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Novel Soluplus®—TPGS mixed micelles for encapsulation of paclitaxel with enhanced *in vitro* cytotoxicity on breast and ovarian cancer cell lines

Ezequiel Bernabeu[a,c], Lorena Gonzalez[b], Maximiliano Cagel[a,c], Esteban P. Gergic[a], Marcela A. Moretton[a,c], Diego A. Chiappetta[a,c,*]

[a] Departamento de Tecnología Farmacéutica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina
[b] Departamento de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina
[c] National Science Research Council (CONICET), Buenos Aires, Argentina

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ABSTRACT

The aim of this work was to develop mixed micelles based on two biocompatible copolymers of polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol (Soluplus®) and D-α-tocopheryl polyethylene-glycol 1000 succinate (TPGS), to improve the aqueous solubility and the *in vitro* anti-tumor activity of paclitaxel (PTX). Pure and mixed nanomicelles were prepared by solvent evaporation method and characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Solubility of PTX was increased 60,000 and 38,000 times, when it was formulated in pure Soluplus® micelles and in mixed micelles (Soluplus®:TPGS; 4:1 ratio), respectively. The *in vitro* PTX release profile from micellar systems was characterized employing the dialysis membrane method where all drug-loaded formulations showed a sustained and slow release of PTX. *In vitro* assays were conducted on human cancer cell lines including ovarian cancer cells SKOV-3, breast cancer cells MCF-7 and triple negative breast cancer cells MDA-MB-231. Cytotoxicity studies showed that mixed micelles exhibited better antitumor activity compared to PTX solution against the three cell lines. Furthermore mixed micelles showed a significant increase on PTX cellular uptake in comparison with pure Soluplus® micelles and free drug in all cell lines assayed. More important, blank mixed micelles have shown cytotoxic activity due to the ability of TPGS to induce apoptosis in cancer cells. This effect was associated with the expression levels of cleaved-PARP, an apoptosis-related protein. On the basis of these results, the mixed micelles developed in this study might be a potential nano-drug delivery system for cancer chemotherapy.

1. Introduction

Cancer remains a leading cause of death worldwide according to the World Health Organization (WHO). Approximately 70% of deaths occur in low- and middle-income countries [1]. By 2050, it is predicted that the number of patients with cancer will double [2]. Thus, this disease is one of major public health problems, which need to be dealt urgently. In this context, cancer therapy requires a careful selection of one or more interventions, such as surgery, radiotherapy and chemotherapy [3]. This last is of vital importance to obtain the cure or considerably prolong life while improving the patient's quality of life [4]. Given that the discovery of new drugs is a long and expensive pathway, one of the most significant approaches to enhance cancer treatments is currently based on the development of new delivery device.

Paclitaxel (PTX) is one of the most important chemotherapeutic drugs used to treat many types of cancer as breast and ovarian cancer [5]. It is a hydrophobic drug with poor solubility in water (0.3–0.5 μg/mL) [6,7]; for this reason, in order to increase the solubility of PTX, it was formulated in a mixture of Cremophor EL® and dehydrated alcohol (1:1, v/v); registered under the name of Taxol® [5]. Unfortunately, this vehicle is associated with a variety of side effects, such as hypersensitivity, nephrotoxicity and neurotoxicity, attributable mainly to Cremophor EL® [8]. With the goal of improving the solubility and avoiding the use of Cremophor EL®, various PTX delivery systems based on nanotechnology have been studied in the last years, including liposomes [9,10], solid lipid nanopar-

* Corresponding author at: Departamento de Tecnología Farmacéutica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 956 Junín St., 6th Floor, Buenos Aires CP1113, Argentina. Fax: +54 11 4964 8371.
E-mail address: diegochiappetta@yahoo.com.ar (D.A. Chiappetta).

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ticles [11,12], polymeric nanoparticles [3,13], polymeric micelles [14,15] and drug conjugates [16,17]. Few years ago, the Food and Drug Administration (FDA) has approved the first Cremophor® free formulation of PTX for recurrent metastatic breast cancer based in nanotechnology [18]. In this nanoformulation, named Abraxane®, PTX is formulated within albumin particles to retain the pharmacotherapeutic benefits of PTX and eliminate the adverse effects associated with Cremophor® [19]. However, it has been demonstrated that Abraxane® shows a rapid elimination of PTX from the blood circulation and does not improve the pharmacokinetics of PTX from Taxol® [19]. Recently, micelles of monomethoxy-PEG-*b*-poly(D,L-lactide) (mPEG-PDLLA) incorporating PTX in their hydrophobic core (Genexol-PM) have been clinically approved in Bulgaria, Hungary and South Korea and are being evaluated in Phase II trials in the US [2]. This formulation showed a similar toxicity profile than Taxol® against different human cancer cells, including breast, colon, ovarian and non-small cell lung cancer cells [20]. Besides, Genexol-PM presented a similar area under the plasma concentration (AUC) curve than that of Taxol® in a murine melanoma mice model [21]. This formulation was assayed in a phase II trial to explore the efficacy and safety of Genexol in patients with metastatic breast cancer. Genexol-PM was effective and well tolerated with an overall response rate of 59% and 9-month median time to progression [22]. These innovative strategies have replaced Cremophor in their formulations, making the intravenous administration of PTX easier and safer. Nonetheless, there are still some main problems that affect the outcome of drug chemotherapy, as the lack of response due to drug resistant. Then, acquired resistance to PTX has become a serious clinical issue in a high percentage of patients and remains the major obstacle that needs to be urgently coped.

Among the emerging nanoscopic carrier systems, polymeric micelles (PM) are one of the most promising and well-investigated approaches that have improved the solubility and stability of hydrophobic drugs [23]. These carriers are nanoscopic structures formed by amphiphilic block copolymers which are self-aggregate in aqueous media, exposing their hydrophilic head outside and hiding their hydrophobic segments in the interior core region [24]. Consequently, this structure favors the solubilisation of hydrophobic drugs within the micelle core. Additionally, given the composition of the copolymers, these systems can reduce the opsonization and nonspecific uptake by reticuloendothelial system (RES), and favor the tumor targeting by the enhanced permeability and retention (EPR) effect [25].

