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Mixed micelles for encapsulation of doxorubicin with enhanced *in vitro* cytotoxicity on breast and ovarian cancer cell lines *versus* Doxil®

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ABSTRACT

Doxorubicin (DOX) is used as a "first-line" antineoplastic drug in ovarian and metastatic breast cancer. However, serious side effects, such as cardiotoxicity have been reported after DOX intravenous administration. Hence, we investigated different micelle-former biomaterials, as Soluplus®, Pluronic F127, Tetronic T1107 and d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) to develop a potential mixed micellar nanocarrier for DOX delivery. Since DOX hydrochloride is a poor candidate to be encapsulated inside the hydrophobic core of the mixed micelles, we assayed a hydrophobic complex between DOX and sodium deoxycholate (NaDC) as an excellent candidate to be encapsulated within polymeric micelles. The combination of T1107:TPGS (1:3, weight ratio) demonstrated the best physicochemical properties together with a high DL capacity (6.43% w/v). Particularly, DOX *in vitro* release was higher at acidic tumour microenvironment pH value (5.5) than at physiological counterpart (7.4). The hydrodynamic diameter of the DOX/NaDC-loaded mixed micellar system was 10.7 nm (PDI = 0.239). The *in vitro* cytotoxicity of the mixed micellar formulation resulted significantly ($p < 0.05$) higher than Doxil® against ovarian (SKOV-3) and triple-negative breast cancer cells (MDA-MB- 231). Further, the *in vitro* cellular uptake assays demonstrated a significant increment ($p < 0.05$) of the DOX intracellular content for the mixed micelles *versus* Doxil® for both, SKOV-3 (at 2, 4 and 6 h of incubation) and MDA-MB-231 (at 4 h of incubation) cells. These findings suggest that T1107:TPGS (1:3) mixed micelles could be employed as a potential nanotechnological platform for drug delivery of DOX.

1. Introduction

Doxorubicin (DOX) hydrochloride, an anthracycline antibiotic, is one of the most effective antineoplastic agents used as a "first-line" drug in various types of malignancies, including ovarian and metastatic breast cancer [1]. Nevertheless, due to its short biological life span and nonspecific distribution, it exhibits serious adverse effects (hepatotoxicity, dose-limiting myelosuppression and lethal cardiotoxicity) [2], which limit its clinical application as a free-drug. In order to decrease drug cardiotoxicity, a DOX-loaded pegylated liposomal formulation (Doxil®) was approved by the Food and Drug Agency of the United States (FDA) in 1995 [3]. In comparison to free DOX, Doxil® exhibits a slower plasma clearance and smaller volume of distribution [4] and it has proved to reduce the number of cardiac events in patients with metastatic breast cancer. However, it has not shown significant improvements in terms of survival [5]. On the other hand, it has been reported that "pegylation" strongly inhibits the cellular uptake, making these liposomes to present poor cellular drug internalization [6,7]. In this context, the study of other hydrophilic materials that could replace polyethylene-glycol (PEG), represents an interesting alternative to improve nanoparticle cellular uptake.

Recently, one of the biomaterials successfully used for this purpose is the D-α-tocopheryl polyethylene glycol (PEG) 1000 succinate (TPGS). It is an amphiphilic water-soluble derivative of natural source vitamin E and PEG, that has been widely employed as a micelle-former biomaterial. Also, it has been reported that TPGS can inhibit the efflux pump that mediates multidrug resistance in tumour cells, known as P-glycoprotein (P-gp) [8]. Besides, the *in vitro* and *in vivo* antineoplasic activity of TPGS has been studied in different cancer cell lines, as it has been stated that it promotes cellular apoptosis [9]. In this context, TPGS has

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been employed for DOX encapsulation within polymeric micelles [10–12].

Among nanocarriers, polymeric single micelles have emerged as one of the most promising approaches, as they are capable of increasing the solubility and stability of hydrophobic drugs, enhancing cellular uptake and achieving *in vivo* advantages [13,14]. Recently, the combination of two or more block co-polymers to obtain "mixed micelles", has been an excellent approach to enhance the single micelle properties [15], as enhanced thermodynamic and kinetic stabilities, higher drug loading (DL) capacity, more accurate size control and easier ways to modify their surface with different moieties [16].

One of the most employed amphiphilic biomaterials used to prepare mixed systems, are the derivatives of poly(ethylene oxide)–poly(propylene oxide) (PEO–PPO–PEO) block copolymers [17]. They can be classified in two marketed families: (i) the linear and bifunctional triblocks of PEO-PPO-PEO or poloxamers (Pluronic®) and their 4-armed branched counterparts, poloxamines (Tetronic®). This last exhibit two tertiary amine groups in the middle of the molecule, that confer both temperature and pH sensitiveness [18]. A relatively new amphiphilic polymer that has successfully enhanced the solubility of some hydrophobic drugs is the polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer (Soluplus®) [19]. The low critical micellar concentration (CMC) of this polymer (0.76×10^{-3}% w/v) confers high stability to its forming aqueous micellar dispersions [20].

In this framework, hydrophilic DOX hydrochloride is a poor candidate to be encapsulated inside the hydrophobic core of the mixed micelles. Alternatively, a hydrophobic complex formed by electrostatic-interactions between DOX and sodium deoxycholate (NaDC, a negatively charged surfactant) could be an excellent candidate to be encapsulated within polymeric micelles [21,22]. Moreover, DOX complex with NaDC is easy to prepare and does not require chemical reactions to obtain the DOX base.

Hereby, the objective of the present study was to develop mixed micelles consisting of a drug–surfactant complex encapsulated in a novel mixed micellar formulation composed of different block copolymers and TPGS, for enhancing the *in vitro* antitumoural efficacy of DOX. The physicochemical parameters of the DOX-loaded mixed micelles, DL, entrapment efficiency (EE), particle size and *in vitro* release profile were explored. Finally, the *in vitro* cytotoxicity and cellular uptake were also investigated in breast (MDA-MB-231) and ovarian (SKOV-3) human cancer cell lines, *versus* Doxil® and free DOX.

2. Experimental

2.1. Materials

Doxorubicin hydrochloride (99.9%) was purchased from LKM Laboratories (Argentina). Pluronic® F127 (F127, MW ~12.6 kDa, 70 wt % PEO), Tetronic® 1107 (T1107, MW ~15.0 kDa, 70 wt% PEO) and Soluplus® (MW ~ 120 KDa) were a gift of BASF (Argentina). TPGS (MW ~1513 g/mol) was purchased from Eastman Chemical Company (USA), sodium deoxycholate (NaDC) was purchased from Riedel-de Häen® (Germany). Doxil® was purchased from Raffo Laboratories (Argentina). Tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium], inner salt (MTS) and phenazinemethosulfate (PMS) were purchased from Promega Corporation (USA). All solvents were analytical or high performance liquid chromatography (HPLC) grade and were used following the manufacturer's instructions.

2.2. Preparation of single and mixed micelles

Mixed F127:TPGS, T1107:TPGS and Soluplus®:TPGS micelles (2% w/v total polymer concentration) were prepared by dissolving the required amount of each polymer in distilled water at room temperature and equilibrating the system at 25 °C, at least 24 h before use. Binary systems with polymer weight ratios of 3:1, 2:2 and 1:3 were assayed. Single F127, T1107, Soluplus® and TPGS micelles (2% w/v) were used as controls.

2.3. Critical micellar concentration (CMC)

The CMC values of the formulations in aqueous solution were determined by dynamic light scattering (DLS, Zetasizer Nano-ZSP, ZEN5600, Malvern Instruments, United Kingdom). Measurements were conducted at a scattering angle of $\Phi = 173°$ to the incident beam. The micellar dispersions were prepared and diluted with an appropriate media to get a polymer concentration range between 1.10^{-5} and 1% w/v. Samples were equilibrated 24 h at 25 °C before the analysis. The copolymer concentration at which a sharp increase in the scattering intensity was observed was considered as the CMC value, employing the derived count rate *versus* the polymer concentration (%w/v) graph.

2.4. Cloud point (CP)

CP measurements were conducted by submerging sealed glass vials that contained each single and mixed micellar preparation (2% w/v) in a glycerin oil bath at room temperature. Then, the temperature was gradually increased from room temperature (1 °C/min) until the point of abrupt change in the visual appearance of the system from clear to turbid [23]. Once the temperature exceeded the CP, the system was cooled down and the whole process was repeated to check the reproducibility of the measurement. The maximum uncertainty in the CP measurement was ± 1 °C.

