtures, in tests made in our laboratories, the mixtures of P123 with copolymer $E_{45}S_{17}$ showed stable thermoresponsive properties only when the ratio was 50/50 at concentration 29% (m/m), with a transition temperature fluid-gel-fluid in the range 18-47 °C. In addition, the systems P123/ $E_{45}S_8$ 50/50 and 70/30 presented stable thermoresponsive properties in

the concentrations 25-29% (50/50) and 23-29% (70/30), showing the potential to use such mixtures in subcutaneous administration of drugs, since they may form gel at body temperature (unpublished results).

Critical micelle concentration (cmc)

The arrows in the plots of fluorescence intensity of DPH against copolymer concentration (log scale) of two copolymer systems ($E_{45}S_{17}$ and P123/ $E_{45}S_{17}$) in Figure 2 indicates the points at which a sharp increase in fluorescent intensity takes place, estimated as the cmc, due to the transference of DPH from the aqueous polar medium to the hydrophobic environment of the cores of the micelles.²⁸

As seen in Table 2, the obtained values of cmc were in the range 1×10^{-2} - 2×10^{-4} wt.%. The much higher concentration of the systems used in the solubilisation experiments (1.0 wt.%) is consistent with effectively complete micellization.

There was no considerable variation of cmc of diblocks $E_{45}S_8$ and $E_{45}S_{17}$ and their mixtures as the temperature

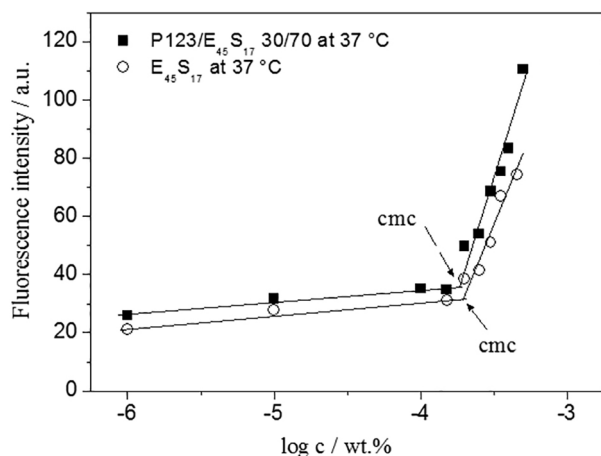


Figure 2. Fluorescence intensity (DPH) versus concentration (log scale) for aqueous solutions of (o) $E_{45}S_{17}$ and (■) P123/ $E_{45}S_{17}$ 30/70 mixture at 37 °C.

changed from 25 to 37 °C (see Table 2 and Figure 3). Typically, the cmc at 37 °C was lower than at 25 °C, especially for P123 (Table 2). Previous studies show that $E_nP_mE_n$ copolymers have a more endothermic micellization process (ΔH_{mic}° around 200 kJ mol^{-1})²⁶ than E_nS_m , $E_nS_mE_n$ copolymers (ΔH_{mic}° ranging from 4 to 40 kJ mol^{-1}).^{31,32}

Values of cmc for $E_{45}S_8$ and $E_{45}S_{17}$ expressed in mol dm^{-3} are much lower than those of P123 ($E_{21}P_{67}E_{21}$). Diblock copolymers have cmc values lower than their correspondent triblock copolymers (with the same molar mass and block composition), and the more hydrophobic is the copolymer, the lower its cmc.³³ This explains why the cmc of P123 ($E_{21}P_{67}E_{21}$) is the highest, since P123 is the less hydrophobic copolymer. The relative hydrophobicity between P and S units in a diblock copolymer is 1:12.¹¹ From this concept, we find that the EP copolymers which are equivalent to $E_{45}S_8$ and $E_{45}S_{17}$ would be $E_{45}P_{96}$ and $E_{45}P_{204}$, respectively, and then we can compare the hydrophobicities of the three copolymers by comparing their HLB's: 6.4 for P123, 5.2 for $E_{45}P_{96}$ and 2.8 for $E_{45}P_{204}$. The lower the HLB, the higher is the hydrophobicity and the lower is the cmc. Therefore, it was expected that $E_{45}S_{17}$ would have the lowest cmc. Besides this, diblock copolymers have cmc values lower than their correspondent triblock copolymers (with the same molar mass and block composition),³³ contributing to the lower cmc value of $E_{45}S_{17}$ in comparison to the triblock P123.

