INTRODUCTION

The pharmaceutical industry constantly faces challenges in obtaining new drugs. These challenges are usually attributed to the low solubility of the drug, that can lead to low bioavailability resulting in suboptimal drug delivery. Among numerous studies, binary micelles of amphiphilic copolymers have been explored, owing to the many advantageous features as drug delivery systems. The success of those binary mixtures is related to the versatility of the chemical composition of block-type copolymers, where the micelle core polarity can be readily tailored according to the choice of the chemical composition of block-type copolymers, where the micelle core polarity can be readily tailored according to the choice of hydrophobic and hydrophilic portions and their relative lengths.2,3

Micelles have aroused interest due to their ability to assist in the transport of poorly soluble drugs. In this study the mixture of copolymers F127/E45S8 in different proportions (F/ES 30/70, 50/50 and 70/30) was performed to improve the bioavailability of griseofulvin and quercetin. The results of cytotoxicity (MTT assay) revealed that the copolymers F127 and E45S8 had considerable biocompatibility and did not affect the metabolism of human neutrophils. The binary systems were also evaluated by critical micellar concentration (CMC) and thermoresponsive behavior. The CMC values were intermediate to those of the isolated copolymers. The systems maintained the thermoresponsive properties present in F127 making the systems interesting for subcutaneous administration. The systems presented small size, an average range in size from 17 to 38 nm, and the samples prepared with higher hydrophobic proportion presented more uniform sizes. Results suggest stability and the increasing of the nanosystems circulation time. The F/ES 30/70 system has polydisperity smaller than 0.1 and showed an increase of 129 times for quercetin solubility. Thus, it is possible to consider F127/E45S8 micelles as potential nanosystems for poorly soluble drug delivery.

Keywords: Atomic Force Microscopy; drug solubilisation; cytotoxicity assay; tube inversion.

EXPERIMENTAL

Materials

Copolymer E45S8 (ES) was synthesized by anionic polymerization of styrene oxide followed by ethylene oxide and donated by the
School of Chemistry, Manchester University. The copolymer E₄₅S₈ (F127) was purchased from Uniqema LTD. Molecular characteristics of the copolymers are shown in Table 1. The fluorescent dye DPH (1,6-diphenyl-1,3,5-hexatriene) was supplied by Biochemika. Human leucocyte-rich blood from healthy adults was obtained from blood bank – HEMOCÉ (Fortaleza, Brazil). The model drug griseofulvin (352.8 g mol⁻¹) was supplied by Sigma-Aldrich (Poole Dorset, UK), and quercetin (302.2 g mol⁻¹) was donated by PADETEC – UFC (Fortaleza, Brazil). Both drugs were used as received. All other reagents were used in analytical grade.

### Copolymers characterization

**MTT assay**

The neutrophils were exposed to E₄₅S₈ and F127 (10, 50 and 100 μg mL⁻¹) water (vehicle, control), Hanks’ balanced salt solution (HBSS, culture medium, negative control) or Triton X-100 (0.2%, cytotoxic standard) for 15 min at 37 °C and then 200 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added. After 3 h the cells were washed with phosphate buffer solution, and the DMSO (100 μL) was added for the solubilisation of the formazan product. The absorbance was measured at 540 nm.³⁵

**Binary systems**

**Binary mixtures**

The copolymers mixtures F127/E₄₅S₈ were prepared by dissolving the desired concentrations. The systems followed the ratios 30/70, 50/50, 70/30 and were denominated F/ES 30/70, F/ES 50/50, F/ES 70/30, respectively.

**Critical micelle concentration (CMC)**

Firstly, stock solutions were prepared by dissolving the systems in Milli-Q water, allowing 24 h for complete dissolution before diluting to required concentrations within the range 0.0001 – 1 g L⁻¹. The 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe to determine micelles formation. The DPH fluorescent dye was dissolved in methanol (0.4 mmol l⁻¹) in the dark and then added (30 μL DPH in 3 mL solution) to the solutions of the copolymers. The solutions containing DPH were kept in the dark and the absorbance measurements of the systems were taken 12 h after the addition of DPH. This well-established methodology was first reported by Alexandridis et al.,³⁰ where in this assay, an F-4500 Hitachi Fluorescence Spectrophotometer was used to determine the onset of micellisation for each copolymer solution. During the whole experiment, the temperature was kept at 25 °C and 37 ± 0.2 °C, and the fluorescence emission at 428 nm, being measured with an excitation wavelength at 350 nm.