2.5. Measurement of the micellar size, zeta potential and morphological characterization

The average hydrodynamic diameter (D_h) and micellar size distribution of single and mixed micelles (2% w/v polymer concentration) were measured by DLS (scattering angle of $\theta = 173°$ to the incident beam, Zetasizer Nano-ZSP, Malvern Instruments, United Kingdom) at 25 and 37 °C. Samples were filtered (0.45 μm acetate cellulose filters, Microclar, Argentina) and equilibrated for 5 min at each temperature before the measurements. The results were expressed as the average of three measurements ± standard deviation (S.D.). Zeta potential of the single and mixed micelles was measured using the same analyzer at 25 °C. Experimental values were the average of three different formulations. Then, T1107:TPGS (1:3, weight ratio) mixed (2% w/v) micelles were visualized by means of transmission electron microscopy (TEM, Philips CM-12 TEM apparatus, FEI Company, The Netherlands). An aliquot (5 μL) was placed onto a clean grid and covered with Fomvar film. Then, samples were negatively stained with phosphotungstic acid solution (5 μL, 1% w/v), washed with distilled water (5 μL) and dried into a silicagel container before the analysis.

2.6. Preparation of DOX/NaDC complex-loaded micellar systems

2.6.1. Complex preparation

DOX-NaDC complex was prepared by dissolving 50 mg of DOX hydrochloride in 10 mL of distilled water and adding 90 mg of NaDC to the solution under magnetic stirring. After 2 h of stirring, the aqueous insoluble DOX-NaDC complex suspension was transferred to a Falcon® conical tube and centrifuged (5600 rpm, 10 min, 20 °C, Refrigerated Centrifuge Combi 514R, Hanil Science Industrial Co., Korea). The supernatant was discarded and the precipitate was re-dispersed with 10 mL of distilled water and homogenized with vortex (Vortex Mixer Wizard, Velp Scientifica, Italy).

2.6.2. DOX/NaDC complex-loaded single and mixed micelles

The obtained DOX-NaDC complex suspension was added in excess

to the single and mixed (2% w/v) micellar dispersions and left under magnetic stirring (2 h). The resulting dispersions were filtered (0.45 μm acetate cellulose filters, Microclar, Argentina) to remove insoluble DOX-NaDC and stored at 4 °C. DOX concentration was determined by UV–vis spectrophotometry (λ: 500 nm, UV-260, UV–vis Recorder Spectrophotometer, Shimadzu, Japan) in *N,N*-Dimethylformamide at room temperature. The linearity range was established between 5 and 63 μg/mL (R^2: 0.9986) in *N,N*-Dimethylformamide. DOX-free micellar dispersions and an aqueous NaDC control solution (without DOX and copolymer) were used as controls. Assays were done by triplicate and the results were expressed as the average ± S.D.

2.7. Characterization of DOX/NaDC complex-loaded pristine and mixed micelles

2.7.1. Size, size distribution of micelles and zeta potential

As previously described, the D_h, micellar size distribution and zeta potential of DOX/NaDC complex-loaded single and mixed systems (2% w/v) were measured by DLS under the same conditions of the unloaded samples.

2.7.2. Evaluation of drug loading and entrapment efficiency

To determine the DL (%) and EE (%) of the systems, DOX/NaDC complex-loaded micelles were frozen (−20 °C) and lyophilized (FIC-L05, shelf temperature −14 °C, condenser temperature −39 °C, Scientific Instrumental Manufacturing, Argentina) for 48 h. Then, the DL and EE were calculated according to the following equations:

$$DL(\%) = \frac{\text{mass of drug encapsulated in micelles}}{\text{mass of drug} - \text{loaded micelles}} \times 100$$

$$EE(\%) = \frac{\text{mass of drug encapsulated in micelles}}{\text{initial mass of drug used}} \times 100$$

All samples were done in triplicate and the results were expressed as mean ± S.D.

2.8. In vitro drug release

In vitro release of DOX from mixed F127:TPGS, T1107:TPGS and Soluplus®:TPGS (1:3) micellar systems were performed using the dialysis method (96 h). Dispersions were placed into a dialysis bag (regenerated cellulose dialysis membranes; molecular weight cut off of 3500 g/mol; Spectra/Por® 3 nominal flat width of 45 mm, diameter of 29 mm, Spectrum Laboratories, Inc., USA), sealed and placed in a Falcon® conical tube (15 mL) containing the release medium (PBS, pH 7.4 or pH 5.5). Then, each Falcon® conical tube was placed in an orbital gyratory device at 40 rpm and 37 °C. At every time intervals (1, 2, 4, 8, 24, 48, 72 and 96 h), the whole medium was withdrawn and replaced with an equal volume of fresh medium pre-heated at 37 °C. The released DOX amounts were quantified by UV–vis as described above with correction for the volume replacement. (n = 3 ± S.D.)

2.9. In vitro cytotoxicity

MDA-MB 231 and SKOV-3 human cancer cell lines were obtained from the American Type Culture Collection (ATCC) (USA). Cells were maintained in Dulbecco's minimum essential medium (DMEM®, 37 °C, 5% CO_2) supplemented with 10% fetal bovine serum (FBS), 50 μg/mL gentamycine and 2 mM L-glutamine (Invitrogen, Argentina). For *in vitro* cytotoxicity assays, cells were seeded in 96-well plates (Corning Costar, Fisher Scientific, USA) at a density of 5000 cells/well and incubated 24 h. Then cells were incubated with free DOX (2 mg/mL), blank mixed micelles, DOX-loaded (2 mg/mL) T1107:TPGS (1:3) micelles and Doxil® for 72 h. After incubation, the medium was removed, the wells were washed with PBS and fresh medium was added. Finally, the water soluble tetrazolium salts (WST) solution prepared according to manufactures instructions (CellTiter 96® aqueous non-radioactive cell proliferation assay, Promega) was added and cells were incubated for 2 h. The absorbance at 490 nm was measured using a microplate reader (Biotrak II Plate Reader, Amershan Bioscienes, USA). Triplicates were run for each treatment. DOX concentrations leading to 50% cell-killing (IC_{50}) were determined from concentration-dependent cell survival curves. Values were expressed in terms of percent of untreated control cells set as 100%. Statistical analysis was performed using one-way ANOVA test ($p < 0.05$). (n = 3 ± S.D.)

2.10. In vitro cellular uptake

Briefly, MDA-MB 231 or SKOV-3 cells were seeded in 6-well plates at the density of 4×10^5 cells/well and cultured at 37 °C in the presence of 5% CO_2 for 24 h. Then cells were incubated with free DOX, blank mixed micelles, Doxil® and DOX-loaded T1107:TPGS (1:3) mixed micelles at 25 μg/mL of DOX for 0.5, 2, 4 and 6 h, respectively. Untreated cells acted as a control. At predetermined time-points, the cells were washed with 1.5 mL cold PBS. Then, cells were washed with PBS and 0.25 mL trypsin PBS solution (2.5 μg/mL) was added. The cell lysate was centrifuged (13,000 rpm, 10 min, MiniSpin® plus™, Eppendorf, Germany). Drug content in the supernatants was measured by high performance liquid chromatography (HPLC) Instrument (Shimadzu SCL-10A, Japan) equipped with a plus autosampler (Shimadzu SIL-10A, Japan) and UV-detector (Shimadzu SPD-10A, Japan). Chromatographic separations were performed on a reversed phase C18 column (4.6 mm × 250 mm, 3.5 μm, Xterra RP18, Waters, Ireland). The mobile phase of acetonitrile:water (30:70, v/v) at pH = 3 (adjusted with phosphoric acid) was delivered at flow rate of 0.6 mL/min at ambient temperature. Detection wavelength was 233 nm. Sample solution was injected at a volume of 10 μL. Values were normalized by protein content in each sample determined using BCA protein assay kit (Pierce Corporation, China) according to the manufacturer's protocol. Statistical analysis was performed using one-way ANOVA test ($p < 0.05$). (n = 3 ± S.D.)

3. Results and discussion

3.1. Self-aggregation of mixed micelles

In the present study, amphiphilic block copolymers (Pluronic® F127, Tetronic® T1107 and Soluplus®) were used to produce mixed micelles with TPGS as potential nanocarriers for DOX. F127 is a highly hydrophilic linear thermo-responsive copolymer, while T1107 is a 4-armed branched copolymer with temperature and pH sensitiveness [18]. Both are derivatives of similar hydrophilicity and different PPO block molecular weights [17]. On the other hand, Soluplus® is an amphiphilic graft copolymer that has successfully increased the solubility of some hydrophobic drugs [19,20,24,25]. Finally, TPGS was chosen for its properties as P-gp inhibitor and its *in vitro* and *in vivo* antineoplastic activity [9].

To evaluate the self-aggregation capacity of single and mixed micelles, we defined the CMC of each copolymer and mixtures using DLS (Table 1). This technique has demonstrated to be as sensitive as fluorescence measurements (using pyrene as a probe) for the CMC determination of amphiphilic copolymers [26].

