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Development and characterization of miltefosine-loaded polymeric micelles for cancer treatment

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ABSTRACT

Miltefosine presents antineoplastic activity but high hemolytic potential. Its use in cancer has been limited to treating cutaneous metastasis of breast cancer. To decrease hemolytic potential, we developed a formulation of miltefosine-loaded polymeric micelles (PM) of the copolymer Pluronic-F127. A central composite design was applied and the analysis of variance showed that the optimum level of hydrodynamic diameter and polydispersity index predicted by the model and experimentally confirmed were 29 nm and 0.105, respectively. Thermal analyses confirmed that miltefosine was molecularly dispersed within PM. Pluronic-F127 PM with miltefosine 80 μM presented a significant reduction of hemolytic effect (80%, $p < 0.05$) in comparison to free drug. *In vitro* assays against HeLa carcinoma cells demonstrated similar cytotoxicity to free miltefosine and PM. Our results suggest that, by lowering hemolytic potential, miltefosine-loaded Pluronic-F127 PM a promising alternative to broaden this drug use in cancer therapy, as well as of other alkylphosphocholines.

1. Introduction

Miltefosine (MTF) is an alkylphospholipid drug (APL) used orally for the treatment of leishmaniasis and topically to treat skin metastases of breast cancer [1]. This drug class also has potential to treat fungal and bacterial infections, as well as Chagas' disease [2]. Unlike other DNA-targeting anticancer agents, APL drugs are involved in phospholipid metabolism, non-vesicular cholesterol transport and homeostasis, biochemical survival pathways (for example, Akt-mTOR pathway), and interaction with membrane signal transduction proteins, such as phospholipase C, phospholipase D and protein kinase C. However, the precise mechanism of action has not been fully elucidated yet [3].

Miltefosine presents potent antitumor activity *in vitro* [4] and in experimental animal models. Nevertheless, clinical use is limited due to side effects associated with its amphiphilic nature [5–7]. More specifically, MTF is highly hemolytic when administered intravenously and orally it was associated with cumulative gastrointestinal toxicity. These side effects, correlated to the drug aggregation into micelles, have limited the maximum daily dose by these routes (maximum tolerated dose: 200 mg/day), preventing *in vivo* observation of antiproliferative effects [6–8]. One strategy to improve the efficacy and safety of oral and/or intravenous cancer treatment with MTF refers to nanotechnology. More specifically, MTF incorporation in nanocarriers may prevent its self-aggregation into micelles and, therefore, the above-mentioned side effects.

In the last decade, nanocarriers have been widely used in anticancer systems/formulations to avoid drug contact with healthy tissues and allow drug accumulation in tumor cells [8]. The incorporation of drugs in nanocarriers enhances solubility and blood half-life, promoting controlled and site-specific release. In addition, combined therapy can be achieved by incorporation of more than one drug [9–11]. The first application of nanocarriers for MTF delivery dates back to 1991 when it

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was incorporated in liposomes [12]. Thereafter, a series of formulations based on liposomes, pegylated or not, were developed with phospholipids and MTF as bilayer forming constituents [7,13–15]. However, poor stability of liposomal delivery systems limits their use in drug delivery; few liposome-based marketed products are available regardless of extensive and long research in this area [2]. On the other hand, polymeric micelles are an interesting alternative to prevent MTF side effects and, to the best of our knowledge, there are no previous reports using polymeric micelles for the delivery of MTF.

Polymeric micelles (PM) are unique core-shell nanostructures formed by amphiphilic copolymers aggregation that can incorporate poorly soluble drugs [16–20]. Amphiphilic block copolymers have the ability to self-assemble into PM in aqueous media and they have been widely studied in the field of nanomedicine, biomedicine and pharmacy [20–22]. The PM represent thermodynamic aggregations of multi amphiphilic macromolecules above their critical micelle concentration (CMC) [23].