**Thermoresponsive behavior**

Aqueous solutions (0.5 g with concentration range from 15 – 35 wt% of copolymers/system) were prepared, enclosed into small tubes (10 mm in diameter) and slowly heated (0.1 °C min⁻¹) in a water bath through the temperature range 10 – 90 °C. Gelation was recognized by immobility of the solution when the tube was inverted at intervals of ±1 °C.²²

**Drug solubilisation**

In this assay, copolymers and their mixtures were dissolved in Milli-Q water (1% w/w, 10 mL), where a further mass of drug (w = 10 mg) was added. Then, the prepared system was slowly stirred at 25 ± 0.1 °C in a thermostatic bath for 4 days. The supernatant was filtered (0.45 μm Millipore) to remove any non-solubilised drug and diluted with methanol. The drug concentration was monitored by UV/Visible spectroscopy (Thermo Scientific Genesys 6) at 292 and 375 nm for griseofulvin and quercetin, respectively, using a calibration curve, as described in Crothers et al.² Blank experiments, without copolymer, were done to determine the solubility of the drug in water. All measurements were carried out in triplicate and the results are shown averaged with standard deviation.

**Particle size**

Dynamic light scattering (DLS) technique was used to determine the hydrodynamic diameter (Dₜ) of the copolymers micelles in diluted solution, with and without drug. Aliquots were filtered in Millipore 0.45 μm and analyzed using a Malvern Zetasizer Nano ZS90 equipment. The systems were investigated using 30 scans with 30 s a acquisition time allowed for each scan. All measurements were made in triplicate.

### RESULTS AND DISCUSSION

**Cytotoxicity tests**

Figure 1 shows the effect of copolymers on human neutrophil viability evaluated through MTT test. The results demonstrated that both E₄₅S₈ and F127 at concentrations from 10 to 100 μg mL⁻¹ did not reduce the viability of cells when compared to the control group (vehicle, DMSO 1%). The MTT test measured cell viability evaluating its ability to reduce tetrazolium compound to formazan crystals by mitochondrial succinate dehydrogenase.³⁴ The results suggest that E₄₅S₈ or F127 did not affect the metabolism of human neutrophils at the evaluated concentrations. These results were corroborated by previous studies where E₄₅S₈ and its mixtures with Pluronic® P123 were not toxic for plasma membrane of human neutrophils.³⁵ The absence of toxic effect of E₄₅S₈ and F127 is very important considering that human neutrophils are the most abundant white blood cells having a central role on innate immune response.³⁵

**Critical micelle concentration (CMC)**

The fluorescence emission spectra of DPH in diluted polymer systems was used to plot graphs of DPH emission intensity at a wavelength of 428 nm versus the logarithm of the concentration (g L⁻¹) of polymer systems. The CMC of each system was estimated as the point where it starts a sharp increase in emission intensity of DPH, due to its solubilisation in the hydrophobic cores of the micelles.³³

### Table 1. Molecular characteristics of copolymers E₄₅S₈ and F127

| Copolymer       | Mᵃ | Wₓ | Wᵧ | Mᵯ/Mᵃ | Reference |
|-----------------|----|----|----|--------|-----------|
| E₄₅S₈ (ES)      | 2940 | 0.673 | 0.327 | 1.06   | ²         |
| E₄₅P₆₇E₄₅ (F127)| 12510 | 0.689 | 0.311 | 1.20   | ³¹        |

¹Average number of molecular weight (g mol⁻¹) by Nuclear Magnetic Resonance (¹³C NMR); ²Wₓ is the mass fraction of hydrophilic block in the copolymer chain; ³Wᵧ is the mass fraction of hydrophobic block; ⁴Polydispersity index by Gel Permeation Chromatography (GPC).
Binary micelles (E₄₅S₈/F₁₂₇) for quercetin and griseofulvin solubilisation

Values of CMC of ES expressed in mmol L⁻¹ are much lower than those for F₁₂₇ (Table 2). The length of Eₘ does not affect the CMC, but the hydrophobic character of hydrophobic block does.¹⁷,³⁶,³⁷ So, the higher is the hydrophobic effect, the lower is the surfactant’s CMC. This explains why the CMC of E₄₅S₈ is lower. The relative hydrophobicity between P and S units is 1:12.⁷