For single micelles, the CMC values of F127 and T1107 were in agreement with previous studies using DLS and surface tension, respectively [18,27]. For Soluplus® and TPGS, their CMC values were similar to our previously reported values [28,29]. CMC values resulted as follows: Soluplus® < TPGS < T1107 < F127 (Table 1).

For mixed micelles, the incorporation of TPGS to F127:TPGS and T1107:TPGS mixed systems led to a gradual decrease of the CMC experimental values, as it was expected. Mingkwan et al. and Gao et al. observed a similar behaviour for poloxamers:TPGS mixtures [30,31]. This is probably due to the increase in hydrophobic interactions

Table 1
CMC and cloud point (CP) values for micellar systems in water at 25 °C.

Copolymers	Mixture composition[a]	CMC (%w/v)	CP (°C)
F127	–	0.55353	115
T1107	–	0.37675	> 120
Soluplus®	–	0.00053	40
TPGS	–	0.06420	113
F127:TPGS	3:1	0.14000	114
	2:2	0.10100	119
	1:3	0.05288	> 120
T1107:TPGS	3:1	0.22200	> 120
	2:2	0.18100	119
	1:3	0.07750	117
Soluplus®:TPGS	3:1	0.00109	33
	2:2	0.00119	33
	1:3	0.04810	32

[a] Polymer weight ratios.

between the PPO segment of Pluronic® and vitamin E portion of TPGS in the core of micelles [31]. In addition, the content of PPO units in these copolymers could provide a synergistic effect with the hydrophobic portion of TPGS, improving system stability and drug solubilization. Interestingly, an opposite effect was obtained for Soluplus®:TPGS systems. In this case, we observed higher CMC experimental values when the TPGS concentration increased, as we previously reported [19]. This behaviour is due to the higher hydrophilic nature of TPGS, when compared to Soluplus®, negatively affecting the self-aggregation tendency of Soluplus® [19].

3.2. Cloud point

The temperature at which a homogeneous aqueous dispersion of amphiphilic substances is separated into a surfactant-rich phase and a surfactant-poor phase is known as cloud point (CP) [16]. This physical change is produced when the hydrophilic portions of non-ionic surfactants are dehydrated. For this reason, in mixed micelles it is to be expected that this phenomenon substantially differ from that of single micelles [32]. Table 1 summarizes the CP values observed for single and mixed micelles. The high CP value displayed by F127, T1107 and TPGS relies on the high content of their hydrophilic portion, PEO for the PEO-PPO derivatives and PEG for vitamin E derivative. Conversely, Soluplus® showed a low CP value, due to the high hydrophobicity that presents its chemical structure. These values are in good agreement with previous reports [19,27,33]. In the case of the binary samples using poloxamer or poloxamine with TPGS, the CP values were equal to or higher than those of pristine components, indicating the hydrophilic nature of the mixtures. Contrary, the mixtures Soluplus® and TPGS exhibited CP values lower than pure components, indicating a higher hydrophobicity of the mixtures. Finally, it should be noted that all systems showed one single CP, indicating the formation of a mixed micellar system [34].

3.3. Size, size distribution, zeta potential and morphology of drug free single and mixed micelles

The D_h and size distribution of the drug free single and mixed micelles were characterized by DLS (Table 2, Figs. 1 and 2). On the one hand, F127 single micelles showed a smaller fraction (peak 1, D_h = 6.3 nm, 24%) that would correspond to singly dispersed polymeric chains (unimers) and the major one (peak 2, D_h = 36.5 nm, 76%) to polymeric micelles. A PDI value of 0.462 was consistent with a bimodal distribution. The four-arm counterpart T1107 also presented a bimodal size distribution (PDI: 0.296) (Table 2, Figs. 1 and 2). The presence of two size populations could be related with the presence of residual materials from the synthetic obtaining process of both polymers, indicating that both F127 and T1107 micellized partially [27,33,35]. On the other hand, Soluplus® and TPGS single micelles presented a unimodal distribution with D_h values of 69.8 nm and 11.7 nm, respectively. Both systems showed small PDI value of 0.029, which is consistent with a complete micellization.

Interesting results were observed for the mixed systems. F127:TPGS mixtures generated smaller micelles of D_h between 13.4–18.9 nm, sizes gradually shrank when increasing TPGS concentrations. In addition, PDI values decreased from 0.108 to 0.044 with the increase of TPGS. These findings would suggest that TPGS enhances the F127 self-aggregation with a complete micellization [30]. T1107:TPGS mixed micelles showed a similar trend though sizes were slightly smaller than those of F127:TPGS (Table 2). As with the poloxamer, the poloxamine:TPGS mixtures presented small PDI values, indicating a complete micellization. These results could be associated with an improvement of the hydrophobic interactions in the micellar core upon TPGS incorporation, enhancing the self-aggregation tendency of the biomaterials [10]. Also, with a higher proportion of TPGS, the mixture is composed by a higher amount of TPGS unimers, being this last that governs the micellar structure and size. These results were in line with the decrement of the CMC values observed as the TPGS concentration increased (Table 1).

On the other hand, Soluplus®:TPGS systems presented an interesting behaviour, according to the proportion employed of both biomaterials. For copolymer mixtures of 3:1 and 2:2, we observed an increase in the micellar size (88.8 nm and 137.7 nm, respectively), as the TPGS concentration increased. This behaviour was previously observed by our group [19] and, also, a similar phenomenon was found with Soluplus® and Solutol® HS-15 mixed micelles [36]. It could be explained by the intercalation of TPGS chains in the corona and core of the mixed micelles, being Soluplus® a more hydrophobic micelle-former biomaterial than TPGS. Also, vitamin E has been used to increase the micellar size in previous reports [37]. Particularly, for the 2:2 ratio, the mixed system exhibited the highest PDI value and a bimodal size distribution (Table 2, Figs. 1 and 2). These results clearly indicated that this proportion of biomaterials is the less favoured to conform mixed micelles.

However, an opposite behaviour was observed for 1:3 ratios, where the size of the mixture decreases to 20.8 nm. Probably, this effect is due to a greater proportion of TPGS in the mixture, generating the same effect that we observed with PEO-PPO derivatives. All mixed systems showed unimodal distributions with small PDI values between 0.044–0.170, being consistent with a complete micellization.

Zeta potential values were neutral for both, single and mixed micelles, as it is shown in Table 2. These neutral potential values were expected, due to the presence of PEG (a non-ionic hydrophilic polymer) in the micelles corona.

Finally, mixed micelles demonstrated a spherical morphology with a narrow size distribution (Fig. 3).

3.4. Drug loading and entrapment efficacy of DOX into pure and mixed micelles

DOX is commercially available as hydrochloride salt. Generally, in order to be encapsulated within micelles, it is neutralized with alkaline base in several steps overnight [38]. Alternately, we used a simpler method using a hydrophobic complex of DOX and NaDC, with improved DOX affinity for the hydrophobic core of the polymeric micelles [21]. It is worth stressing that NaDC is a water-soluble bile salt that it is currently employed in the mixed micellar formulation of Amphotericin B (Fungizone®) [39]. Moreover, we confirmed that the presence of NaDC did not affect DOX quantification by UV/Visible spectrophotometry (λ: 500 nm) in *N,N*-dimethylformamide.

The DL content of different systems is shown in Fig. 4. The results indicate that DOX was successfully incorporated into single and mixed micelles at a polymer concentration of 2% w/v (> > > CMC). PEO-PPO block copolymers presented the lower DL values (0.21% and 0.17% for F127 and T1107, respectively), while Soluplus® showed an

Table 2
Micellar size, size distribution (PDI) and zeta potential of single and mixed micelles in absence and presence of DOX at 25 °C. Results are expressed as mean ± S.D. (n = 3).