Recently, a large number of studies on mixed polymeric micelles have appeared due to the possibility of combining prominent advantages of different types of single polymeric micelles [26–28]. Parameters as solubility and stability of drug in mixed micelles can be greatly improved with different kinds of copolymers, as compared with single micelles [29,30].

A new polymer with amphiphilic properties, which shows excellent solubilising properties for poorly water-soluble drug substances, is the polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer (Soluplus®) [31–33]. This polymer is capable of forming micelle structures in solution with a low critical micellar concentration (CMC) value (0.76×10^{-3}% w/v) [34], providing a high stability to dilution. Recently, few research groups have used Soluplus® to successfully enhance the solubility of some drugs [32,34,35]. On the other side, D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) is a PEGylated-vitamin E, which has been used as solubilizer and absorption enhancer in some drug delivery formulations [36]. Also, it has been shown that TPGS can inhibit the *P*-glycoprotein (*P*-gp), known as an efflux pump which mediates multidrug resistance in tumor cells [37]. Moreover, it has been reported that TPGS exhibited *in vitro* and *in vivo* cytotoxic activity on different cancer cell lines by promoting cellular apoptosis [38].

The objective of the present study was to develop a novel mixed polymeric micellar formulation comprised of Soluplus® and TPGS, for increasing the aqueous solubility and enhancing *in vitro* antitumor efficacy of PTX. The physicochemical characteristics of PTX-loaded mixed micelles, such as particle size, morphology and *in vitro* release profile were investigated. In comparison to Taxol® and single Soluplus® micelles, *in vitro* cytotoxicity and cell uptake of PTX loaded mixed micelles were evaluated.

2. Experimental

2.1. Materials

Paclitaxel (PTX) of purity 99.9% was purchased from Rhenochem AG (Basel, Switzerland), D-α-tocopheryl polyethylene-glycol (PEG) 1000 succinate (TPGS, MW ~1513 g/mol) was from Eastman Chemical Company (Kingsport, TN, USA) and Soluplus® was from BASF (Ludwigshafen, Germany). Tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) and phenazine methosulfate (PMS) were purchased from Promega Corporation (Madison, Wisconsin, USA). All solvents such as acetone and acetonitrile were of analytical or high performance liquid chromatography (HPLC) grade and were used following the manufacturer's instructions.

2.2. Preparation of pristine and mixed micelles

Mixed Soluplus®:TPGS micelles (5% w/v total polymer concentration) were prepared by dissolving the required amount of each polymer in water distilled at room temperature and equilibrating the system at 25 °C, at least 24 h before use. Binary systems with polymer weight ratios of 4:1, 3:2, 2:3 and 1:4 were included in this study. Single Soluplus® and TPGS micelles (5% w/v) were used as controls.

2.3. Critical micellar concentration determination

The critical micellar concentration (CMC) of each single and mixed system, at 25 °C ± 0.5 °C was determined by means of surface tension using the du Nöuy ring method (Fernández Berlusconi y Rocca SRL, Argentina).

2.4. Cloud point

Cloud point (CP) measurements were performed by submerging a sealed glass tube containing each 5% micellar system (pristine and mixed) in a paraffin oil bath. The temperature was increased gradually from room temperature (1 °C/min) until the point of abrupt change in the visual appearance of the micellar system from clear to turbid [39]. Once the temperature exceeded the CP, the system was cooled down and the whole process was repeated to check the reproducibility of the measurement.

2.5. Morphological characterization

To evaluate the morphology of single and mixed systems, we prepared Soluplus® 5% p/v, TPGS 5% p/v and Soluplus®:TPGS (3:2) 5% p/v, and then, they were studied by means of transmission electron microscopy (Philips CM-12 TEM apparatus, FEI Company, Eindhoven, The Netherlands). Samples (5 μL) were placed onto a grid covered with Fomvar film. After 30 s, the excess was carefully removed with filter paper and phosphotungstic acid (2% w/v) was added. After 30 s, the excess was removed and 5 μL of water was

added, left for 30 s and removed. Finally, samples were dried in a closed container with silica gel and analyzed.

2.6. Preparation of PTX-loaded pristine and mixed micelles

PTX (30 mg/mL) was solubilized in acetone (3 mL) and added drop wise to aqueous micellar systems (10 mL) under magnetic stirring (10 h) at 25 °C. The resulting suspensions were filtered (0.45 μm, cellulose nitrate) to remove insoluble PTX. The drug concentration was determined by high performance liquid chromatography (HPLC) using an analytical method previously validated [40]. The HPLC instrument consisted of a pump (Shimadzu SCL-10A, Japan), an autosampler (Shimadzu SIL-10A, Japan), a reversed phase C18 column (4.6 mm × 250 mm, 5 μm, Fluophase PFP, Thermo, USA) and an UV-detector (Shimadzu SPD-10A, Japan) set at 227 nm. Mobile phase was a mixture of acetonitrile:water (50:50, v/v), filtered through 0.45 μm membrane filter and eluted at a flow rate of 1.0 mL/min. PTX concentrations were determined using 20 μL of injection volume at room temperature.

Solubility factors (f_s) were calculated according to the equation. $f_s = \frac{S_a}{S_{water}}$ where S_a and S_{water} are the apparent solubility of PTX in each micellar system and the intrinsic solubility of the drug in a polymer-free distilled water (0.19 μg/mL), respectively.

2.7. Characterization of PTX-loaded pristine and mixed micelles

The average hydrodynamic diameter (D_h) and the size distribution of PTX-loaded micelles were determined in pristine (Soluplus® and TPGS) and a mixed system (Soluplus®:TPGS, 4:1) by dynamic light scattering (DLS; Zetasizer Nano-Zs, Malvern Instruments, UK) provided with a He–Ne (633 nm) laser and a digital correlator ZEN3600. Measurements were conducted at a scattering angle of $\theta = 173^\circ$ to the incident beam. Samples were equilibrated at 25 °C for at least 5 min prior to the analysis. The results were expressed as the average of five measurements. Then, PTX-loaded mixed micellar system was visualized by TEM (as described above).