An important property of PM is their size, which usually ranges from 10 to 80 nm, thereby closing the gap between individual macromolecule drug carriers (albumin, dextran, and antibodies) with sizes bellow 10 nm; and nanocarriers such as liposomes and nanocapsules with sizes of 100–200 nm [24]. Closing this gap is relevant for selected drug administration routes, such as percutaneous lymphatic delivery, or extravasation into solid tumors. Comparing to surfactant micelles (such as MTF micelles), PM are much less hemolytic and kinetically stable. They present slow dissociation that allows them to retain integrity and perhaps drug content in blood circulation above or even below the critical micelle concentration for some time, increasing the chances of reaching the target site before decaying into monomers [20].

One of the most commonly employed polymer class to prepare PM is the Pluronics [25], triblock copolymers of poly(ethylene oxide)–poly (propylene oxide)–poly(ethylene oxide) (PEO-PPO-PEO) with very low CMC values and a solution-gel transition behavior depending on the temperature [26]. Pluronic-based nanocarriers are safe, Food and Drug Administration (FDA) approved and undergo less opsonization than other nanocarriers (since they are sterically stabilized), preventing the subsequent recognition and uptake by macrophages of the reticuloendothelial system (RES). As a consequence, PM of Pluronics may have a reasonably longer half-life in circulation and may deliver the payload to desired sites of action more efficiently [27,28].

Considering the advantages of PM as drug carriers and the potential of MTF as a chemotherapeutic agent for cancer therapy, we developed MTF-loaded PM of Pluronic F127 that showed to be significantly less hemolytic than the free drug. To develop the formulation, a factorial design was employed to evaluate the effect of hydration temperature, stirring speed and stirring time on nanostructure mean size (D_h) and polydispersity index (PI). Our results of *in vitro* activity show that MTF-loaded PM are less hemolytic than the free drug while preserving the antiproliferative activity against tumor cell lines.

2. Materials and methods

2.1. Materials

The miltefosine (MTF) was purchased from Avanti® Polar Lipids, Inc. (Alabama, USA). Pluronic® F127 ($(PEO)_{100}$-$(PPO)_{65}$-$(PEO)_{100}$; Mw: 12,600 Da), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and phosphotungstic acid were supplied by Sigma-Aldrich® (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was supplied by Gibco® Laboratories (USA). Defibrinated sheep blood was obtained from New Prov® (Pinhais, Brazil). The human cervical carcinoma cell line (HeLa) and the human bronchioalveolar carcinoma cell line (H-358) were obtained from ATCC® (Manassas, VA, USA). All other chemicals were of analytical grade and used without further purification. All experiments were carried out with ultra-purified water (Milli-Q, Millipore®, Bedford, MA, USA).

2.2. Preparation of miltefosine-loaded polymeric micelles

MTF-loaded PM were prepared by thin-film hydration method [29]. Briefly, 3.6 mg of MTF and 90.7 mg of Pluronic F127 were dissolved in 5 mL of chloroform in a round-bottom flask. The solvent was extracted by rotary evaporation (Buchi Rotavapor R-210/215, Buchi®, Switzerland) at 50 °C, 150 rpm, and 100 mBar for 30 min to obtain MTF:polymer matrix forming a thin film. Then, the film was hydrated with phosphate buffer saline (PBS), 10 mM, pH 7.4, at different temperatures, stirring rates and stirring times according to the experimental design detailed in the following section. The micellar dispersions were filtered through a 0.22 μm membrane to remove aggregates. After defining the optimal micellar dispersion, prepared according to the experimental design, the system was freeze-dried (24 h at 0.120 mBar, after freezing at −70 °C overnight) in a Liotop® L101 equipment (Liobras, Brazil).