Copolymers	Mixture composition[a]	DOX/NaDC complex	Peak 1[a]		Peak 2[b]		PDI	Zeta potential (mV)
			D_h	%	D_h	%		
F127	–	–	6.3 (0.2)	24.3	36.5 (1.8)	75.7	0.462 (0.024)	−2.7 (0.7)
T1107	–		6.4 (0.1)	84.5	313.6 (35.4)	15.5	0.296 (0.009)	−8.3 (1.2)
Soluplus®	–		69.8 (0.4)	100.0	–	–	0.029 (0.013)	0.3 (0.1)
TPGS	–		11.7 (0.1)	100.0	–	–	0.029 (0.004)	−1.5 (1.7)
F127:TPGS	3:1		18.9 (0.1)	100.0	–	–	0.108 (0.002)	−2.4 (0.9)
	2:2		13.7 (0.1)	100.0	–	–	0.059 (0.002)	−4.7 (1.0)
	1:3		13.4 (0.1)	100.0	–	–	0.044 (0.004)	−8.7 (4.2)
T1107:TPGS	3:1		15.7 (0.1)	100.0	–	–	0.137 (0.004)	−6.9 (3.0)
	2:2		14.0 (0.2)	100.0	–	–	0.074 (0.008)	−5.9 (2.7)
	1:3		12.7 (0.2)	100.0	–	–	0.044 (0.014)	−5.0 (1.5)
Soluplus®:TPGS	3:1		88.8 (0.7)	100.0	–	–	0.074 (0.018)	0.3 (0.2)
	2:2		225.0 (33.0)	59.0	> 1 µm	41.0	0.447 (0.014)	−0.2 (0.1)
	1:3		20.8 (0.4)	100.0	–	–	0.170 (0.006)	−1.1 (0.6)
F127	–	✓	6.1 (1.2)	14.8	38.8 (5.7)	85.2	0.142 (0.018)	−0.6 (0.3)
T1107	–		390.5 (26.2)	100.0	–	–	0.386 (0.058)	−5.7 (0.2)
Soluplus®	–		79.1 (2.5)	100.0	–	–	0.142 (0.018)	−2.5 (0.2)
TPGS	–		11.0 (0.1)	100.0	–	–	0.154 (0.010)	−3.2 (0.2)
F127:TPGS	3:1		15.1 (0.5)	56.5	495.3 (98.9)	43.5	0.398 (0.042)	−0.9 (0.1)
	2:2		11.8 (0.2)	76.4	382.3 (80.3)	23.6	0.257 (0.028)	−1.1 (0.3)
	1:3		11.6 (0.1)	100.0	–	–	0.248 (0.010)	−1.4 (0.1)
T1107:TPGS	3:1		11.2 (0.1)	24.4	448.3 (31.2)	75.6	1.000 (0.001)	−2.5 (0.2)
	2:2		11.4 (0.3)	100.0	–	–	0.285 (0.014)	−2.7 (0.3)
	1:3		10.7 (0.2)	100.0	–	–	0.239 (0.010)	−2.9 (0.6)
Soluplus®:TPGS	3:1		96.8 (0.9)	100.0	–	–	0.103 (0.007)	−0.5 (0.1)
	2:2		232.4 (18.1)	100.0	–	–	0.292 (0.026)	−1.3 (0.2)
	1:3		240.7 (3.0)	100.0	–	–	0.220 (0.014)	−1.8 (0.4)

[a] Smaller size population.
[b] Higher size population.

intermediate value (1.50%) and TPGS the highest increment (5.27%). These results indicate that the DOX/NaDC complex has more affinity to the core of TPGS micelles than to the rest of the amphiphilic surfactants, favouring its loading into these nanocarriers. When there is not enough affinity between the drug and the polymeric micelle core, the drug will not be successfully loaded into the micelle [25,40]. As it was expected, an increase in the TPGS concentration on the binary systems resulted in higher DL values. A similar behaviour was observed for F127:TPGS and P123:TPGS mixtures employing the same polymer ratios [30]. Therefore, out of the 3 combinations used, the ratio 1:3 presented the greater DL% values (6.74, 6.45 and 6.23% for F127:TPGS, T1107:TPGS and Soluplus®:TPGS, respectively). This is probably due to the good hydrophobic interactions within the mixed micelles core composed of TPGS hydrophobic portion (vitamin E succinate) and poly(propylene oxide) moieties of PEO-PPO copolymers, being this interaction weaker between the hydrophobic portions of TPGS and Soluplus®. For instance, for 2:2 ratios, the mixed systems demonstrated DL values of 5.20, 5.03 and 4.75% for F127:TPGS,

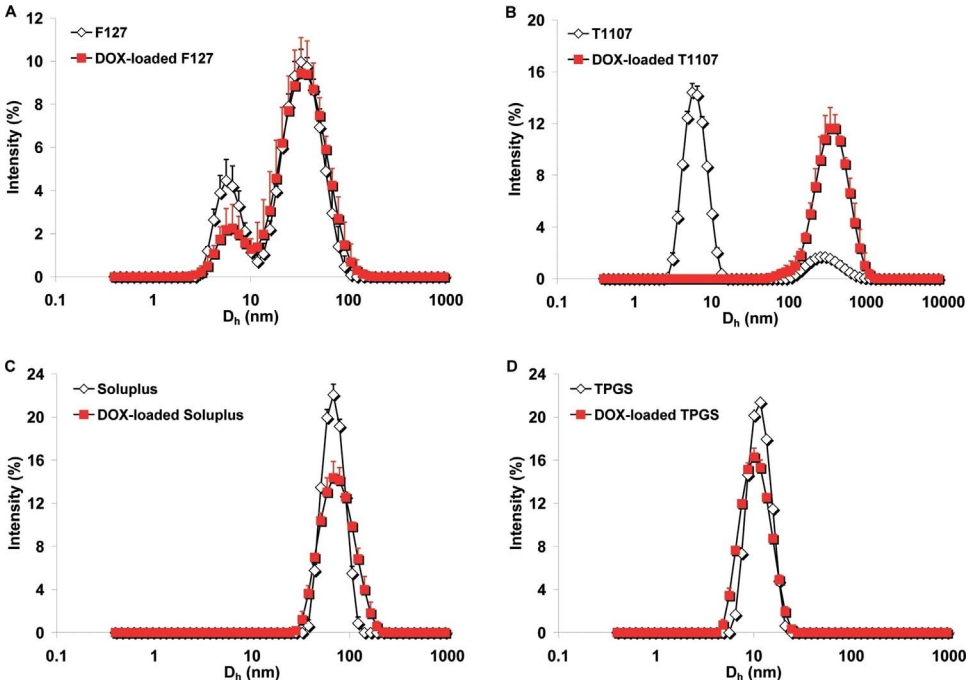

Fig. 1. Micellar size and size distribution graphs of single micelles in absence and presence of DOX at 25 °C. Results are expressed as mean ± S.D. (n = 3).