The cmc's (mol dm^{-3}) of the mixtures are similar to those of the pure copolymers $E_{45}S_8$ and $E_{45}S_{17}$ with the same order of magnitude (see Table 2). At 37 °C all systems, including P123, have similar cmc's. The low cmc values of these systems bring potential stability of their micelles after blood dilution, turning these mixtures promising drug carriers in pharmacological applications.

The change in standard Gibbs free energy of micellization *per* mole of copolymer unimer can be related to cmc as shown in the following equation:

$$\Delta G_{mic}^\circ = RT (\ln \text{cmc}) \quad (1)$$

Table 2. Values of cmc and standard Gibbs free energy of micellization for $E_{45}S_8$, $E_{45}S_{17}$ and their mixtures with P123 at 25 °C and 37 °C

System	cmc / (mmol dm^{-3})		$\Delta G_{mic}^\circ / (\text{kJ mol}^{-1})$	cmc / (mmol dm^{-3})		$\Delta G_{mic}^\circ / (\text{kJ mol}^{-1})$
	25 °C	37 °C		25 °C	37 °C	
P123 ^a	2.44×10^{-2}		-26.3	7.00×10^{-4}		-36.5
P123/ $E_{45}S_8$ 50/50	9.20×10^{-4}		-34.4	4.80×10^{-4}		-37.5
P123/ $E_{45}S_8$ 30/70	6.60×10^{-2}		-24.8	4.20×10^{-4}		-37.8
$E_{45}S_8$	1.02×10^{-3}		-34.2	6.80×10^{-4}		-36.6
P123/ $E_{45}S_{17}$ 50/50	3.50×10^{-4}		-36.8	3.10×10^{-4}		-38.6
P123/ $E_{45}S_{17}$ 30/70	3.50×10^{-4}		-36.8	2.90×10^{-4}		-38.8
$E_{45}S_{17}$	4.70×10^{-4}		-36.1	4.50×10^{-4}		-37.6

^aLiterature values.²⁸

It was observed that the values of ΔG_{mic}° at both temperatures for the mixtures P123/E₄₅S₈ and P123/E₄₅S₈ were lower than the values for P123 and E₄₅S₈ indicating that the micellization process of mixtures is more spontaneous than the micellization process of pure surfactants, demonstrating a synergistic effect (Table 2). It was observed that all the values of ΔG_{mic}° were lower at body temperature than at room temperature, indicating that these systems may be used to spontaneously solubilize drugs in the body.

Table 3 shows the cmc values at 25 °C for diblock copolymers with similar ethylene oxide block length and different styrene oxide (S_n) block lengths. The cmc of E₅₀S_{3.5} is much higher than the expected. Mai *et al.*³⁴ explain that the relatively high values of cmc found for this copolymer are consistent with their wider distribution of apparent hydrodynamic radius.

Figure 3 shows the plot of cmc (mol dm⁻³) versus hydrophobic block length of diblock copolymers with similar ethylene oxide (E_m) block length and different styrene oxide (S_n) block lengths. Data from copolymers studied in the literature and data from this work were used to plot the graph.^{23,31,34} To plot the graph, the values of log₁₀ (cmc; mol dm⁻³) of the copolymers were adjusted to a common E block length, since the number of E units affect the cmc within a series of copolymers. We used the equation:

$$d(\log \text{ cmc})/dv = 0.004(100 - v) \quad (2)$$

This equation, proposed by Booth, Atwood and Price,³⁵ where *v* is the number of E units in the copolymer, was used to correct each value for a change in E block length from 100 units. It can be noted that the increase of hydrophobic block length reduces the cmc of the copolymers. Drug solubilizing agents with low values of cmc are interesting since they present higher micellar stability after blood dilution.

Table 3. Solubilisation capacities S_{cp} and S_h (mg g⁻¹) of griseofulvin in 1 wt.% solutions of P123, E₄₅S₁₇, E₄₅S₈ and their mixtures at 25 °C and 37 °C. S₀ = 1.4 e 1.9 mg dL⁻¹ at 25 °C and 37 °C, respectively. Data obtained by UV-Vis spectroscopy