2.8. In vitro *PTX release*

In vitro release of PTX from pristine Soluplus® and Soluplus®:TPGS (4:1) micellar systems were performed using the dialysis method over 120 h. Micellar systems were dispersed in phosphate buffer USP 30 (pH 7.4, 2 mL) containing 0.5% v/v of polysorbate 80. The resulting dispersion was 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 and volume/length ratio of 6.4 mL/cm; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), sealed, and placed in a Falcon® conical tube (50 mL) containing the release medium (PBS, pH 7.4 containing 0.5% v/v of polysorbate 80, 48 mL). Polysorbate 80 was added to increase the intrinsic solubility of PTX in the release medium and to ensure sink conditions [41]; as already mentioned, PTX is poorly water soluble. Then, each Falcon® conical tube was placed in an orbital water bath shaking at 40 rpm at 37 °C. At every time intervals (1, 2, 4, 6, 8, 24, 48, 72, 96, and 120 h), 48 mL aliquots were withdrawn and replaced with an equal volume of fresh medium pre-heated at 37 °C. The released PTX amounts were quantified by HPLC as described above with correction for the volume replacement. Assays were carried out in triplicate and the results are expressed as mean ± S.D. The analysis of the release kinetics and the fitting for order zero model was conducted with Microsoft® Excel 2010.

2.9. In vitro *cytotoxicity*

MCF-7, MDA-MB 231 and SKOV human cancer cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were maintained in Dulbecco's minimum essential medium (DMEM®) supplemented with 10% fetal bovine serum (FBS), 50 μg/mL gentamycine (Invitrogen, Argentina) and 2 mM L-glutamine (Invitrogen, Argentina). The cells were maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO_2. For *in vitro* cytotoxicity assays, cells were seeded in clear 96-well plates (Corning Costar, Fisher Scientific, USA) at a density of 5000 cells/well and incubated 24 h to allow cell attachment. The cells were then incubated with PTX, blank single micelles, blank mixed micelles, PTX-loaded Soluplus® micelles and PTX-loaded Soluplus®:TPGS mixed micelles for 48 h. After the incubation period, 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, Piscataway, New Jersey, USA). Triplicates were run for each treatment. PTX 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%.

2.10. Western blot analysis

MCF-7, MDA-MB231 and SKOV cells were treated with blank Soluplus® micelles, blank Soluplus®:TPGS mixed micelles or TPGS at a same concentration of material as the tested PTX concentration of 25 μg/mL for 48 h. Untreated cells acted as a control for this experiment. Cells were homogenized in buffer composed of 1% v/v Triton, 0.1 M Hepes, 0.1 M sodium pyrophosphate, 0.1 M sodium fluoride, 0.01 M EDTA, 0.01 M sodium vanadate, 0.002 M PMSF, and 0.035 trypsin inhibitory units/mL aprotinin (pH 7.0) at 4 °C. Cell homogenates were centrifuged at 15,000 × *g* for 40 min at 4 °C to remove insoluble material. Protein concentration of supernatants was determined by the BCA protein assay kit. Equal protein aliquots of solubilized cells were diluted in Laemmli buffer, boiled for 5 minutes, and stored at −20 °C until electrophoresis. Samples were subjected to electrophoresis in SDS-polyacrylamide gels. Electrotransference of proteins from gel to PVDF membranes and incubation with anti-PARP and anti β-actin antibodies were performed as already described [42]. Immunoreactive proteins were revealed by enhanced chemiluminescence. Band intensities were quantified using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The expression of PARP protein was normalized to that of β-actin as a control.

2.11. In vitro *cellular uptake*

To evaluate the cellular uptake of PTX encapsulated within Soluplus® or Soluplus®:TPGS micelles, we determined PTX by HPLC assay of collected MCF-7, MDA-MB 231 and SKOV cells, which were incubated with different PTX formulations for different incubation times. Briefly, MCF-7, MDA-MB231 or SKOV cells were seeded in 6-well plates at the density of 4×10^5 cells/well and allowed to attach for 24 h at 37° in CO_2. The cells were then incubated with PTX, PTX-loaded Soluplus® micelles and PTX-loaded Soluplus®:TPGS mixed micelles at 25 μg/mL of PTX for 0.5, 2, 4 and 6 h, respectively. Untreated cells acted as a control. At predetermined time-points, the cells were rinsed with 1.5 mL ice cold PBS and then, 0.25 mL trypsin PBS solution (2.5 μg/mL) was added. The cell lysate was centrifuged at 13,000 rpm (MiniSpin® plus™, Eppendorf, Germany) for

10 min. Drug content in the supernatant after centrifugation was measured by HPLC method and obtained values were normalized by protein content in each sample determined using BCA protein assay kit (Pierce Corporation, Beijing local agent, China) according to the manufacturer's protocol. Statistical analysis of intracellular/cell PTX levels as delivered by the different nanoformulations, and the PTX solution at 6 h was performed by one-way ANOVA test and Dunnett's Multiple Comparison *post-hoc* test using GraphPad Prism version 5.02 for Windows (GraphPad Software, USA). Statistical significance was defined as $p < 0.05$. All experiments were repeated in triplicate.

3. Results and discussion

3.1. Self-aggregation of mixed micelles

In the present study, we explored and characterized for the first time, the generation of mixed Soluplus®:TPGS micelles. Aiming to study these mixed micelles, four different weight ratios were employed. TPGS has been chosen for its excellent amphiphilic properties, its capacity as potent inhibitor of P-gp and more interestingly, for its *in vitro* and *in vivo* cytotoxic activity on different cancer cell lines [38]. On the other hand, Soluplus® is a relatively new hydrophilic graft copolymer, which can easily form colloidal micelles with very good solubilization ability and stability, due to its low value of CMC [32].