2.3. Central composite design (CCD)

Preliminary experiments at a fixed Pluronic F127 concentration (7.2 mM) and varying MTF concentration were conducted to define the maximum drug concentration to be incorporated in the polymeric micelles. Then, a central composite design (CCD) was used for the optimization of three independent variables, namely hydration temperature (X_1), stirring speed (X_2) and stirring time (X_3). The micelles mean hydrodynamic diameter (D_h) and polydispersity index (PI) were selected as dependent variables, according to a 2^3 full factorial design matrix generated by Minitab® software (Trial version 17). Response surface methodology (RSM) was used in the optimization of response variables of D_h and PI. For the estimation of the significance and validity of the model, analysis of variance (ANOVA) was applied with 5% significance level, and regression coefficients were calculated. Fisher's *F*-test was performed to test the adequacy of the model.

The D_h and PI values were measured by dynamic light scattering (DLS) in a Zetasizer® Nano ZS (Malvern Instruments, Worcestershire, UK). Analyses were performed at a scattering angle of 90° at 25 °C. Previous to measurement, each freshly prepared sample was diluted two times in PBS to avoid multi-scattering phenomena. The results were expressed as a mean size ± SD for three separate experiments.

2.4. Surface morphology by transmission electronic microscopy (TEM)

The morphology of the MTF-loaded PM obtained from the optimized formulation was evaluated by TEM (JEM-2100, Jeol® Tokyo, Japan). Samples were dropped onto copper coated carbon grids, then stained with phosphotungstic acid solution 2% (w/v) and the excess was wiped by filter paper [30].

2.5. Thermal analysis

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were carried out using TA-Instruments® (New Castle, DE, USA) calibrated with indium. Freeze-dried MTF-loaded PM were weighed to place 4 mg for DSC and 3 mg for TGA, and the samples were retained in hermetically sealed aluminum pans. As a control, a pan containing the same amount of free MTF was prepared. Thermal analysis of isolated copolymer Pluronic F127 and the physical mixture of MT and Pluronic F127 (a blend of both solids without interaction media) were also carried out. The dynamic scans were taken in N_2 atmosphere at the heating rate of 10 °C/min.

2.6. Hemolytic potential

The effect of the MTF-loaded PM on erythrocyte membranes integrity was investigated by *in vitro* hemolysis assay [31]. The systems were prepared with MTF-loaded PM and free MT at different drug

concentrations, in a range of 30 to 350 μM in PBS with 5% (v/v) of defibrinated sheep blood. The samples were incubated at 37 °C for 1 h under gentle agitation and after that were centrifuged at 25 °C, 1788 × *g* for 5 min. The percent of hemolysis (HP %) in the supernatant was spectrophotometrically (Molecular Devices®) measured at 540 nm, according to Eq. (1). Distilled water and PBS were used as positive and negative control of hemolysis, respectively.

$$HP\% = \frac{\text{Abs sample}}{\text{Abs distilled water}} \times 100 \quad (1)$$

Statistical analysis by comparison of different experimental conditions according to the evaluation of mean values assessed by analysis of variance (two-way ANOVA) with the Bonferroni's *post hoc* test using Graph Pad® software. A p-value < 0.05 or less was considered significant.

2.7. Cell toxicity assays

Human epithelial cervix carcinoma (HeLa) and human bronchioalveolar carcinoma (H358) cell lines were used to perform cytotoxicity assays according to ISO standards (Third ed., 2009). Cells were cultured in DMEM supplemented with fetal bovine serum to a final concentration of 10%, pH 7.2. Cells were placed on 96-well plates at a density of 6×10^3 cells/well in 100 μL of complete media and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Next, the cells were treated with 100 μL of complete medium containing free MTF or MTF-loaded PM with a gradient at specific concentrations. Unloaded PM was also tested at 6 μM (equivalent to the concentration of polymer present in the MTF-loaded PM evaluated). Untreated cells were used as controls.