System	S _{cp} / (mg g ⁻¹)		S/S ₀	S _h / (mg g ⁻¹)		S/S ₀
	25 °C			37 °C		
P123	3.30 ± 0.07	4.9	3.6	5.10 ± 0.21	7.5	3.7
P123/E ₄₅ S ₈ 50/50	4.00 ± 0.13	7.9	3.8	7.50 ± 0.16	14.9	4.9
P123/E ₄₅ S ₈ 30/70	3.80 ± 0.06	8.7	3.7	5.60 ± 0.23	12.9	3.9
E ₄₅ S ₈	7.40 ± 0.20	22.4	6.3	8.70 ± 0.08	26.4	5.6
P123/E ₄₅ S ₁₇ 50/50	2.80 ± 0.15	4.7	3.0	8.10 ± 0.17	13.6	5.3
P123/E ₄₅ S ₁₇ 30/70	2.40 ± 0.12	4.3	2.7	2.80 ± 0.19	5.0	2.5
E ₄₅ S ₁₇	5.20 ± 0.07	10.2	4.7	7.40 ± 0.24	14.5	8.6

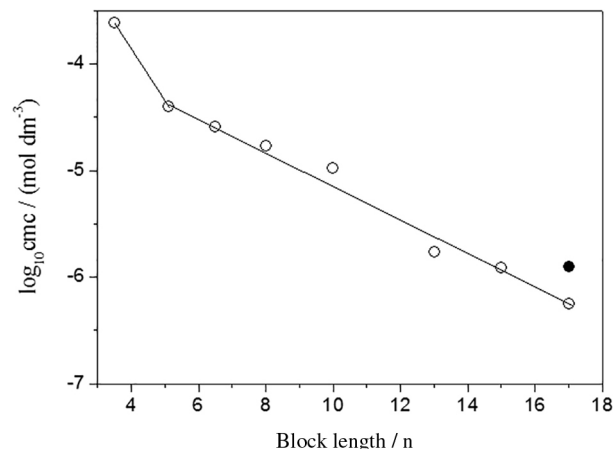


Figure 3. Critical micelle concentration (cmc) values at 25 °C for diblock copolymers with similar ethylene oxide (E_m) block length and different styrene oxide (S_n) block lengths. Literature values (o)^{23,26,34} and this work (•).

Solubilisation of griseofulvin and micellar size

With the data of drug solubilities obtained by UV-Vis spectroscopy, two parameters were investigated for the copolymers and their mixtures: S_{cp}, the solubilisation capacity expressed in mg of drug *per* g of polymer, and S_h, the solubilisation capacity expressed in mg of drug *per* g of hydrophobic block, where S_{cp} = S - S₀/m_{cop} and S_h = S_{cp}/W_h (S: solubility of drug in the micellar solution; S₀: aqueous solubility of drug; W_h: mass fraction of hydrophobic block). These values are shown in Table 4.

E₄₅S₈ and E₄₅S₁₇ presented satisfactory solubilisation capacity values when compared to Pluronic® P123 (E₂₁P₆₇E₂₁). This is due the higher hydrophobicity of their poly(styrene oxide) blocks in comparison with the poly(propylene oxide) block (P₆₇) of Pluronic® P123 (see Table 6). The values found for the P123 and E₄₅S₈ are in agreement with the data found in the literature: S_{cp} = 3.0 mg g⁻¹ at 25 °C and S_{cp} = 3.8 mg g⁻¹ at 37 °C for P123;²² and S_{cp} = 7.5 mg g⁻¹ at 25 °C for E₄₅S₈.

Table 4. Results of solubilisation capacity (S_{cp}) of griseofulvin in the copolymer solutions of $E_{45}S_{17}$ in the concentrations 0.1, 0.5 and 1 wt.% at 25 °C and 37 °C measured by UV-Vis spectrometry. $S_0 = 1.4$ e 1.9 mg dL⁻¹ at 25 °C and 37°C, respectively

Concentration / wt.%	$S_{cp} / (\text{mg g}^{-1})$	$S_h / (\text{mg g}^{-1})$	$S_{cp} / (\text{mg g}^{-1})$	$S_h / (\text{mg g}^{-1})$
	25 °C		37 °C	
0.1	14.20 ± 0.18	28	22.20 ± 0.15	44
0.5	7.60 ± 0.09	15	8.70 ± 0.17	17
1.0	5.20 ± 0.07	10	7.40 ± 0.05	15

Table 5. Solubilisation capacity of griseofulvin (S_{cp} and S_h) at 25 °C, micellar association number (N_w) and ethylene oxide weight percentage (%E) of diblock copolymers with similar ethylene oxide (E_n) block lengths and different styrene oxide (S_n) block lengths