The systems presented a decrease in CMC values with the temperature increase, especially for F₁₂₇, representing a higher micelle formation capacity at higher temperatures, due to dehydration of the ethylene oxide chains leading to increased segregation between the PEO and PPO blocks.⁴,³⁸ This behavior also can be explained by previous studies that show a more endothermic micellisation process for EₙPₘEₙ type triblocks, with micellisation ∆H° values around 200 kJ mol⁻¹ or more. This results in instability of micelles in aqueous solution, increasing the number of micelles with increasing temperature.³³,³⁷

The copolymers EₙSm type presented a reduced micellisation enthalpy (∆H_m ≈ 0), which can be related to the interaction of the S block with water hydrophobic effect. This results in stability of micelles over a wide temperature range.³⁶,³⁹

These standard micellisation enthalpy values are calculated from the log ratio (CMC) versus 1/T (Equation 1):

\[ \Delta H_m = \text{RT}^2 (\text{dln CMC/dT}) \]  

However, the contribution of entropy generally dominates the micellisation process in aqueous surfactant solutions with the enthalpy playing a minor role. The unfavorable enthalpy of triblocks copolymers EₙPₘEₙ type is outweighed by a stronger entropy effect. The presence of hydrocarbon molecules in water causes a reduction in water entropy, inducing an increase in the degree of structuring of the water molecules due to cavity formation. However, this decrease in entropy is restored when hydrocarbon molecules aggregate to form micelles due to hydrogen bond formation.³⁹

The mixtures presented intermediate CMC values to the isolated copolymers and the increased proportion of the less hydrophobic core copolymer in the mixture results in an increase in the CMC value of the systems. Similar results were found in the literature.²²,⁴⁰–⁴²

Yet the mixtures retain low values of CMC, which make them promising for pharmacological applications due to the potential stability of their micelles after dilution in the blood, causing a greater circulation time of the drug in the blood,⁴³,⁴⁴ in addition of minimizing the side effects caused by the drug in its free form.

| Systems | CMC (g L⁻¹) | 25 °C | 37 °C |
|---------|-------------|-------|-------|
| F₁₂₇   | 0.2300      | 0.0790|
| F/ES 70/30 | 0.0074  | 0.0074|
| F/ES 50/50 | 0.0220  | 0.0190|
| F/ES 30/70 | 0.0047  | 0.0031|
| E₄₅S₈  | 0.0030      | 0.0020|

Taken together the results, it was observed that the CMC of E₄₅S₈ and F₁₂₇ at 37 °C were in the concentration range considered as non-toxic for human neutrophils. However, we are discussing about distinct methods (chemical and biological) being important additional studies to determine the bioavailability of these materials.

Thermoresponsive behavior

Figure 2 shows the phase diagrams of F₁₂₇, diblock ES and their mixtures. The copolymer ES did not present the interesting thermoresponsive properties of F₁₂₇, which was already expected for copolymers with poly(styrene oxide).⁵¹,⁴⁵ For the mixtures of F₁₂₇ and ES, it was not possible to produce a stable curve of mobile-hard transition when the ratio of F₁₂₇ was 30% (F/ES 70/30), therefore the results are not shown in Figure 2.

The F₁₂₇’s thermoresponsive property is a capacity of reversibly transforming from moving fluids to immobile gels and to return to moving fluids in the range temperature and can be related to the trichlocks EₙPₘEₙ type with high ∆H_m value. Due to the endothermic nature of the micellisation process, the number of micelles increases with increasing temperature and a compacted micellar gel forms at a critical gelling temperature.³⁷

However, EₙSm copolymers do not have thermoresponsive properties due to their low ∆H_m value, presenting micellar stability over a wide temperature range, in addition to the kinetic stabilization of micelles by vitrification of the S block core when the temperature tends to 0 °C.³⁹

The systems F/ES 50/50 and F/ES 70/30 had curves of transition...
which almost overlap, retaining the thermoresponsive properties of F127, gelling upon heating. The gel formation of these systems with a transition temperature in the range between room temperature and body temperature (25 – 37 °C) makes them interesting for application in subcutaneous drug delivery.