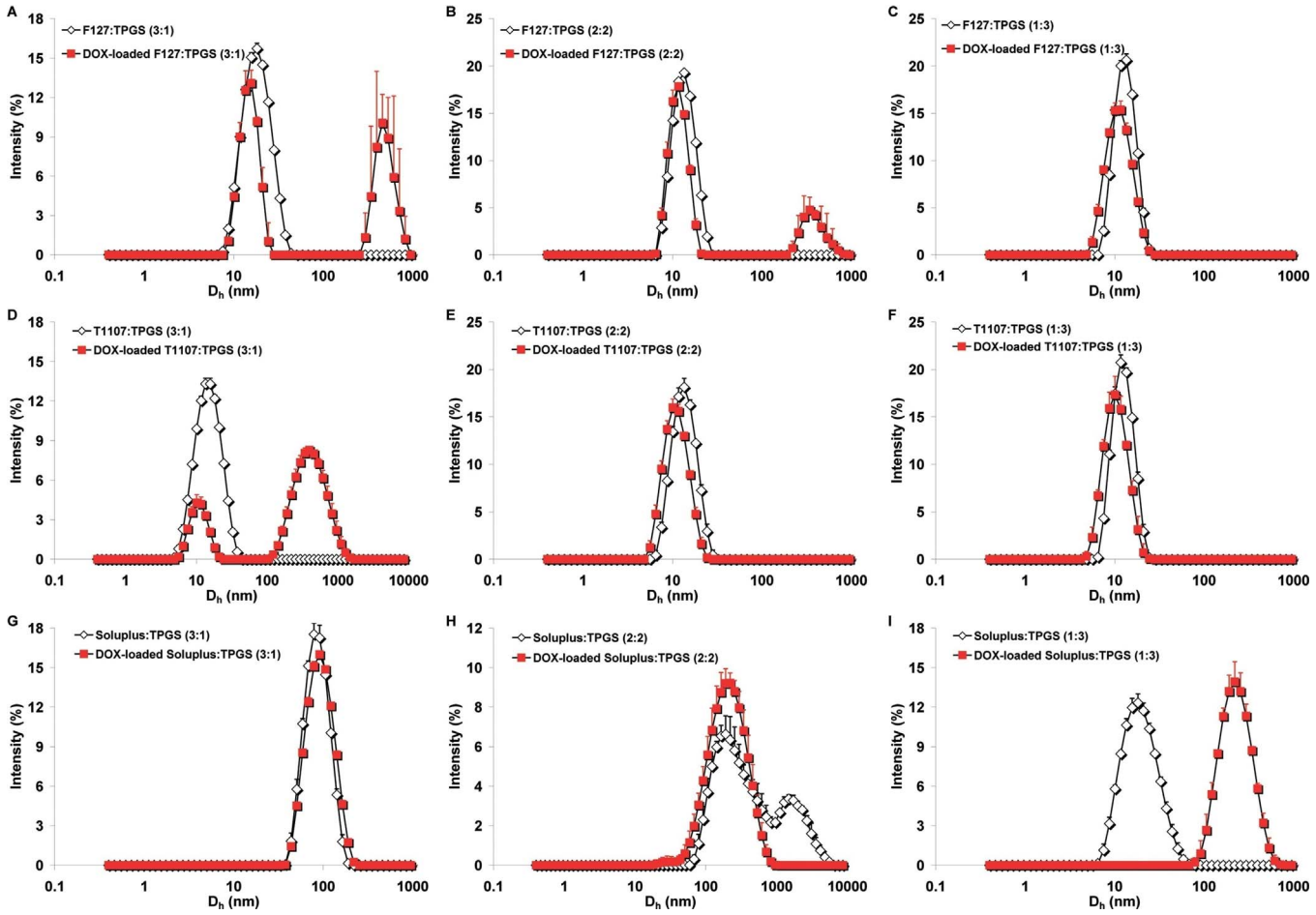

Fig. 2. Micellar size and size distribution graphs of mixed micelles in absence and presence of DOX at 25 °C. Results are expressed as mean ± S.D. (n = 3).

T1107:TPGS and Soluplus®:TPGS, respectively (Fig. 4).

Furthermore, a decrease in the amount of TPGS in the mixtures leads to a decrement in the DOX cargo (Fig. 4). For example, the DL values obtained for T1107:TPGS mixed micelles were 3.26, 5.03 and 6.45% for 3:1, 2:2 and 1:3 ratios, respectively.

Finally, if we compared the colloidal systems with the lowest amount of TPGS (3:1 ratio), Soluplus®:TPGS formulation exhibited the highest DL value (3.55%), followed by T1107:TPGS (3.26%) and F127:TPGS (2.81%). For this ratio, we observed that the DL is influenced by the greater affinity of DOX/NaDC complex for Soluplus® than for PEO-PPO copolymers as we observed in the single micelles.

In the case of EE% values, a similar behaviour was observed (Fig. 4) for all the assayed systems.

3.5. *Effect of DOX/NaDC complex on the aggregation and zeta potential of single and mixed micelles*

As shown in Table 2, Figs. 1 and 2, for linear PEO-PPO copolymer (F127) the presence of DOX/NaDC complex exhibited similar sizes, in comparison to their free-drug counterparts. A similar behaviour was observed for Soluplus® and TPGS. In this case, for DOX/NaDC complex loaded Soluplus® single micelles the value of D_h increased slightly in comparison to unloaded micelles, maintaining a unimodal distribution (PDI: 0.142). For TPGS, we did not observe differences of size between loaded and unloaded single micelles. However, T1107 micelles loaded with this complex presented remarkably higher D_h values (one size population 390.5 nm, PDI 0.386), when compared to the unloaded system. In this case, insoluble drug-complex has been incorporated in the micelles forming larger micelle clusters. A similar behaviour was previously observed for triclosan [18].

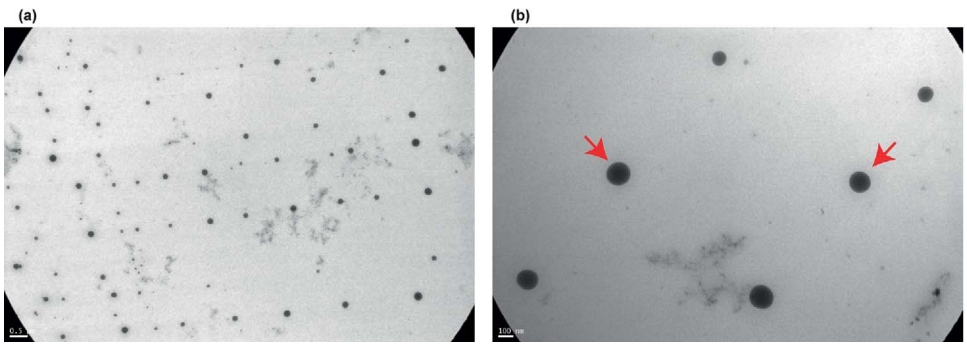

Fig. 3. TEM micrograph of T1107:TPGS (1:3, weight ratio) mixed micelles in distilled water and negatively stained with phosphotungstic acid solution (1% w/v). Scale bar in (a) 0.5 μm and (b) 100 nm. Red arrows in (b) point out the spherical mixed micelles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

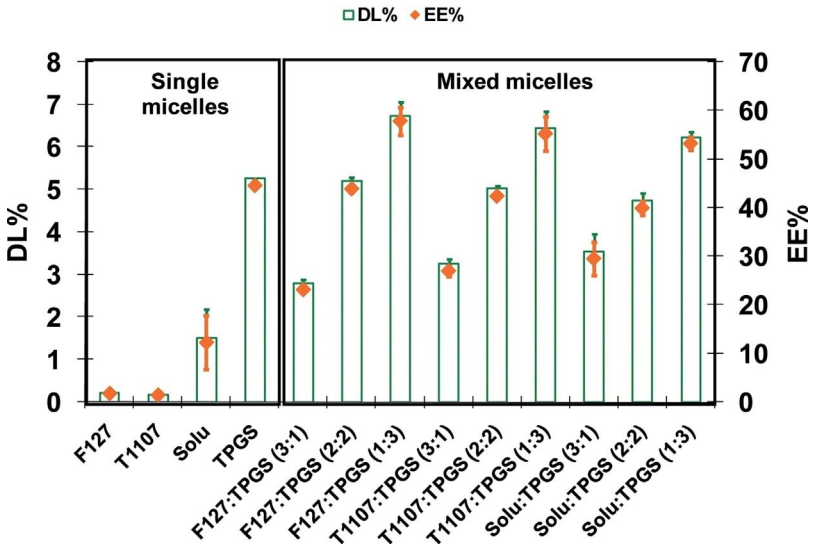

Fig. 4. DOX loading content (%) and entrapment efficacy (%) of single and mixed micelles (at different weight ratios). Results are expressed as mean ± S.D. (n = 3).

In the case of F127:TPGS mixtures and compared to unloaded micelles, we observed the apparition of a second peak by the 3:1 and 2:2 ratio. Probably, this second size population represents large micelle clusters. The increase of TPGS in these mixtures decreases the size of the second peak from ~495 to ~380 nm, and the intensity from 43.5 to 23.6%, until the total disappearance by the 1:3 ratio. In this last case, we only observed a single peak, where the D_h value remained almost unchanged versus their DOX-free counterparts (Table 2, Figs. 1 and 2). These results evidenced that the presence of DOX/NaDC complex affected the micellization for those mixtures with higher amount of poloxamer (3:1 and 2:2 ratio) in a greater extent. Nevertheless, for T1107:TPGS systems, the bimodal size distribution was only observed for the 3:1 ratio (11.2 and 448.3 nm), where the highest peak probably corresponded to larger micelle clusters. By contrast, 2:2 and 1:3 ratio presented a single peak, where the value of D_h decreased slightly in comparison to the unloaded mixed micelles (Table 2, Figs. 1 and 2). These results demonstrated that the increment of TPGS in the mixtures improves the self aggregation tendency of the mixture.

Surprisingly, Soluplus®:TPGS systems showed an increase in the size from 96.8 to 240.7 nm, when increasing the concentration of TPGS in the mixtures. However, unlike the other PEO-PPO copolymers:TPGS mixtures, Soluplus®:TPGS systems presented only one size population for every assayed ratio. The values of D_h increased in comparison to unloaded micelles (Table 2, Figs. 1 and 2). This effect could be related to the negatively effect of TPGS on Soluplus® self-aggregation.

Finally, zeta potential values were neutral for every drug-loaded micellar system assayed in a similar manner to the drug-free colloidal dispersions (Table 2).

3.6. In vitro drug release

The DOX *in vitro* release studies from the micellar reservoirs were performed at pH 7.4 (physiological pH) and 5.5 (similar to the acidic tumour microenvironment pH) [10], in comparison to Doxil® and a 2 mg/mL solution of DOX. For the 1:3 mixed micelles, results demonstrated that the studied systems exhibited a similar release pattern, as they presented an initial burst release of DOX the first 8 h, followed by a steady increase until the end of the study, regardless the pH value (Fig. 5). Besides, it was observed that at a more acidic pH (5.5), the mixed micelles released about 40% of DOX, while at pH 7.4 the mixed micellar formulations released about 24–28% of the drug after 96 h. Furthermore, the 2 mg/mL solution of DOX released almost 80% of the drug at 96 h at pH 5.5, compared to 67.5% of the drug released at the same time (pH 7.4), probably due to higher partitioning of DOX at acidic pH [10]. On the other hand, Doxil® only released ~2.5% of the drug at pH 7.4 and ~3.0% at pH 5.5 after 96 h (Fig. 5).