With the aim of evaluating the self-aggregation capacity of single and mixed micelles, we defined the CMC of each copolymer and mixtures. The CMC of TPGS (0.1322 mM) determined by surface tension was identical to the value that we reported in a previous study using the hydrophobic probe solubilization method [24]. However, Soluplus® presents a smaller value of CMC (0.0003 mM) with a minimum variation [34].

The theoretical CMC value for a mixed surfactant system, CMC^*, can be calculated using the following equation [43]:

$$\frac{1}{CMC^*} = \frac{X_1}{CMC_1} + \frac{X_2}{CMC_2}$$

Being X_1 and X_2 the molar fractions of the components 1 and 2, and CMC_1 and CMC_2 the CMC values of components 1 and 2, respectively. The theoretical and experimental data at 25 °C is shown in Table 1. In general, the incorporation of TPGS to Soluplus® micelles resulted in a gradual increase of the theoretical and experimental CMC of the mixtures. At 25 °C, the experimental CMC was lower than the theoretical one, except for the ratio (3:2), where we observed similar values for experimental and theoretical. For Soluplus®:TPGS systems, the experimental CMC values were smaller than the theoretical ones, presenting a negative deviation from the ideal behavior. A similar tendency was observed with mixed systems formed by Pluronic® F88 and P123 [39]. A negative deviation from ideality indicates a favorable mixing process, while a positive deviation an erratic process. Unfavorable interactions lead to disadvantaged micellization and greater CMC values. The increase of TPGS in the mixtures resulted in a steady augment of the CMC^*, suggesting that the incorporation of TPGS into Soluplus® micelles has an unfavorable effect on the Soluplus® self-aggregation. This effect is probably related to the fact that TPGS could decrease the hydrophobic interactions among the polymer chains at the micellar core. Similar behavior was observed by Huang et al. with mixed micelles of mPEG-PLA and TPGS [44]. On the other hand, almost all the binary systems displayed CMC values that were lower than those of single TPGS. This is a very important aspect, because low CMC values provide some advantages, such as good stability and great resistance against dissociation and precipitation of drugs in blood due to dilution in the organism.

3.2. Cloud point

Clouding behavior is a physical change that usually occurs in homogeneous solutions of amphiphilic substances, where the solution is separated into a surfactant-rich and a surfactant-poor phase at a defined temperature, known as the cloud point (CP). This phenomenon is produced by the dehydration of hydrophilic portion of nonionic surfactants at higher temperature condition. CP is a particular characteristic of nonionic surfactants. As the temperature rises and the CP approaches, micelles attract each other and may form large aggregates, indicating the instability of the formulation. The inter-micellar interactions have a critical impact in the CP of a system. For this reason, this physicochemical characteristic of the binary systems may substantially differ from that of single micelles [43]. Table 1 summarizes the data of pure and mixed micelles. Soluplus® showed a CP of 36 °C, in good agreement with a previously report [31], whereas TPGS exhibited a higher CP (110 °C). Then, the increase of the TPGS concentration in the binary system resulted in the decrease of the CP with respect to pristine Soluplus®, except for ratio 1:4 (Table 1). In the case of binary systems, as the TPGS concentration increases, CP values decreases (ratio 4:1, 3:2 and 2:3) and then augments again (ratio 1:4). The initial decrease of CP for the binary systems (ratio 4:1, 3:2 and 2:3) is due to the presence of TPGS, which penetrates into Soluplus® micelles and expels the water molecules from the core, enhancing hydrophobic interactions. This effect is completely attenuated when the proportion of TPGS is much greater than Soluplus® in the mixed micelles (ratio 1:4). The high CP displayed by TPGS relies on the high hydrophilicity of this substance. The results were congruent with the co-micellization process. As expected, when the Soluplus® concentration in the mixed micelles increased, the system became more hydrophobic and, thus, the CP decreased.

3.3. Morphology of pristine and mixed polymeric micelles

TEM analysis was utilized to confirm the spherical morphology of single and mixed micelles, as exemplified for Soluplus®, TPGS and Soluplus®:TPGS (3:2) in Fig. S1.

3.4. Solubilization of PTX into pure and mixed polymeric micelles

The solubilization capacity of single and mixed micelles of Soluplus® and TPGS was evaluated at a copolymer concentration (5% w/v) far above the CMC of each one of them. To investigate the impact of different proportions of Soluplus® and TPGS on the PTX solubility, this parameter was determined in the mixed micelles with varied weight ratio of Soluplus® and TPGS (4:1; 3:2; 2:3 and 1:4).

As shown in Fig. 1, the micelles comprised of pure Soluplus® displayed the highest S_a, while pristine TPGS micelles presented the lowest, at the same concentration. The lower S_a for TPGS (0.30 mg/mL) could be explained by the fact that it has a smaller lipophilic portion, when compared to Soluplus®. The hydrophobic blocks of the copolymer form the core of the micelle that serves as a microenvironment for incorporation and accommodation of the lipophilic molecules. In addition, the solubility of the hydrophobic drug is enhanced by the nature of the interior of the micelles through hydrophobic-hydrophobic interactions [45]. When there is not enough affinity between the drug and the copolymer inside the core, the drug will not be well loaded into the micelle [46]. All micellar systems led to sharp increases in drug solubility at least two orders of magnitude, as compared with PTX aqueous solubility. Soluplus® solely micelles substantially improved the water solubility of PTX. S_a value for the pure Soluplus® micelles was 11.60 mg/mL, representing more than 60000-fold increase. At the moment, this solubilization improvement is the highest

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

Copolymers	Mixture composition (soluplus®:TPGS)[a]	CMC		CP (°C)
		Experimental value[b] (mM)±S.D.	Theoretical value[c] (mM)	
Soluplus®	5:0	0.0003 ± 0.0001	–	36
Soluplus®:TPGS	4:1	0.0042 ± 0.0003	0.0066	34
	3:2	0.0162 ± 0.0004	0.0159	29
	2:3	0.0160 ± 0.0012	0.0308	34
	1:4	0.0424 ± 0.0022	0.0589	53
TPGS	0:5	0.1322 ± 0.0096	–	110

[a] Soluplus® %w/v:TPGS %w/v.
[b] Experimental value determined by surface tension.
[c] Theoretical value calculated by Eq. (1).