**Drug solubilisation**

The aqueous solubilities \( S_0 \) for both drugs were obtained according to the well-established solubilisation procedure “Shake-Flask”,\(^{14}\) For quercetin \( S_0 \) was 0.05 mg dL\(^{-1} \) at 25 °C similar to that obtained by Saija et al.,\(^{26} \) 0.0514 mg dL\(^{-1} \) at room temperature. For griseofulvin \( S_0 \) was 3.6 mg dL\(^{-1} \) at 25 °C, also compatible with the literature.\(^4\)

After obtaining the drug solubilities data, two parameters were investigated for the copolymers and their mixtures: \( S_{cp} \), the solubilisation capacity expressed in mg of drug per g of polymer (Equation 2), and \( S_h \), the solubilisation capacity expressed in mg of drug per g of hydrophobic block \( (W_h) \) (Equation 3), where \( S \) is the solubility of drug in micellar solution, \( S_h \) is the aqueous solubility of drug and \( m_{cop} \) is the mass of copolymer used in the solution composition. The results are shown in Figure 3 and Table 3.

\[
S_{cp} = \frac{S - S_0}{m_{cop}} \tag{2}
\]

\[
S_h = \frac{S}{W_h} \tag{3}
\]

The values found for the mixtures, F/ES, approached an average of the copolymers alone. For instance, for griseofulvin, the F/ES 70/30 system showed \( S_h \) values of 18.2, which is in accordance to comiscellisation process of diblock with F127. Besides, the same profile was also observed for the drug quercetin. All \( S_h \) values were much lower when compared to griseofulvin values, being attributed to the lower water solubility of quercetin, and also showing the dependence between the \( S_h \) of copolymer and the drug, as observed by Crothers et al.\(^{15} \)

Solubilisation can be considered as a drug partition between two phases: aqueous and micellar. The partition coefficient (P) is the ratio of drug concentration in micelle phase to the drug concentration in water for a specific surfactant concentration,\(^{46} \) according to Equation 4.

\[
P = \frac{S_{cp}}{S_0} \tag{4}
\]

The solubilisation results show an increase of \( P \) with increasing hydrophobicity in the systems, a result similar to the literature.\(^{4,40} \) This suggests a direct relationship between the values of \( S_h \) and \( P \).

The variation of Gibbs energy \((\Delta G°)\) of solubilisation can be calculated as a function of temperature and partition coefficient (Equation 5), where \( R \) is gas constant, \( T \) is temperature in Kelvin and \( P \) is partition coefficient.

\[
\Delta G° = -RT \ln P \tag{5}
\]

The results indicate spontaneous solubilisation for the systems, at standard conditions, manifested by the negative values of \( \Delta G° \), except for the F127 system with griseofulvin. The obtained data indicate that the increase of the hydrophobic character of the micelles decreases the \( \Delta G° \), favoring the spontaneity of the solubilisation. This is in accordance with the literature.\(^{40} \)

The hydrophobicity effect is usually even more pronounced for process of micellisation for non-ionic surfactants.\(^{44,4} \) This effect is greater for diblock due the relative hydrophobicity between \( P \) and \( S \) units is 1:12.\(^7 \) The presence of hydrophobes causes a reduction in the entropy of water that is restored when the surfactant molecules aggregate to form micelles. The objective of this aggregation is to restore hydrogen bonds and the degree of freedom of the hydrophobe, as well as to increase the entropy of water making the phenomenon of micellisation entropy favorably.\(^{33,37,49} \) Thus, systems with molecules of greater hydrophobic character form systems more spontaneously.
Table 3. Solubility parameters of 1 wt.% solutions of F127, E₄₅S₈ (ES) and their mixtures at 25 °C for griseofulvin (S₀ = 3.6 mg dL⁻¹) and quercetin (S₀ = 0.05 mg dL⁻¹)

| Systems          | Griseofulvin       | Quercetin         |
|------------------|--------------------|-------------------|
|                  | Sₐ(mg dL⁻¹)        | Sₐ(mg dL⁻¹)       | ΔG° (kJ mol⁻¹) | Sₐ(mg dL⁻¹)        | Sₐ(mg dL⁻¹)       | ΔG° (kJ mol⁻¹) |
| F127             | 3.0                | 0.8               | +0.462        | 0.8               | 2.6               | -6.87          |
| F/ES 70/30       | 5.8                | 1.8               | -1.18         | 1.8               | 5.8               | -8.88          |
| F/ES 50/50       | 6.3                | 3.2               | -0.87         | 1.04              | 3.26              | -7.52          |
| F/ES 30/70       | 7.8                | 2.17              | -1.92         | 6.4               | 19.86             | -12.0          |
| E₄₅S₈           | 9.3                | 2.58              | -2.35         | 3.2               | 9.9               | -10.3          |

*Solubilisation capacities expressed in mg of drug per g of polymer; *Solubilisation capacities expressed in mg of drug per g of hydrophobic block; *Increased solubilities; *Partition coefficient.