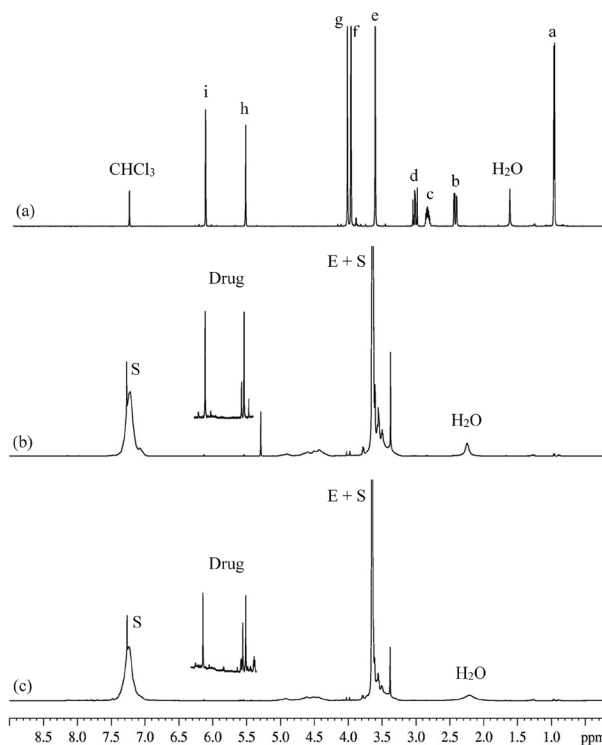
Copolymer	$S_{cp} / (\text{mg g}^{-1})$	$S_h / (\text{mg g}^{-1})$	N_w	%E	HLB	Reference
$E_{50}S_{3.5}$	2.8	16	20	84	16.8	34
$E_{50}S_{5.1}$	4.0	17	35	78	15.6	34
$E_{51}S_{6.5}$	4.9	18	50	74	14.8	34
$E_{45}S_8$	7.5	22	80	67	13.4	23
$E_{45}S_{10}$	11.2	29	103	62	12.4	23
$S_{13}E_{60}$	14.1	37	100	63	12.6	34
$S_{15}E_{63}$	11.2	28	140	61	12.2	23
$E_{45}S_{17}$	5.2	10	155	49	9.8	present work
$S_{17}E_{65}$	11.7	27	150	58	11.6	23
$S_{20}E_{67}$	12.4	27	189	55	11.0	23

It is interesting to evaluate the dependence of the drug solubilisation capacity (S_{cp}) with temperature and cmc. Low values of cmc mean that the copolymers are more hydrophobic, what confers to them higher solubilisation capacities. Values of cmc of $E_{45}S_8$ and $E_{45}S_{17}$ are similar to each other and lower than that of P123 (see Table 3); this explains the higher solubilisation capacities of $E_{45}S_8$ and $E_{45}S_{17}$ in comparison to P123.

Observing the chemical structure of $E_{45}S_{17}$, it was expected that its solubilization capacity would be higher than that of $E_{45}S_8$, due its higher hydrophobicity (it contains 9 units of S more than $E_{45}S_8$). However this was not observed. The reason may be the limited solubility of this polymer, since it aggregates itself forming a turbid system at 1.0 wt.%, what affects the solubilisation of griseofulvin in the system. This observation suggests that a %E of 49% (the %E of $E_{45}S_{17}$) is too low to turn a copolymer of E_mS_n type soluble in water.

Quantification experiments using ¹H NMR were performed to confirm the results obtained at 37 °C, and the values of S_{cp} found using this technique were 8.7 mg g⁻¹ for $E_{45}S_8$ and 6.8 mg g⁻¹ for $E_{45}S_{17}$, in agreement with the UV-Vis quantification. Figure 4 shows the spectra of griseofulvin and the copolymers encapsulated with griseofulvin. Attributions were based on the work of Rekatás *et al.*³⁶ and Ribeiro *et al.*⁷ See Figure 1 for griseofulvin proton attributions.

Silva *et al.*³⁷ observed that, in aqueous solutions, P123 ($E_{21}P_{67}E_{21}$) and F127 ($E_{98}P_{67}E_{98}$) form micelles, while L121

**Figure 4.** ¹H NMR spectrum in CDCl₃ of griseofulvin in (a), griseofulvin and $E_{45}S_8$ recovered from solubilisation at 37 °C in (b) and griseofulvin and $E_{45}S_{17}$ recovered from solubilisation at 37 °C in (c). Expansion of the drug peaks was made in the region 5.3-6.3 ppm.