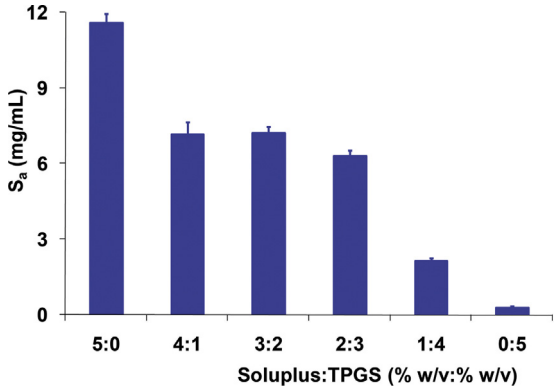

Fig. 1. Apparent solubility (S_a) of PTX in single and mixed micelles composed by Soluplus® and/or TPGS. Error bars represent S.D. ($n = 3$).

Table 2
PTX apparent solubility (S_a) and solubility factors (f_s) for micellar and mixed micellar dispersions in distilled water at 25 °C. Data represents mean ± standard deviation (S.D.) of the mean, $n = 3$.

Soluplus®:TPGS concentration (% w/v:% w/v)	Distilled water	
	S_a (mg/mL) (±S.D.)	f_s
5:0	11.60 (0.34)	61052
4:1	7.16 (0.48)	37684
3:2	7.22 (0.26)	38000
2:3	6.32 (0.20)	33263
1:4	2.15 (0.10)	11316
0:5	0.30 (0.04)	1579

ever reported for PTX by any micellar system in aqueous medium composed by FDA-approved biomaterials. The high solubility of Soluplus® can be probably a consequence of the increase in the length of the hydrophobic region, which facilitates the solubilization of PTX inside the micellar core. Another feature that may influence the PTX solubility is the size of the micelles. The increase in the micellar size usually leads to an augment in the solubilizing capacity. In this case, pure Soluplus® micelles showed a hydrodynamic diameter of ~90 nm, while pristine TPGS micelles showed a size of ~10 nm.

On the other hand, mixed micelles were prepared in four different Soluplus®:TPGS ratios (4:1, 3:2, 2:1 and 1:4) to determine their effects in PTX solubility. Considering the extremely low solubility of PTX in water, all the binary systems displayed enhancement in solubility of the drug. Soluplus®—containing mixed micelles displayed a statistically significant increase in the encapsulation capacity, in comparison to pure TPGS ($p < 0.05$). For example, Soluplus®:TPGS (3:2) and (4:1) combinations increased the S_a 38000 and 37684-times, respectively, while pristine TPGS showed the smallest increase (1579-fold) (Table 2). Also, the increase of TPGS in mixed micelles had a detrimental effect, S_a being always substantially lower than that of pure Soluplus®. This phenomenon was particularly remarkable at the highest TPGS content; *e.g.*, S_a values for 1:4 ratio was 2.15 mg/mL ($f_s = 11316$). In spite of the fact that there was an increment in the Soluplus® content, similar S_a levels for 2:3, 3:2 and 4:1 systems were observed. Thus, S_a values for mixed micelles were not further improved, when the total concentration of Soluplus® was higher than 20 mg/mL. These results could be attributed to the increased hydrophilic portion, due to the presence of a great amount of TPGS in the micellar system. Therefore, the probability of interactions between hydrophilic and hydrophobic segments augments, leading to changes on the hydrophobicity of the core and, consequently, altering their ability to encapsulate the drug. A similar effect was observed with mixed micelles prepared with Pluronic® F127 and TPGS [47]. Results suggested that the component that played the most dominant role in PTX solubilization was Soluplus®.

3.5. Characterization of PTX-loaded pristine and mixed micelles

The size and the size distribution of drug-loaded nanocarriers are essential parameters that may affect the course in which they interact with different cell types and may be critical in charting the path of these systems *in vivo*, no matter the administration route [48]. In our case, the size of our micelles must be suitable for an adequate intravenous administration. The evaluated drug-loaded formulations exhibited a narrow and unimodal size distribution with a D_h of 89.2 ± 4.6 nm (PDI: 0.216), 12.3 ± 2.1 nm (PDI: 0.160) and 119.7 ± 5.6 nm (PDI: 0.122) for pristine Soluplus®, single TPGS and mixed micelles (ratio 4:1), respectively. The unimodal size distributions in the systems are illustrated in Fig. 2, indicating the acceptable formation of micelles. Interestingly, the incorporation of TPGS in the mixed micelle produces an increase in the micellar size, despite the lower size of single TPGS micelles. Similar behavior was observed with micelles of Pluronic® F127 and TPGS [47]. DLS determines the D_h, which includes the associated solvent molecules in the corona. Consequently, if there are any changes in this outer

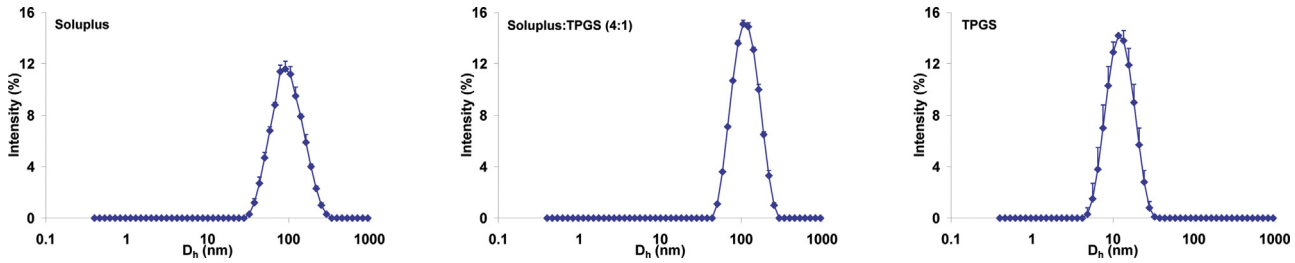

Fig. 2. Size distribution of PTX-loaded micelles of Soluplus®, Soluplus®:TPGS (4:1) and TPGS.

region, the measured diameter will be affected. In our study, this increase might be attributed to the location of TPGS in the micellar corona. Besides, the spherical morphology of drug-loaded copolymeric mixed micelles was confirmed by TEM analysis, as displayed in Fig. S2.