Further, we also assayed the DOX *in vitro* release from all the mixed systems with a copolymer ratio of 2:2 (Data not shown). However, we found out that DOX was released from these micellar systems in a greater extent than from the 1:3 systems at pH 7.4. For example, the DOX released from the 2:2 systems and the 1:3 systems of T1107:TPGS was 30% and 22%, respectively at 48 h. A similar behaviour was observed for the F127:TPGS mixed systems. In this case, the DOX released at 48 h was 28% and 25% for the 2:2 and the 1:3 ratios, respectively.

In the case of DOX, it is important that the amount of drug released at pH 7.4 could be the lowest possible to avoid or minimize DOX side effects. Taking into account this, T1107:TPGS 1:3 system exhibits different benefits, as: i) the lowest DOX release at physiological pH (24.3% at 96 h) and ii) the highest difference of released DOX amount between both pH values, which could improve drug intracellular delivery in cancer cells. Particularly, after 8 h, the amount of released DOX was 18.4 and 26.0% at pH 7.4 and 5.5, respectively. In contrast, the amount of released drug was 19.4 (pH 7.4) and 25.5% (pH 5.5) for F127:TPGS and 18.4 (pH 7.4) and 22.0% (pH 5.5) for Soluplus®:TPGS mixed micelles. These benefits could be associated to the pH-dependent nature of T1107, unlike F127, TPGS and Soluplus®.

Taking into account the *in vitro* release results and the physicochemical characterization of the mixed micelles, we have chosen the T1107:TPGS (1:3) system for the *in vitro* citotoxicity and cellular uptake assays.

3.7. In vitro anticancer activity

To compare the *in vitro* anticancer performance of the T1107:TPGS (1:3) mixed micelles *versus* free DOX and Doxil®, SKOV-3 and MDA-MB-231 cells were exposed to different DOX concentrations encapsulated within the nanocarrier or Doxil® and drug solution. These tumour cells were selected, considering the clinical application of DOX in cancer therapy. Further, free-loaded mixed micelles were evaluated for comparison.

Promising results were observed for MDA-MB-231 cells, since there was a significant ($p < 0.05$) decrement of the IC_{50} values for the DOX-loaded mixed micelles (0.11 μg/mL) in comparison with Doxil® (0.76 μg/mL) (Table 3, Fig. 6). These results remain of clinical relevance, due to the lack of expression of estrogen, progesterone and human epidermal growth factor type 2 (HER 2) receptors in MDA-MB-231 cancer cells, also known as the TNBC cell line. In contrast to estrogen receptor expressing tumours, TNBC does not respond to

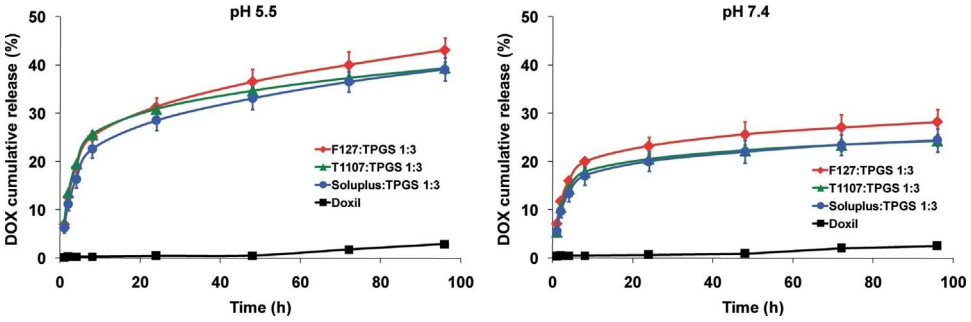

Fig. 5. *In vitro* drug release profiles of DOX-loaded mixed micelles dispersions (1:3, weight ratio) and Doxil® at 37 °C over 96 h (n = 3 ± S.D.).

Table 3
IC_{50} (mean ± S.D.) values in SKOV-3 and MDA-MB-231 after 72 h treatment by Doxil®, DOX solution, free-loaded mixed micelles and DOX-loaded mixed micelles.

IC_{50} (μg/mL)				
Cell line	Doxil®	DOX solution	DOX-loaded T1107:TPGS mixed micelles	DOX free-T1107:TPGS mixed micelles
SKOV-3	0.41 (0.09)	0.58 (0.09)	0.17 (0.03)[a,b,c]	8.74 (1.02)[a,b]
MDA-MB-231	0.76 (0.09)	0.16 (0.04)	0.11 (0.02)[a,c]	15.70 (1.83)[a,b]

Note: Multiple comparisons were performed using one-way ANOVA (n = 3 experiments).
[a] Significant difference compared to Doxil® ($p < 0.05$).
[b] Significant difference compared to DOX solution ($p < 0.05$).
[c] Significant difference compared to DOX-free T1107:TPGS mixed micelles ($p < 0.05$).

hormonal or HER 2 targeting therapies, thus the conventional treatment is chemotherapy [19]. In general, TNBC has a less favourable prognosis than hormone receptor expressing breast cancers [41]. Hence, the enhancement of the *in vitro* anticancer performance of our novel mixed micelles *versus* the commercially available DOX formulation represents a potential alternative for cancer chemotherapy.

Interestingly, the DOX control solution demonstrated a better *in vitro* anticancer performance than the PEGylated commercially available liposomes (Table 3). A similar behaviour was previously reported by Kibria et al. [42].

On the other hand, there were also promising results with the ovarian cancer cell line. In this case, the IC_{50} of the DOX-loaded mixed micellar system resulted lower than that of free DOX and Doxil®, being significantly different in both cases, $p < 0.05$) (Table 3, Fig. 6).

Interestingly, drug-free mixed micelles also exhibited cytotoxic effect (regardless the cell line), which could be attributed to the TPGS presence (Table 3), since this biomaterial presents selective anticancer activity as a pro-apoptotic agent against cancer cells [9,19,43].

Finally, it is worth stressing that the viability for the NaDC control (10 μg/mL, without the presence of DOX and copolymer) was > 90% and > 93% in SKOV-3 and MDA-MB-231, respectively. These results demonstrated that NaDC did not present *in vitro* anticancer activity in any of the cell lines.

Overall, results showed that our DOX-loaded nanotechnological platform could clearly improve the *in vitro* cytotoxicity against breast and ovarian cancer cell lines, when compared to free DOX and Doxil®. To the best of our knowledge, this is the first study in which a DOX-loaded mixed micellar formulation is compared and improves the *in vitro* cytotoxic effect of Doxil® in ovarian and breast human cancer cell lines. The enhanced cytotoxic activity of the mixed micellar formulation might be attributed to the combined effect of both, the encapsulated drug and the selective anti-cancer effect of TPGS.

3.8. In vitro cellular uptake

Since an antineoplastic drug must be accumulated inside tumour cells to get an optimal cytotoxic effect, we assayed the *in vitro* cellular uptake of DOX from a drug solution, Doxil® and the mixed T1107:TPGS (1:3) micelles in MDA-MB-231 and SKOV-3 cancer cell lines over 6 h.

There was a significant ($p < 0.05$) increment of the DOX intracellular levels for the mixed micelles in comparison with Doxil® and the drug control solution at 2, 4 and 6 h of incubation with SKOV-3 (Fig. 7). These results are in good agreement with the *in vitro* cytotoxicity data for this cell line, as the mixed micellar system exhibited the lowest IC_{50}, when compared to the rest of the assayed formulations (Table 3). Interestingly, with the increase of the incubation time, it can be noticed that the intracellular levels of DOX-loaded T1107:TPGS mixed micelles increased sharply, while this behaviour was not observed in the case of free DOX and Doxil®. Such results may suggest that the presence of TPGS could improve the intracellular levels of the encapsulated drug, as previously reported [19,44,45]. Probably, the better DOX uptake results observed for the micellar nanocarrier *versus* Doxil® could be related to the presence of TPGS in our micellar system that enhanced DOX accumulation inside the tumour cells, contrary to the PEGylated-surface of the DOX-loaded commercially available liposomes. Similar results were found for poly(lactic-co-glycolic acid) nanoparticles surface decorated with either, TPGS or poly(vinyl)alcohol and poly(styrene) nanoparticles without an hydrophilic coating in Caco-2 cells [46].