($E_5P_{68}E_5$) form aggregates that eventually separate from the solution forming a crystalline liquid phase.³⁷ This is due to

the very low HLB of L121 (2.0) in comparison to that of F127 (HLB = 14) and that of P123 (HLB = 6.4).

As already mentioned, $E_{45}S_{17}$ at 1 wt.% solutions shows turbidity, a behavior which seems similar to that reported for L121. This behavior is probably due to the high hydrophobicity of the copolymers. Since the relative hydrophobicity between P and S unit in a diblock copolymer is 1:12, we found the E_mP_n copolymer equivalent to $E_{45}S_{17}$ and calculated the HLB of this copolymer in order to compare its hydrophobicity with that of L121 ($E_5P_{68}E_5$). We found that $E_{45}P_{204}$ (12×17 units of S = 204) is the E_mP_n with a hydrophobicity equivalent to $E_{45}S_{17}$. The HLB of $E_{45}P_{204}$ is 2.8, only a little higher than that of L121.

The graphs of scattering intensity *versus* hydrodynamic diameter for the copolymer $E_{45}S_{17}$ are in Figure 5. The wide unimodal peak found for $E_{45}S_{17}$ at 1 wt.% confirms the presence of agglomerates. The average diameter at 25 °C for the copolymer $E_{45}S_{17}$ was 29 ± 3 nm at 0.1 wt.% with a polydispersity of 0.255 and 66 ± 2 nm at 1.0 wt.% with a polydispersity of 0.230, respectively. The average diameter at 25 °C for the copolymer $E_{45}S_8$ was 19 ± 2 nm at 0.1 wt.% with a polydispersity of 0.200 and 17 ± 2 nm with a polydispersity at 1.0 wt.% of 0.091, respectively.

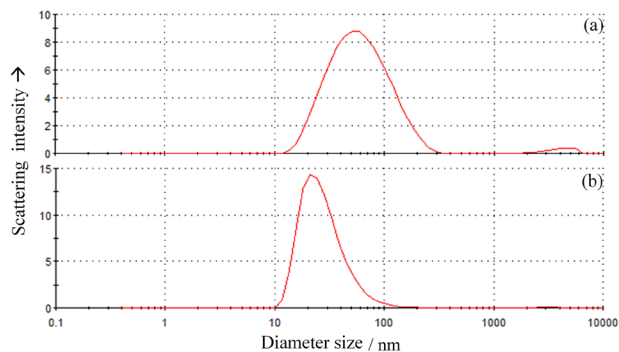


Figure 5. Scattering intensity *versus* hydrodynamic diameter at 25 °C for the copolymer $E_{45}S_{17}$ at 0.1 wt.% in (a) and 1.0 wt.% in (b).

With the aim to evaluate the influence of $E_{45}S_{17}$ agglomerates on the solubilisation capacity, griseofulvin solubilisation experiments were also performed in 0.1 wt.% and 0.5 wt.% solutions of this copolymer. The results are shown in Table 4.

The aggregation presented by diblock $E_{45}S_{17}$ adversely affects the solubility of griseofulvin. When the concentration of $E_{45}S_{17}$ is 0.1 wt.%, the aggregation phenomenon seems to be absent and relatively high solubilisation capacity is reached. The low cmc values of $E_{45}S_{17}$ (Table 2) ensures a virtually complete micellization at 0.1 wt.%. Observing the substantial increase of S_{cp} in this concentration, we may infer that as the polymer concentration increases,

the percentage of polymer molecules in aggregate form increases and the percentage of molecules in micellar form, available to solubilise the drug, decreases, causing a relatively low S_{cp} for $E_{45}S_{17}$ at 1.0 wt.%.

Micellar size measurements by light scattering were performed to evaluate if a co-micellization process took place in the binary mixtures of $E_{45}S_{17}$ and $E_{45}S_8$ with P123 at 1 wt.%. As shown in Figure 6, the appearance of a single peak for both mixtures instead of two peaks, confirm the formation of binary micelles in these systems.³⁸ The values of hydrodynamic diameter for P123/ $E_{45}S_8$ 50/50 and P123/ $E_{45}S_{17}$ 50/50 at 1 wt.% were 17 ± 1 nm with a polydispersity of 0.106 and 30 ± 3 nm with a polydispersity of 0.248, respectively.