After 24, 48 and 72 h, the mitochondrial metabolic activity of cells was quantitatively determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [32]. Briefly, cells were washed with PBS at pH 7.4, and complete fresh medium with 5 mg/mL MTT was added, followed by incubation for 1 h at 37 °C. Then, dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan crystals. Finally, MTT reduction to formazan was measured colorimetrically at 570 nm in a spectrophotometer (Biotek®). At each time point, the absorbance of treated samples was normalized by the absorbance of control samples (which was considered as 100% viability). Assays were performed in quadruplicate and statistical significance was assessed by analysis of variance (two-way ANOVA) with the Bonferroni's *post hoc* test using Graph Pad® software. A p-value < 0.05 or less was considered significant.

3. Results

3.1. Development of miltefosine-loaded polymeric micelles

To prepare the PM, we employed 7.2 mM of Pluronic F127 what is about 60 times above the copolymer CMC estimated by us based on the pyrene solubilization method (CMC ~0.118 mM, data not shown). For this fixed concentration of copolymer, we investigated the incorporation of increasing amounts of MTF up to a maximum where pure MTF micelles were still not detected (see MTF DLS at Supplementary material, Fig. S1) and PM characteristics (size, polydispersity) were preserved. The MTF-loaded PM were evaluated by DLS and the scattering profiles are presented as Supplementary material (Fig. S2) and summarized in Table 1.

As can be seen, Pluronic F127 micelles of approximately 30 nm of D_h were obtained in the absence of MTF. At increasing drug concentrations, the D_h of PM was ~25 nm, and at 12 mM of MTF, a second peak corresponding to nanostructures of D_h ~4.9 nm was observed. We believe these smaller structures could be pure MTF micelles formed by unloaded drug aggregation. Since the drug is amphiphilic, we assumed it behaved as a co-surfactant aggregating with the Pluronic F127 into PM. However, at high concentrations (above 9 mM) part of the MTF molecules was not able to accommodate in the PM and aggregated as pure MTF micelles. Therefore, we considered 9 mM as the maximum concentration of MTF to be incorporated in 7.2 mM of Pluronic F127 PM without resulting in drug aggregation in pure MTF micelles. In other words, for systems with a copolymer:drug molar ratio up to 1:1.3, MTF monomer concentration was considered to be the drug CMC, reported in the literature as approximately 0.05 mM [33]. Considering the co-micellization process and free MTF concentration approximately the drug CMC, we assumed that 99.4% of miltefosine was in aggregates with Pluronic F127.

Table 1
Hydrodynamic diameter (D_h) and polydispersity index (PI) of F127 polymeric micelles (7.2 mM) with different concentrations of miltefosine.

Miltefosine (mM)	Peak 1 D_h (nm)	Intensity peak 1 (%)	Peak 2 D_h (nm)	Intensity peak 2 (%)	PI ± SD
0	30.96	100	–	–	0.109 ± 0.01
1	30.30	100	–	–	0.093 ± 0.01
3	25.25	100	–	–	0.169 ± 0.01
6	25.92	100	–	–	0.121 ± 0.01
9	36.46	98	–	–	0.245 ± 0.02
12	26.17	94.1	4.9	2.6	0.253 ± 0.02

The effects of hydration temperature (X_1), stirring speed (X_2) and stirring time (X_3) on D_h and PI of PM were studied by a 2^3 full factorial design, and the results are presented in Table 2.

Response surface plots were obtained according to regression equations with Minitab™ 17, and the three factors were found to influence 1/PI ($p < 0.05$), while they did not significantly affect D_h. Lower temperatures led to a very slight and statistically insignificant average size increase (21 nm to 24 nm). Considering that only the 1/PI was influenced by the factors under study, in Fig. 1 we present the surface plot for 1/PI as a function of temperature (X_1), stirring speed (X_2), and stirring time (X_3). As can be seen, the surface plotted indicates an optimal region (red color) where the factors evaluated allow obtaining higher 1/PI values, corresponding to lower PI.