A different behaviour was observed for MDA-MB-231 cells. In this

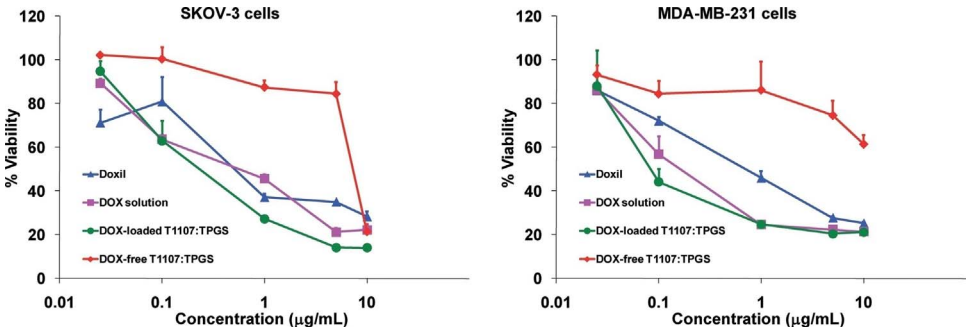

Fig. 6. Cell viability of SKOV-3 and MDA-MB-231 cells after 72 h of treatment with blank mixed micelles T1107:TPGS (1:3, weight ratio), DOX-loaded T1107:TPGS (1:3, weight ratio), DOX solution and Doxil®. Results are expressed as mean ± S.D. (n = 3).

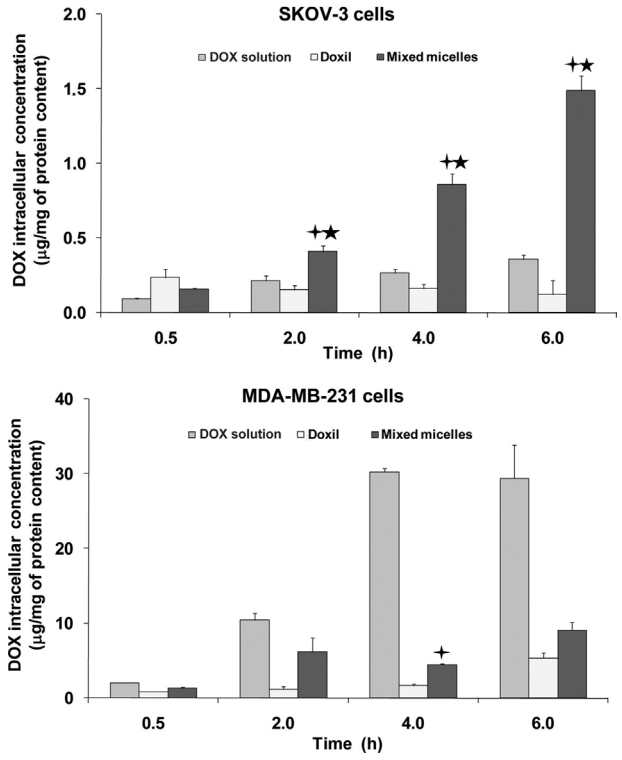

Fig. 7. Time-dependent intracellular/cell DOX levels in SKOV-3 and MDA-MB-231 cell lines for drug-loaded T1107:TPGS (1:3, weight ratio) mixed micelles in comparison with DOX solution and Doxil®. DOX amount was normalized by protein concentrations of the cell lysates. Results are expressed as mean ± S.D. (n = 3).
★ The intracellular/cell DOX levels are significantly ($p < 0.05$) higher for mixed micelles *versus* the drug solution.
✦ The intracellular/cell DOX levels are significantly ($p < 0.05$) higher for mixed micelles *versus* Doxil®.

case, there was a gradual increase on the intracellular/cell DOX content over time for both, the mixed micelles and Doxil®. However, a significant ($p < 0.05$) increment of the drug intracellular concentration for the mixed nanocarrier (4.47 ± 0.14 μg DOX/mg of protein) *versus* Doxil® (1.65 ± 0.23 μg DOX/mg of protein) was only observed after 4 h of incubation (Fig. 7). Being these results in good correlation with the *in vitro* anticancer performance, where the decrement of the IC_{50} values observed for the mixed system were significant ($p < 0.05$) in comparison with Doxil® (Table 3).

On the other hand, the highest intracellular/cell DOX levels were achieved with the drug control solution, where it was observed a clear increase of the intracellular DOX levels over time (Fig. 7). These results could be related with the cell response (motility increment) to the antineoplastic drug over the first 6 h of exposure. However, this clear increment of the drug intracellular/cell levels of DOX did not lead to higher *in vitro* anticancer performance in comparison with the nanocarrier (Table 3). Similar results with DOX were observed by Shin et al. for MDA-MB-231 cells in comparison with MCF-7 cells.

In this context, data confirms that our novel DOX-loaded mixed micellar system could be efficiently *in vitro* internalized by two different cancer cell lines to promote an efficient cytotoxic effect.

4. Conclusions

In the present investigation we successfully developed a novel DOX-loaded mixed micellar nanocarrier based on two commercially available biomaterials: T1107 and TPGS. This micellar system demonstrated excellent colloidal properties and high DOX cargo. Moreover, our micellar nanoformulation exhibited a better *in vitro* anticancer performance than Doxil® in two cancer cell lines (SKOV-3 and MDA-MB-231). Further, the *in vitro* cellular uptake of DOX was enhanced with the mixed micelles *versus* the DOX commercially available liposome-formulation. Then, our micellar system represents a feasible nanotechnological platform to improve ovarian and breast cancer chemotherapy.

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References

[1] P. Mohan, N. Rapoport, Doxorubicin as a molecular nanotheranostic agent: effect of doxorubicin encapsulation in micelles or nanoemulsions on the ultrasound-mediated intracellular delivery and nuclear trafficking, Mol. Pharm. 7 (2010) 1959–1973.
[2] G. Damodar, T. Smitha, S. Gopinath, S. Vijayakumar, Y. Rao, An evaluation of hepatotoxicity in breast cancer patients receiving injection Doxorubicin, Ann. Med. Health Sci. Res. 4 (2014) 74–79.
[3] M. Cagel, E. Grotz, E. Bernabeu, M.A. Moretton, D.A. Chiappetta, Doxorubicin: nanotechnological overviews from bench to bedside, Drug Discov. Today 22 (2017) 270–281.
[4] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, et al., Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes, Cancer Res. 54 (1994) 987–992.
[5] M.E. O'Brien, N. Wigler, M. Inbar, R. Rosso, E. Grischke, A. Santoro, et al., Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX/Doxil) versus conventional doxorubicin for first-line treatment of metastatic breast cancer, Ann. Oncol. 15 (2004) 440–449.
[6] S.T. Duggan, G.M. Keating, Pegylated liposomal doxorubicin: a review of its use in metastatic breast cancer ovarian cancer, multiple myeloma and AIDS-related Kaposi's sarcoma, Drugs 71 (2011) 2531–2558.
[7] H. Zhao, Q. Li, Z. Hong, Paclitaxel-Loaded mixed micelles enhance ovarian cancer therapy through extracellular pH-triggered PEG detachment and endosomal escape, Mol. Pharm. 13 (2016) 2411–2422.
[8] Z. Zhang, S. Tan, S.S. Feng, E. Vitamin, TPGS as a molecular biomaterial for drug delivery, Biomaterials 33 (2012) 4889–4906.
[9] C.M. Neophytou, C. Constantinou, P. Papageorgis, A.I. Constantinou, D-alpha-tocopheryl polyethylene glycol succinate (TPGS) induces cell cycle arrest and apoptosis selectively in survivin-overexpressing breast cancer cells, Biochem. Pharmacol. 89 (2014) 31–42.
[10] A.M. Butt, M.C.I.M. Amin, H. Katas, N. Sarisuta, W. Witoonsaridsilp, R. Benjakul, In vitro characterization of Pluronic F127 and d-α-tocopheryl polyethylene glycol 1000 succinate mixed micelles as nanocarriers for targeted anticancer-drug delivery, J. Nanomater. 2012 (2012) 916573.
[11] F. Danhier, T.T. Kouhé, N. Duhem, B. Ucakar, A. Staub, N. Draoui, et al., Vitamin E-based micelles enhance the anticancer activity of doxorubicin, Int. J. Pharm. 476 (2014) 9–15.
[12] A.M. Butt, M.C. Mohd Amin, H. Katas, Synergistic effect of pH-responsive folate-functionalized poloxamer 407-TPGS-mixed micelles on targeted delivery of anticancer drugs, Int. J. Nanomed. 10 (2015) 1321–1334.
[13] J. Gong, M. Chen, Y. Zheng, S. Wang, Y. Wang, Polymeric micelles drug delivery system in oncology, J. Control. Release 159 (2012) 312–323.
[14] K. Kataoka, A. Harada, Y. Nagasaki, Block copolymer micelles for drug delivery: design: characterization and biological significance, Adv. Drug Deliv. Rev. 47 (2001) 113–131.
[15] A.B.E. Attia, Z.Y. Ong, J.L. Hedrick, P.P. Lee, P.L.R. Ee, P.T. Hammond, et al., Mixed micelles self-assembled from block copolymers for drug delivery, Curr. Opin. Colloid Interface Sci. 16 (2011) 182–194.
[16] M. Cagel, F.C. Tesan, E. Bernabeu, M.J. Salgueiro, M.B. Zubillaga, M.A. Moretton, et al., Polymeric mixed micelles as nanomedicines: achievements and perspectives, Eur. J. Pharm. Biopharm. 113 (2017) 211–228.
[17] D.A. Chiappetta, A. Sosnik, Poly(ethylene oxide)-poly(propylene oxide) block copolymer micelles as drug delivery agents: improved hydrosolubility, stability and bioavailability of drugs, Eur. J. Pharm. Biopharm. 66 (2007) 303–317.
[18] D.A. Chiappetta, J. Degrossi, S. Teves, M. D'Aquino, C. Bregni, A. Sosnik, Triclosan-loaded poloxamine micelles for enhanced topical antibacterial activity against biofilm, Eur. J. Pharm. Biopharm. 69 (2008) 535–545.
[19] E. Bernabeu, L. Gonzalez, M. Cagel, E.P. Gergic, M.A. Moretton, D.A. Chiappetta, Novel Soluplus(®)-TPGS mixed micelles for encapsulation of paclitaxel with enhanced in vitro cytotoxicity on breast and ovarian cancer cell lines, Colloids Surf. B Biointerfaces 140 (2016) 403–411.
[20] L. Dian, E. Yu, X. Chen, X. Wen, Z. Zhang, L. Qin, et al., Enhancing oral bioavailability of quercetin using novel soluplus polymeric micelles, Nanoscale Res. Lett. 9 (2014) 684.
[21] J. Bhattacharjee, G. Verma, V.K. Aswal, A.A. Date, M.S. Nagarsenker, P.A. Hassan, Tween 80-sodium deoxycholate mixed micelles: structural characterization and