3.6. In vitro *release*

Based on the previously solubilization studies, the release of PTX from pristine Soluplus® and Soluplus®:TPGS (4:1) micellar systems was investigated. The PTX release profiles are shown in Fig. S3. Results demonstrated that the amount released drug was slightly lower for mixed micelles than for simple system over 72 h. Then, the mixed system showed a higher release. For example, the amount of drug released from the systems in the first 24 h was ~17% and ~13% for simple and mixed micelles, respectively. Whereas at 96 h, the PTX release was ~38% for pure micelles and ~40% for mixed formulation. Both micellar formulations exhibited a sustained release of PTX. However, mixed micelles showed a higher rate release, for example, the zero order release constant of the systems was 0.383 (R^2: 0.9717) and 0.426 (R^2: 0.9956) for single and mixed micelles, respectively. The rate difference could be attributed to the presence of TPGS, which would facilitate the release of PTX for its greater hydrophilicity in the micellar system. The hydrophobic core of single and mixed micelles restricted the drug's release, as PTX is physically entrapped in this inner fraction. In consequence, the hydrophobic core widely affects the *in vitro* release profile of the antineoplasic agent from the micellar formulation. As a result of the strong hydrophobic interactions between polyvinyl caprolactam portions of Soluplus® and PTX, longer diffusion times may be obtained and, thus, a sustained release may be observed. Similar behavior was found by Dian et al. where quercetin-loaded Soluplus® micelles presented significant sustained-release property [34]. These results demonstrated that single and mixed micelles were able to achieve not only an increase in the solubility of PTX, but also a maintained release.

3.7. In vitro *cytotoxicity*

To determine anti-tumor efficacy of the micellar systems, cell proliferation was performed in three different cancer cell lines by WTS assay (Fig. 3). The study was conducted on human cancer cell lines including ovarian cancer cells SKOV-3, breast cancer cells MCF-7 and triple negative breast cancer cells MDA-MB-231. These tumor cells were chosen for the *in vitro* treatment experiments based on the clinical oncology application of PTX. The results showed that PTX formulated in mixed micelles appeared equivalent to or have better effects against the cancer cells than free drug after 48 h of incubation. On the other hand, PTX-loaded single Soluplus® micelles exhibited low percentages of cell growth inhibition in all cancer cell lines. Therefore, their IC_{50} values could not be precisely determined (Table 3). The reduction in the cytotoxicity of PTX formulated in micelles may be partially due to their poor cellular uptake. Interestingly, blank mixed micelles showed cytotoxicity in all tumor cells, as the Soluplus®:TPGS concentration increased, whereas blank single micelles displayed extremely low cytotoxicity even at high concentrations. This effect could be attributed to the presence of TPGS, since it has been demonstrated that this biomaterial is selectively cytotoxic in cancer cell lines [3,38]. Thus, the low IC_{50} values of blank mixed micelles might be caused by the cancer specific cytotoxic property of TPGS to serve as a pro-apoptotic agent against cancer cells [38,49].

Line MCF-7 expresses markers of the luminal epithelial phenotype of breast cells and is used as a model for estrogen receptor (ER) positive tumor. Cytotoxicity caused by drug-loaded mixed micelles and PTX exhibited a similar pattern in these cells (Fig. 3). However, the IC_{50} values of PTX-loaded micellar formulation (13.4 ± 0.1 μg/mL) was about 1.5 fold less than the free drug ($p < 0.05$). The other breast cancer cell line assayed was the MDA-MB-231. This is known as the triple-negative breast cancer (TNBC) cell line based on the lack of expression of progesterone, estrogen and human epidermal growth factor type 2 (HER2) receptors. Generally, hormone receptor expressing breast cancers have a more favorable prognosis than TNBC [50]. Unlike tumors that express ER, TNBC does not respond to hormonal therapy or therapies that target HER2 receptors. Thus, TNBC tumors are typically treated with chemotherapy. In our viability assay, the IC_{50} values of free PTX on MCF-7 and MDA-MB-231 cells were 19.6 ± 1.7 μg/mL and 34.3 ± 2.4 μg/mL, respectively. It means that ER positive breast cancer cells are more sensitive to PTX than the MDA-MB-231 cells. In TNBC cells we observed that PTX-loaded mixed micelles presented higher cytotoxicity than PTX solution at the four highest drug concentrations assayed (Figure 3). In addittion, the IC_{50} value of mixed micelles (11.6 ± 0.4 μg/mL) was 2.9-fold lower than the free PTX ($p < 0.05$).

For the ovarian cancer cell line, PTX-loaded mixed micelles exhibited a significant higher cytotoxicity than PTX at the concentrations used as percentage of viability measured by WST assay (Fig. 3). The IC_{50} of PTX incorporated in mixed micelles in SKOV-3 cells was found to be 2.6-fold less than the free drug (27.8 ± 3.2 μg/mL) ($p < 0.05$).

It is worth noting that to induce death on MCF-7, SKOV-3 and MDA-MB-231 cells, PTX-loaded mixed micelles was 32%, 61% and 66% more efficient than the free drug after 48 h treatment, respectively (Fig. 3 and Table 3). It means that the mixed micelles could significantly improve the cytotoxicity in different cancer cell lines with different genetic background and biological behavior. The higher cytotoxicity of the formulation might be attributed to the combined effect of drug encapsulation and the selective chemotherapeutic effect of TPGS.