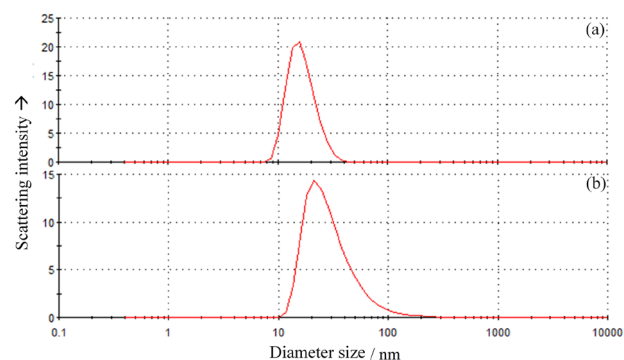


Figure 6. Scattering intensity *versus* hydrodynamic diameter at 25 °C for the mixtures P123/ $E_{45}S_8$ 50/50 at 1 wt.% in (a) and P123/ $E_{45}S_{17}$ 50/50 at 1 wt.% in (b).

Data of griseofulvin solubilisation obtained at 25 °C using UV-Vis for diblock copolymers with similar ethylene oxide (E_m) block length and different styrene oxide (S_n) block lengths are shown in Table 6. These data were used to plot a graph (see Figure 6) that shows the variation of griseofulvin solubilisation capacity (S_h) in relation to the number of S units (hydrophobic block length, n).

The increase in hydrophobic block length (n) in the range 3.5-13 increased the drug solubility and the association micellar number (N_w). However, in the range 13-20, the increase in n leads to a decrease in solubilisation capacity S_h , with a more pronounced effect in $E_{45}S_{17}$. Lee *et al.*³⁹ reported that the formation of molecular aggregates observed for L121 copolymer promotes inefficiency of drug solubilisation. The smooth decrease observed for the copolymers $S_{15}E_{63}$, $S_{17}E_{65}$, $S_{20}E_{67}$ may be associated with a minimum %E, below which the increase of hydrophobic block length does not lead to an increase of solubilisation capacity S_h . For this copolymer series, this %E would be 62% (Figure 7).

In a previous study of a copolymer series of $E_nP_mE_n$ type, an increase on partition coefficient was observed as

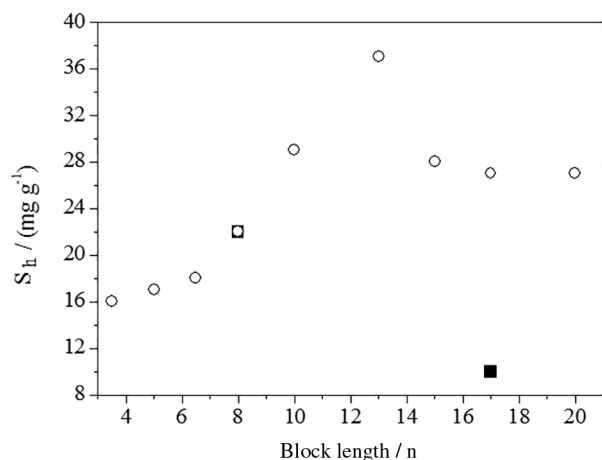


Figure 7. Solubilisation capacity (S_h) of diblock copolymers with similar ethylene oxide (E_m) block lengths and different styrene oxide (S_n) block lengths. 25 °C: (o) literature dates^{23,34} and (■) present work.

the temperature and the hydrophobic block (P_m) length increased.⁴⁰

The mixtures that showed the best S_{cp} results were P123/ $E_{45}S_8$ 50/50 and P123/ $E_{45}S_{17}$ 50/50, with values equal to 7.5 and 8.1 mg g⁻¹, respectively, at 37 °C.

Pierrri and Avgoustakis⁴¹ studied the encapsulation and release of griseofulvin from micelles of polylactide-polyethyleneglycol copolymers named as PLA(X)-PEG(Y), where X and Y are the molecular masses of the blocks in KDa. They found that PLA(4)-PEG(5) encapsulated only 6.5 mg of drug *per* g of copolymer, an amount lower than the S_{cp} values at 37 °C for $E_{45}S_8$, $E_{45}S_{17}$ and the mixtures P123/ $E_{45}S_8$ 50/50 e P123/ $E_{45}S_{17}$ 50/50.