A summary of ANOVA results is shown in Table S1 (Supplementary material), and the mathematical model for PI was well fitted (R-Sq of 0.9753 (adj) and 0.9472 (pred)). Taking higher values of inverse PI (1/PI), the optimal formulation was selected as follows: hydration temperature of 30 °C, stirring speed of 550 rpm, and stirring time of 30 min, according to Eq. (2):

Table 2
Independent variables of the 2^3 full factorial design for miltefosine-loaded Pluronic F127 polymeric micelles preparation with the coded values expressed in brackets and response variables hydrodynamic diameter (D_h) and the inverse of polydispersity index (1/PI).

Experiment number	X_1 Hydration temperature (oC)	X_2 Stirring speed (rpm)	X_3 Stirring time (min)	D_h [nm]	1/PI
1	30 (−1)	550 (−1)	30 (−1)	24	8.8
	30 (−1)	550 (−1)	30 (−1)	24	9.0
2	30 (−1)	550 (−1)	60 (+1)	22	4.5
	30 (−1)	550 (−1)	60 (+1)	21	4.2
3	30 (−1)	750 (+1)	30 (−1)	23	5.2
	30 (−1)	750 (+1)	30 (−1)	22	4.9
4	30 (−1)	750 (+1)	60 (+1)	24	7.2
	30 (−1)	750 (+1)	60 (+1)	23	6.4
5	50 (+1)	550 (−1)	30 (−1)	23	4.4
	50 (+1)	550 (−1)	30 (−1)	23	4.5
6	50 (+1)	550 (−1)	60 (+1)	22	4.0
	50 (+1)	550 (−1)	60 (+1)	22	4.1
7	50 (+1)	750 (+1)	30 (−1)	22	5.0
	50 (+1)	750 (+1)	30 (−1)	23	5.2
8	50 (+1)	750 (+1)	60 (+1)	24	6.6
	50 (+1)	750 (+1)	60 (+1)	22	6.3

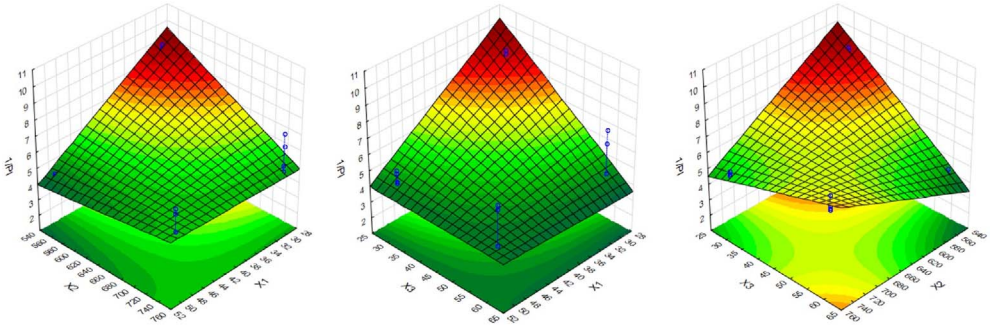

Fig. 1. Response surfaces of the polydispersity index inverse (1/PI) for miltefosine-loaded Pluronic F127 polymeric micelles as a function of temperature (X_1), stirring speed (X_2) and stirring time (X_3). Graphs were plotted using the program Statistica™; higher 1/PI results are represented in red and lower values in darker green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$$\frac{1}{PI} = 91.59 - 1.674 \times X_1 - 0.11863 \times X_2 - 1.562 \times X_3 + 0.002263 \times X_1 \times X_2 + 0.02777 \times X_1 \times X_3 + 0.002188 \times X_2 \times X_3 - 0.000038 \times X_1 \times X_2 \times X_3 \quad (2)$$

The average D_h and PI of experimentally obtained optimized MTF-loaded PM were 24 ± 0.2 nm and 0.112 ± 0.005, respectively. Optimized formulation was submitted to freeze-drying, and both D_h and PI were preserved after this process. The D_h presented minimal variations from 24 nm to 22 nm, and PI slightly increased to 0.181.