application in doxorubicin delivery, J. Phys. Chem. B 114 (2010) 16414–16421.
[22] J. Bhattacharjee, G. Verma, V.K. Aswal, P.A. Hassan, Small angle neutron scattering study of doxorubicin-surfactant complexes encapsulated in block copolymer micelles, Pramana 71 (2008) 991–995.
[23] D. Nandni, K.K. Vohra, R.K. Mahajan, Study of micellar and phase separation behavior of mixed systems of triblock polymers, J. Colloid Interface Sci. 338 (2009) 420–427.
[24] H. Yu, D. Xia, Q. Zhu, C. Zhu, D. Chen, Y. Gan, Supersaturated polymeric micelles for oral cyclosporine A delivery, Eur. J. Pharm. Biopharm. 85 (2013) 1325–1336.
[25] M. Wegmann, L. Parola, F.M. Bertera, C.A. Taira, M. Cagel, F. Buontempo, et al., Novel carvedilol paediatric nanomicelle formulation: in-vitro characterization and in-vivo evaluation, J. Pharm. Pharmacol. 69 (2017) 544–553.
[26] Ö. Topel, B. Acar Çakır, L. Budama, N. Hoda, Determination of critical micelle concentration of polybutadiene-block-poly(ethyleneoxide) diblock copolymer by fluorescence spectroscopy and dynamic light scattering, J. Mol. Liq. 177 (2013) 40–43.
[27] D.A. Chiappetta, G. Facorro, E.R. de Celis, A. Sosnik, Synergistic encapsulation of the anti-HIV agent efavirenz within mixed poloxamine/poloxamer polymeric micelles, Nanomedicine 7 (2011) 624–637.
[28] M.A. Moretton, C. Taira, S. Flor, E. Bernabeu, S. Lucangioli, C. Höcht, et al., Novel nelfinavir mesylate loaded d-α-tocopheryl polyethyleneglycol 1000 succinate micelles for enhanced pediatric anti HIV therapy: in vitro characterization and in vivo evaluation, Colloids Surf. B Biointerfaces 123 (2014) 302–310.
[29] M.A. Moretton, E. Bernabeu, E. Grotz, L. Gonzalez, M. Zubillaga, D.A. Chiappetta, A glucose-targeted mixed micellar formulation outperforms Genexol in breast cancer cells, Eur. J. Pharm. Biopharm. 114 (2017) 305–316.
[30] T. Mingkwan, J. Suksiriworapong, D. Chantasart, Pluronic® P123/TPGS and pluronic® F127/TPGS mixed micelles for the entrapment of itraconazole, Chiang Mai J. Sci. 42 (2015) 946–956.
[31] Y. Gao, L.B. Li, G. Zhai, Preparation and characterization of Pluronic/TPGS mixed micelles for solubilization of camptothecin, Colloids Surf. B Biointerfaces 64 (2008) 194–199.
[32] J.H. Clint, Micellization of mixed nonionic surface active agents, J. Chem. Soc. Faraday Trans. 1 (1975) 1327–1334.
[33] A. Ribeiro, A. Sosnik, D.A. Chiappetta, F. Veiga, A. Concheiro, C. Alvarez-Lorenzo, Single and mixed poloxamine micelles as nanocarriers forsolubilization and sustained release of ethoxzolamide for topical glaucomatherapy, J. R. Soc. Interface 9 (2012) 2059–2069.
[34] R. Cardoso da Silva, W. Loh, Effect of additives on the cloud points of aqueous solutions of ethylene oxide-propylene oxide-ethylene oxide block copolymers, J. Colloid Interface Sci. 202 (1998) 385–390.
[35] A. Ribeiro, I. Sandez-Macho, M. Casas, S. Alvarez-Pérez, C. Alvarez-Lorenzo, A. Concheiro, Poloxamine micellar solubilization of α-tocopherol for topical ocular treatment, Colloids Surf. B Biointerfaces 103 (2013) 550–557.
[36] J. Hou, E. Sun, C. Sun, J. Wang, L. Yang, X.B. Jia, et al., Improved oral bioavailability and anticancer efficacy on breast cancer of paclitaxel via Novel Soluplus (®)-Solutol(®) HS15 binary mixed micelles system, Int. J. Pharm. 512 (2016) 186–193.
[37] M.L. Forrest, C.Y. Won, A.W. Malick, G.S. Kwon, In vitro release of the mTOR inhibitor rapamycin from poly(ethylene glycol)-b-poly(epsilon-caprolactone) micelles, J. Control. Release 110 (2006) 370–377.
[38] X. Jin, B. Zhou, L. Xue, W. San, Soluplus(®) micelles as a potential drug delivery system for reversal of resistant tumor, Biomed. Pharmacother. 69 (2015) 388–395.
[39] C. Alvarez, D.H. Shin, G.S. Kwon, Reformulation of fungizone by PEG-DSPE micelles: deaggregation and detoxification of amphotericin B, Pharm. Res. 33 (2016) 2098–2106.
[40] M.A. Moretton, L. Cohen, L. Lepera, E. Bernabeu, C. Taira, C. Höcht, et al., Enhanced oral bioavailability of nevirapine within micellar nanocarriers compared with Viramune(®), Colloids Surf. B Biointerfaces 122 (2014) 56–65.
[41] K.J. Chavez, S.V. Garimella, S. Lipkowitz, Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer, Breast Dis. 32 (2010) 35–48.
[42] G. Kibria, H. Hatakeyama, Y. Sato, H. Harashima, Anti-tumor effect via passive anti-angiogenesis of PEGylated liposomes encapsulating doxorubicin in drug resistant tumors, Int. J. Pharm. 509 (2016) 178–187.
[43] H. Zhao, L.Y. Yung, Addition of TPGS to folate-conjugated polymer micelles for selective tumor targeting, J. Biomed. Mater. Res. A 91 (2009) 505–518.
[44] M.S. Muthu, S.A. Kulkarni, J. Xiong, S.S. Feng, Vitamin E TPGS coated liposomes enhanced cellular uptake and cytotoxicity of docetaxel in brain cancer cells, Int. J. Pharm. 421 (2011) 332–340.
[45] C. Shi, Z. Zhang, F. Wang, X. Ji, Z. Zhao, Y. Luan, Docetaxel-loaded PEO-PPO-PCL/TPGS mixed micelles for overcoming multidrug resistance and enhancing antitumor efficacy, J. Mater. Chem. B Mater. Biol. Med. 3 (2015) 4259–4271.
[46] K.Y. Win, S.S. Feng, Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs, Biomaterials 26 (2005) 2713–2722.