Cytotoxicity of polymers in human neutrophils

The possible toxic effects of the polymers in human neutrophils were investigated by measurements of LDH activity in cell suspension. We found that diblock copolymers E_mS_n ($E_{45}S_8$ and $E_{45}S_{17}$) and Pluronic® P123 did not induce a significant increase in the LDH activity release by human neutrophils when compared with control group (DMSO 0.4%) (Figure 8). In addition, neither the binary mixtures of copolymers (P123/ $E_{45}S_8$ 50/50 and P123/ $E_{45}S_{17}$ 50/50, named in Figure 8 as P50/S8 and P50/S17, respectively) or their versions loaded with griseofulvin (P123/ $E_{45}S_8$ G and P123/ $E_{45}S_{17}$ G, named in Figure 9 as P50/S8G and P50/S17G, respectively) promoted the increase in the LDH activity (Figure 9). The measurement of LDH activity, enzyme present in the cell cytoplasm, is a marker of intact membrane with considerable sensitivity. In this study the polymers alone or associated with griseofulvin did not affect the cell viability assessed by LDH activity, suggesting the absence of toxicity on the cell membrane human neutrophils.

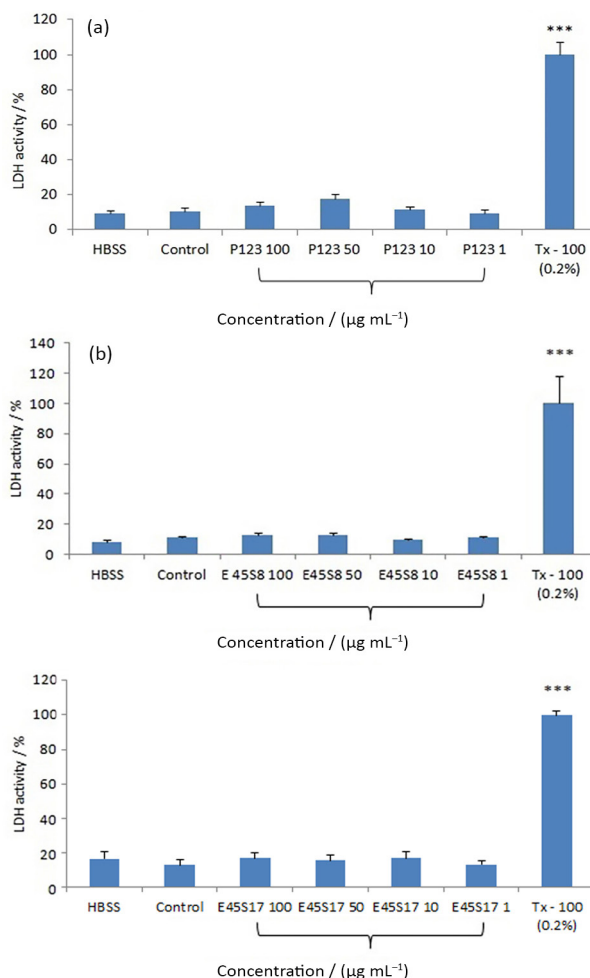


Figure 8. Evaluation of (a) Pluronic® P123, (b) $E_{45}S_8$ and (c) $E_{45}S_{17}$ toxicity measured by lactate dehydrogenase (LDH) activity in human neutrophils. Data from two to eight samples. *Versus control group (DMSO 0.4%, v/v). Results represent means \pm the standard error of the mean (SEM) ($p < 0.05$; ANOVA and Tukey as the *post hoc* test). For more details see item cytotoxicity study of Experimental section.

Conclusions

The E_mS_n type copolymers studied, $E_{45}S_8$ and $E_{45}S_{17}$, and their mixtures with P123 showed low cmc values, an advantage to apply them as drug solubilizing agents for pharmacological applications.

$E_{45}S_{17}$, with a weight percentage of poly(ethylene oxide) (%E) of 49%, formed insoluble/colloidal aggregates that limited the drug solubilisation capacity in comparison to $E_{45}S_8$ at 1.0 wt.%. In $E_{45}S_{17}$ 0.1 wt.% solutions, this phenomenon seemed to be absent and a better solubilisation capacity was observed. An evaluation of data of solubilisation capacity S_h (weight of drug/weight of hydrophobic block) collected from the literature indicated that there is a minimum %E of 62%, below which the increase of hydrophobic block length does not lead to an increase of in S_h in E_mS_n type copolymers.