3.2. Transmission electron microscopy

The morphology of the optimal formulation under TEM presented in Fig. 2 revealed spherical structures that we attributed to the MTF-loaded PM. Based on previous work, we believe that gray spots represent the PPO core of micelles. According to TEM images, the spherical nanostructures presented a mean diameter of about 40 nm, which is slightly higher than the results obtained by DLS (29 nm). The size difference may be due to some degree of nanostructures aggregation on the grids by the absence of solvent.

3.3. Thermal analysis

Fig. 3 shows the DSC curves of free MTF, isolated Pluronic F127, the physical mixture of MTF and Pluronic F127 and the MTF-loaded PM. The DSC curve of MTF presented different endothermic events, and the melting temperature was about 225 °C (ΔH 30.20 J/g), whereas freeze-dried MTF-loaded PM did not present the drug melting endotherm. The Pluronic F127 presented an endothermic event at 48 °C (ΔH 143 J/g); similar results were obtained by other authors [34,35]. The physical mixture exhibited an endothermic event at a lower temperature than free MTF, which could be attributed to the solid free drug in the blend.

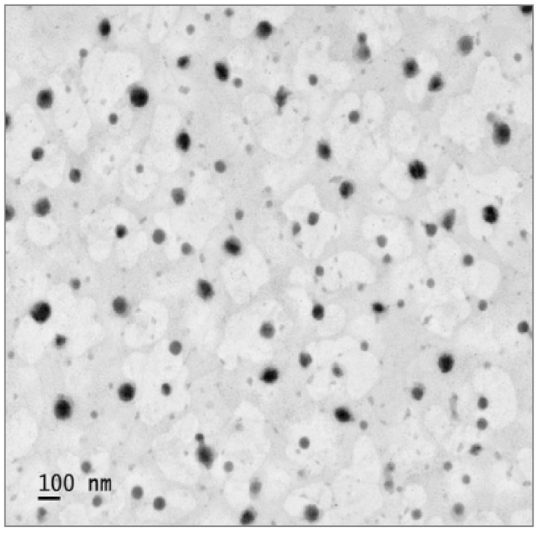

Fig. 2. TEM image of miltefosine-loaded Pluronic F127 polymeric micelles.

Complimentary to DSC, thermogravimetric analysis (Supplementary material, Fig. S3) showed that Pluronic F127 presents high thermal stability, with decomposition temperatures in the range of 330–430 °C. The TGA of MT-loaded PM showed a weight loss of 89% at temperatures higher than 300 °C. Free MT presented a weight loss of 24% at decomposition temperature of 253 °C. The TGA curves of physical mixture exhibited weight loss of 95% in the range of 245–417 °C.

3.4. Hemolytic potential

Fig. 4 shows the hemolytic potential of free MTF and MTF-loaded PM at different concentrations of the drug. For MTF concentrations up to 125 μM the HP% of MTF-loaded PM was significantly lower than free drug ($p < 0.001$). Above this concentration, no significant differences were observed between free drug and MTF-loaded PM. The triblock copolymer Pluronic F127 had no hemolytic effect on red blood cells (empty PM on Fig. 4). In addition, the hemolytic effect was dose-dependent for MTF.

3.5. In vitro cytotoxicity assay

Initially, we determined the drug IC_{50} in human HeLa (Fig. 5A) and H358 (Fig. 5B) cell lines based on MTT assay at 24 h and found an IC_{50} of 6.4 μM HeLa in comparison to 52.4 μM in H358. Giving the higher effect of the drug against HeLa cells, we compared IC_{50} in this cell line for the free MTF and MTF incorporated in PM (Fig. 5C) and found no significant differences in the drug cytotoxic effect. The IC_{50} for MTF-loaded PM was 8.1 μM. We also compared the cytotoxicity of free MTF and MTF-loaded PM at different times, 24, 48 and 72 h (Fig. 5D). For both formulations, cell viability was significantly lower at each time evaluated when compared to the control group ($p < 0.01$). The cytotoxicity of empty Pluronic F127 micelles was also investigated (Supplementary material, Fig. S5) and no significant differences were observed between the empty micelles at 6 μM of Pluronic F127 and the control ($p > 0.05$).