3.8. *Detection of cleaved PARP*

To evaluate the molecular mechanisms of enhanced cytotoxicity observed with TPGS, we examined the levels of cleaved poly(ADP-ribose) polymerase (PARP). The cleaved of PARP into two fragments

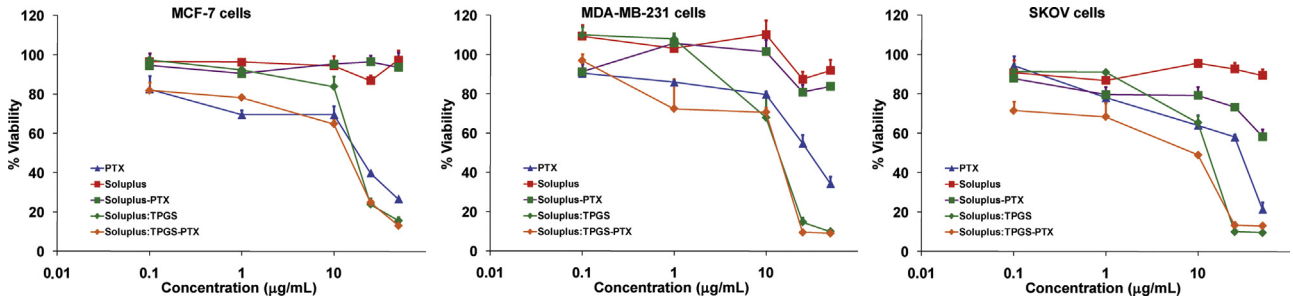

Fig. 3. Cell viability of MCF-7, MDA-MB-231 and SKOV cell after 48 h of treatment with PTX, blank single micelles (Soluplus®), blank mixed micelles (Soluplus®:TPGS), PTX-loaded Soluplus® micelles and PTX-loaded Soluplus®:TPGS mixed micelles.

Table 3
IC_{50} (mean ± S.D.) values in MCF-7, MDA-MB-231 and SKOV cells after 48 h treatment by PTX, blank micelles and PTX-loaded micelles.

Cell line	IC_{50} (μg/mL)				
	PTX	Blank micelles	PTX-loaded micelles	Blank mixed micelles	PTX-loaded mixed micelles
MCF-7	19.6 ± 1.7	>100	>100	17.1 ± 0.7	13.4 ± 0.1*,**
MDA-MB-231	34.3 ± 2.4	>100	>100	14.0 ± 0.8*	11.6 ± 0.4*
SKOV	27.8 ± 3.2	>100	>100	15.3 ± 0.4*	10.8 ± 1.5*

Note: Multiple comparisons were performed using one-way ANOVA with Tukey post hoc test ($n = 3$ experiments).
* Significant difference compared to PTX ($p < 0.05$).
** Significant difference compared to blank mixed micelles ($p < 0.05$).

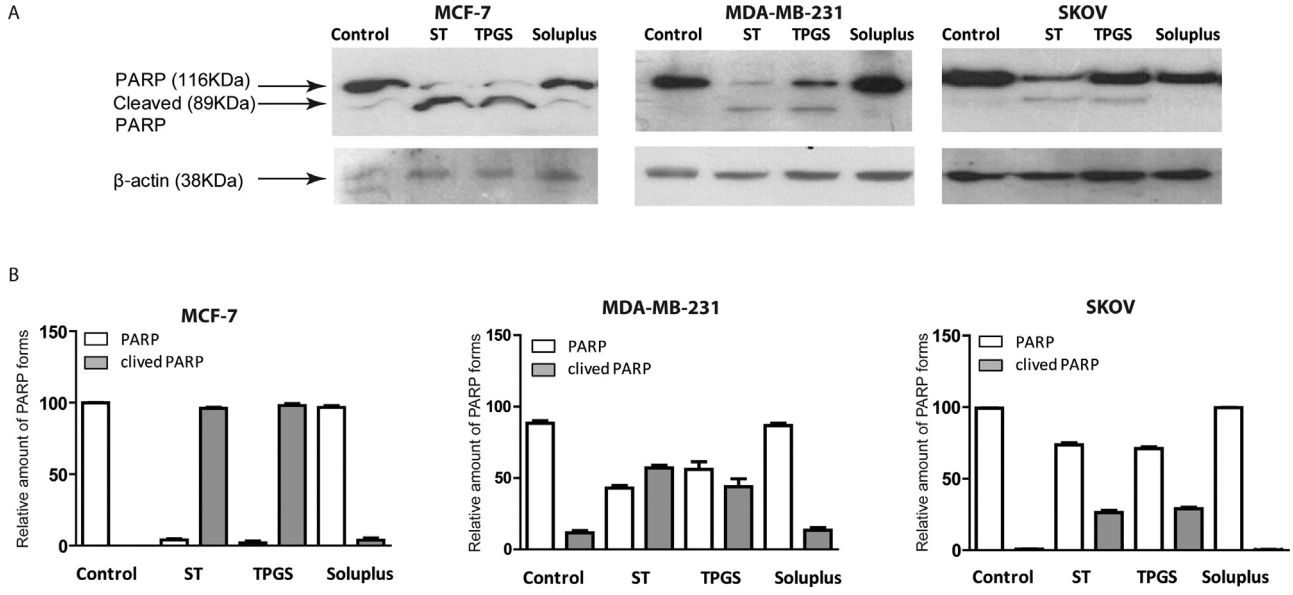

Fig. 4. (Panel A) PARP and cleaved PARP expression determined by western blot analysis. MCF-7, MDA-MB-231 and SKOV cell lysates were prepared from the cells without treatment (control), treated with Soluplus®:TPGS (ST), TPGS or Soluplus® for 48 h. β-actin was used as loading control. An immunoreactive band could be localized at 116 kDa, indicative of total PARP, and a second one at 89 kDa, which represents the cleaved form. Blots are representatives of three independent experiments. (Panel B) Signals of intensities of PARP (open columns) and cleaved-form PARP (closed columns) were quantified and normalized to β-actin. Data are expressed as mean ± S.D. ($n = 3$).