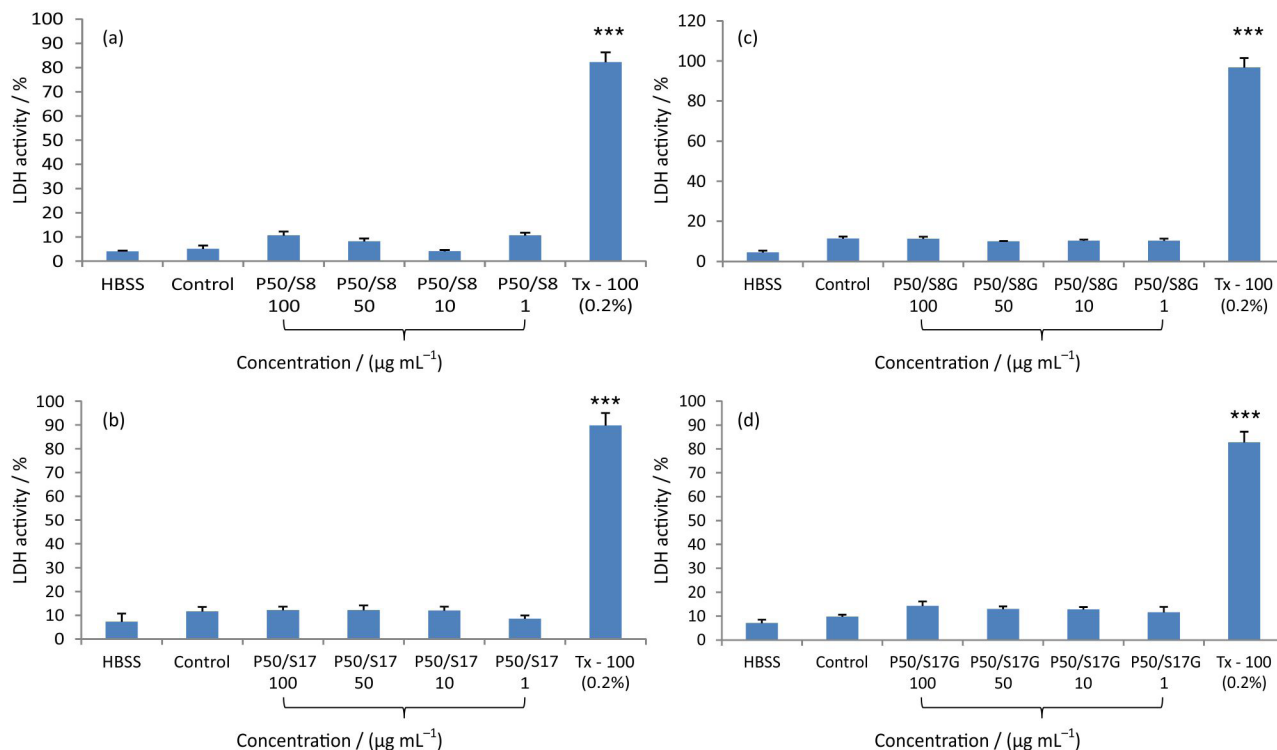


Figure 9. Evaluation of P50/S8 in (a), P50/S17 in (b), P50/S8G in (c) and P50/S17G in (d) toxicity measured by lactate dehydrogenase (LDH) activity in human neutrophils. Data from two to eight samples. *Versus control group (DMSO 0.4%, v/v). Results represent means \pm the standard error of the mean (SEM). ($p < 0.05$; ANOVA and Tukey as the *post hoc* test).

Preliminary studies showed that the systems P123/E₄₅S₈ 50/50, P123/E₄₅S₈ 30/70 and P123/E₄₅S₈ 50/50 presented thermoresponsive gelling properties provided by Pluronic® P123. Among the mixtures, the systems P123/E₄₅S₈ 50/50 and P123/E₄₅S₁₇ 50/50 showed the best solubilisation results. Considering these properties, these binary mixtures have the potential to be used in subcutaneous administration of drugs.

Besides this, E₄₅S₈, E₄₅S₁₇, Pluronic® P123 and their mixtures did not affect the cell viability assessed by LDH activity, suggesting the absence of toxicity on the cell membrane human neutrophils, which supports the use of Pluronic® P123 and copolymers containing poly(styrene oxide) as drug solubilising agents in pharmaceutical formulations.

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