4. Discussion

We developed a Pluronic F127 micellar formulation incorporating the antitumor agent MTF, aiming at lowering its hemolytic potential and improving drug safety. For the pure copolymer, PM with D_h of about 30 nm were obtained. For Pluronic:MTF molar ratios up to 1:1.3, the D_h of MTF-loaded PM was ~25 nm, which is still large enough to avoid first-pass renal clearance that faces particles smaller than 10 nm, thus maintaining their longevity in the bloodstream [36]. As mentioned before, we assumed that MTF co-micellized with Pluronic F127 and free drug concentration was considered approximately the CMC (0.05 mM). Nevertheless, at 1:1.7 Pluronic:MTF molar ratio (12 mM of MTF), a secondary peak corresponding to structures of 4.9 nm diameter was observed. If we consider that pure MTF micelles have a diameter of

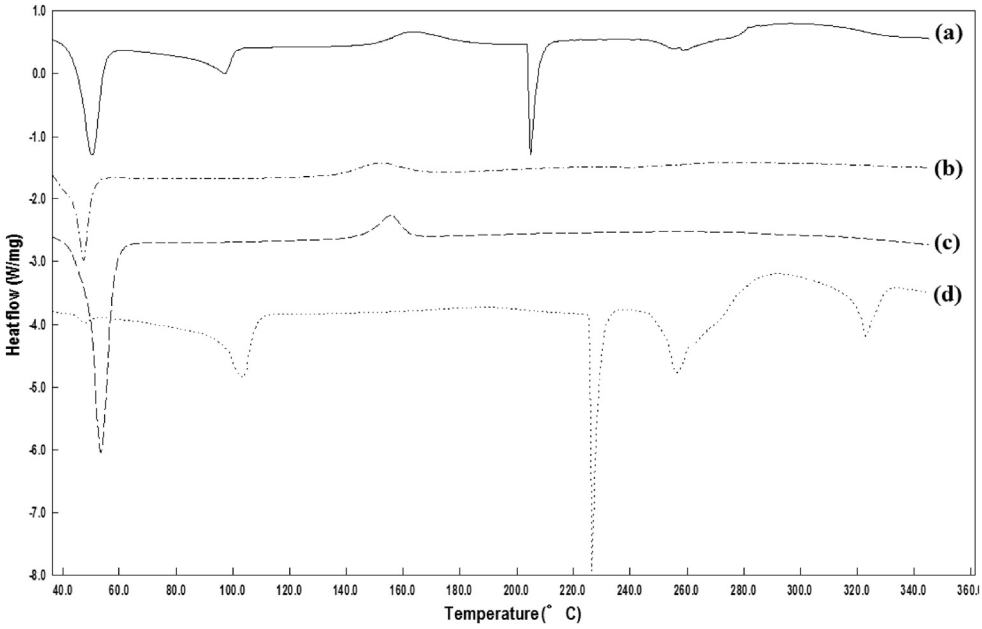

Fig. 3. DSC curves of the physical mixture (a), miltefosine-loaded Pluronic F127 polymeric micelles (b), isolated copolymer Pluronic F127 (c) and free miltefosine (d).

6 nm (Supplementary files, Fig. S1), we can assume that an excess of the drug is aggregating in pure micelles, what is not desirable in terms of drug delivery. Pure MTF micelles are known to be very hemolytic and disaggregate easily under oral or intravenous administration [37]. Therefore, we considered 9 mM as the maximum drug load in 7.2 mM Pluronic F127 PM.