Table 4. Hydrodynamic diameter (Dₙ) and polydispersity of copolymers systems at 25 °C: without and with drugs

| Samples          | Unloaded | Griseofulvin | Quercetin |
|------------------|----------|--------------|-----------|
|                  | Dₙ/nm    | PdI          | Dₙ/nm    | PdI          | Dₙ/nm    | PdI          |
| F127             | 17.43 ± 0.7 | 0.475     | 38.02 ± 4.5 | 0.76        | 28.79 ± 0.8 | 0.50        |
| F/ES 70/30       | 23.52 ± 0.2 | 0.177     | 29.9 ± 0.6  | 0.46        | 25.86 ± 0.6 | 0.31        |
| F/ES 50/50       | 23.13 ± 0.4 | 0.244     | 25.8 ± 1.94 | 0.33        | 29.43 ± 0.6 | 0.47        |
| F/ES 30/70       | 19.15 ± 0.2 | 0.068     | 24.13 ± 0.0  | 0.22        | 22.33 ± 0.2  | 0.28        |
| E₄₅S₈           | 18.30 ± 0.4 | 0.073     | 21.63 ± 0.52 | 0.32        | 18.52 ± 0.4 | 0.18        |

Particle size

Particle size distribution by DLS measurements were performed to evaluate the comiscellisation of ES with F127 at 1 wt.% aqueous solution. The appearance of a single peak for binary mixture samples confirms the formation of micelle self-assembly. Table 4 shows the average hydrodynamic diameter (Dₙ) values for all analyzed systems. The average hydrodynamic diameter (Dₙ) of the systems unloaded has an average size range from 17.43 to 23.53 nm and the systems loaded was between 18.52 to 38.02. The incorporation of drugs results in an increase in the systems’ diameter, which can be associated with drug interaction with the hydrophobic micelle core. The size of systems providing stability and long running time, since nanoparticles ranging in size from 10 to 200 nm favor the accumulation in tumors, since nanoparticles with a diameter between 10 - 200 nm favor the accumulation in tumors via the EPR effect. The F/ES 70/30, F/ES 50/50 and F127 unloaded systems showed moderate polydispersity with PdI between 0.1 – 0.4. The systems loaded with drugs presented more uniform sizes, which can provide stability and increase the nanosystems circulation time. The systems presented better solubilisation capacity for systems with greater hydrophobicity. However, the drugs presented different optimal system. For griseofulvin, they presented E₄₅S₈ and for quercetin they presented F/ES 30/70. Those results may be related to the structural differences of drugs that interact differently with the micelle nucleus. In the particle size study, the systems presented small size and the samples formed with higher hydrophobic proportion presented more uniform sizes, which can provide stability and increase the nanosystems circulation time. Therefore, the micellar nanosystems formed by binary mixture F127 and E₄₅S₈ copolymers are interesting hydrophobic drug nanocarriers.

CONCLUSIONS

In this study, micellar nanosystems formed by copolymers E₄₅S₈, F127 and their binary mixtures were investigated. Research has shown that the mixtures resulted in systems that have a combination of properties of their constituents, such as thermoresponsive property and low CMC values, which make them potential candidates for pharmacological use such as for application in subcutaneous drug delivery. The sample demonstrated better solubilisation capacity for systems with greater hydrophobicity. However, the drugs presented different optimal system. For griseofulvin, they presented E₄₅S₈ and for quercetin they presented F/ES 30/70. Those results may be related to the structural differences of drugs that interact differently with the micelle nucleus. In the particle size study, the systems presented small size and the samples formed with higher hydrophobic proportion presented more uniform sizes, which can provide stability and increase the nanosystems circulation time. Therefore, the micellar nanosystems formed by binary mixture F127 and E₄₅S₈ copolymers are interesting hydrophobic drug nanocarriers.

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