is an indicative of functional caspase activation. Caspases are a family of cysteine acid proteases, which are cleaved following proteolytic activation in apoptosis. The presence of cleaved PARP is a commonly used biomarker for detection of apoptosis in cancer cells [51]. The level of PARP activity in MCF-7, MDA-MB-231 and SKOV cells was detected after 48 h post-incubation (Fig. 4). Similar effects were observed in the three cancer cell lines. The cleaved form of PARP was not observed in lane containing lysate of untreated cells (control) (Fig. 4A). On the other hand, cells treated with TPGS and blank mixed micelles exhibited considerable cleavage of full length PARP (116 K Da) into cleaved PARP (89 K Da), which indicates the cancer cells are undergoing cell death *via* apoptosis pathway (Fig. 4A). In addition, immunoblot results showed that incubation with blank Soluplus® micelles induced negligible PARP cleavage, indicating the superior antitumor efficacy of the formulation made with TPGS. The cells treated with Soluplus®:TPGS mixed micelles presented enhanced apoptosis (Fig. 4B), being this result consistent with its *in vitro* antitumoral activity.

3.9. In vitro *uptake*

Fig. 5 shows the intracellular/cell levels of PTX in MCF-7, MDA-MB-231 and SKOV-3 cell lines at 37 °C. It can be noticed from the Fig. 5A–C that after 4 and 6 h incubation the mixed micelles showed a significantly increase in PTX intracellular levels ($p < 0.05$), as compared to PTX alone in the three cell lines. In addition, among all the formulations studied, it was clearly shown that the cellular uptake of mixed micelles was significantly higher compared with

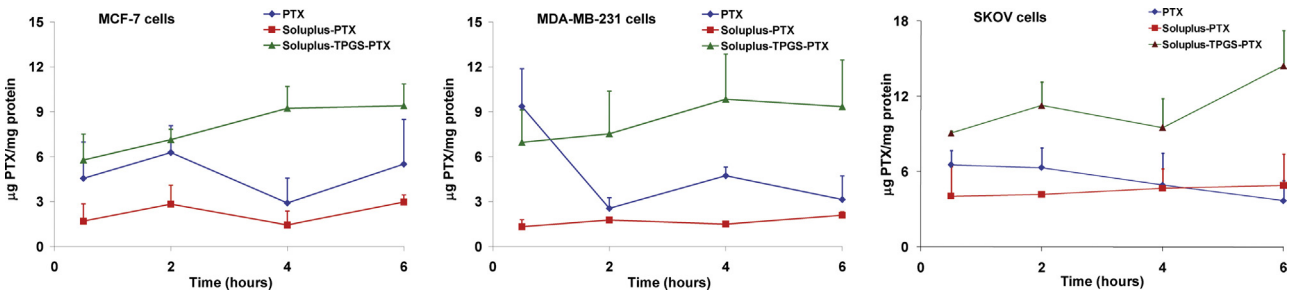

Fig. 5. Time-dependent intracellular/cell PTX levels in MCF-7, MDA-MB-231 and SKOV cancer cell lines for drug-loaded single and mixed micelles in comparison with PTX solution. Drug amount was normalized by protein concentrations of the cell lysates. Results are expressed as mean ± S.D. ($n = 3$).

PTX-loaded single Soluplus® micelles ($p < 0.05$) in cell lines assayed. These results are in agreement with the *in vitro* cytotoxicity assay data since PTX loaded mixed micelles exhibited the lowest IC_{50}. Interestingly, PTX-loaded Soluplus® micelles exhibited the lowest cellular uptake and their intracellular/cell PTX levels remained almost unchanged over 6 h. Thus, the low drug concentration internalized into cancer cells by single Soluplus® micelles could explain that even at very high concentrations of PTX, low cytotoxicity was observed. Such results suggest that the presence of TPGS could enhance the intracellular levels of PTX, which has been previously reported [52,53].

In the case of MDA-MB-231 cells, there was a clear decrease on the intracellular/cell drug levels for PTX solution, as is show on Fig. 5B. For instance, PTX intracellular concentration between 0.5 and 2 h reduces from 9.35 to 2.54 μg/mL. These dates could be associated with the PTX efflux from the cancer cell mediated by Multidrug Resistant Protein. These results are in good agreement with those reported by our research group previously [54].

Taking into account the PTX solubility obtained with the mixed micelles, the FDA-approved biomaterials employed and the *in vitro* citotoxicity performance, our nanosized-system could be a valid strategy to be translated to the clinic. Ongoing experiments are focus on the *in vivo* performance of this nanotechnological platform to evaluate its potential in cancer therapy.

4. Conclusion

In the present investigation, novel Soluplus®:TPGS mixed micelles was successfully employed for the first time for the nanoencapsulation of PTX. A clinically relevant PTX formulation was developed due to the increment on drug apparent solubility for both, single Soluplus® and mixed micelles. On the other hand, these systems presented small size; uniform distribution and an in vitro sustained PTX release, being these adequate for intravenous administration. In vitro cytotoxicity studies showed that the incorporation of TPGS in the micelles allowed a better cytotoxic activity compared to PTX solution and single Soluplus® micelles at different concentrations assayed on MCF-7, MDA-MB-231 and SKOV cancer cells lines. Besides, PTX-loaded mixed micelles were found to be more effective than the other formulations to internalize PTX in all the cancer cell lines employed. These results suggest that Soluplus® and TPGS are convenient biomaterials to be used in a micellar system for antineoplastic therapy, representing an excellent nanotechnological platform to improve the PTX cytotoxicity and accumulation within breast and ovarian cancer cell lines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2016.01.003.

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