The PEO:PPO ratio of the Pluronic, its concentration and formulation parameters such as temperature and ionic strength, are important determinants for drug encapsulation [38,39]. A CCD study allowed us to evaluate the influence of hydration temperature (X_1), stirring speed (X_2) and stirring time (X_3) on D_h (Y_1) and PI (Y_2). Nanostructured formulations for intravenous pharmaceutical applications should present good control of particle size since large particles may result in capillaries obstruction. Our results show that lower temperatures lead to narrower size distribution for MTF-loaded PM, in agreement with previous reports for Pluronic F127 [40]. This may be attributed to the dehydration of the Pluronics PEO heads at higher temperatures, resulting in interactions among the polymeric micelles and, therefore, higher polydispersity.

Regarding MTF incorporation, the effect of temperature on the solubility of drugs depends on its location in the micelle (within the PPO core or in the PEO corona). Drugs that are located preferably in the PEO corona are affected by temperature due to dehydration of PEO groups with increasing temperatures, which reduces the available space for drug incorporation [24]. Since we assumed that MTF behaved as a co-surfactant, its solubility should not change drastically with temperature.

Additionally, lower PI values were observed when higher stirring speed was used to prepare MTF-loaded PM. We believe this is related to an increased probability of micellar aggregation and decrease in bulky polymer at this condition. Regarding stirring time, higher values did not result in lower PI values indicating that kinetics of aggregation is relatively fast for Pluronic 127.

The TEM microscopy analysis showed spherical structures, an important feature to provide long circulation times in the bloodstream and prevent nanotoxicity. Studies reported that spiky nanoparticles could stimulate macrophages within the tissue and nanostructures without roughness or sharp edges avoid the uptake by RES [41,42]. The particle size range observed for MTF-loaded PM (Dh ~25–40 nm) allows incorporation into endocytic vesicles or cellular access to endocytosis pathways [29].

The thermal analysis of the pure polymer, free MTF, physical mixture and MTF-loaded PM by DSC indicated the thermic events and the differences between the curves in presence or absence of MTF. The DSC analysis of MTF-loaded PM showed that MTF melting peak was not present, suggesting the drug dispersion in the micellar formulations. It appears that PEO and PPO groups interact with MTF avoiding phase separation. The DSC curves for physical mixture showed an endothermic event at lower temperatures in comparison to free MTF, confirming the presence of free drug and polymer. As mentioned above, for the MTF-loaded PM this event was not observed suggesting the interaction between the polymeric matrix and the drug. The DSC analysis

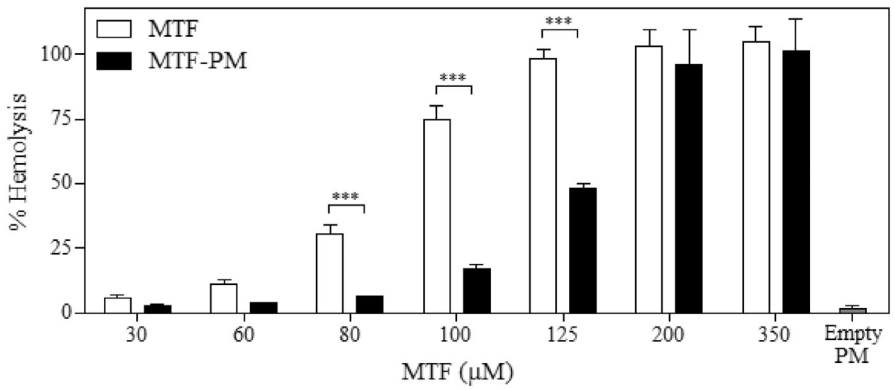

Fig. 4. Hemolytic potential (HP%) of free miltefosine (MTF), MTF-loaded Pluronic F127 polymeric micelles (PM) at different MTF concentrations, and empty PM (7.2 mM of Pluronic F127). Asterisks indicate a significant difference between the indicated groups ($p < 0.001$).

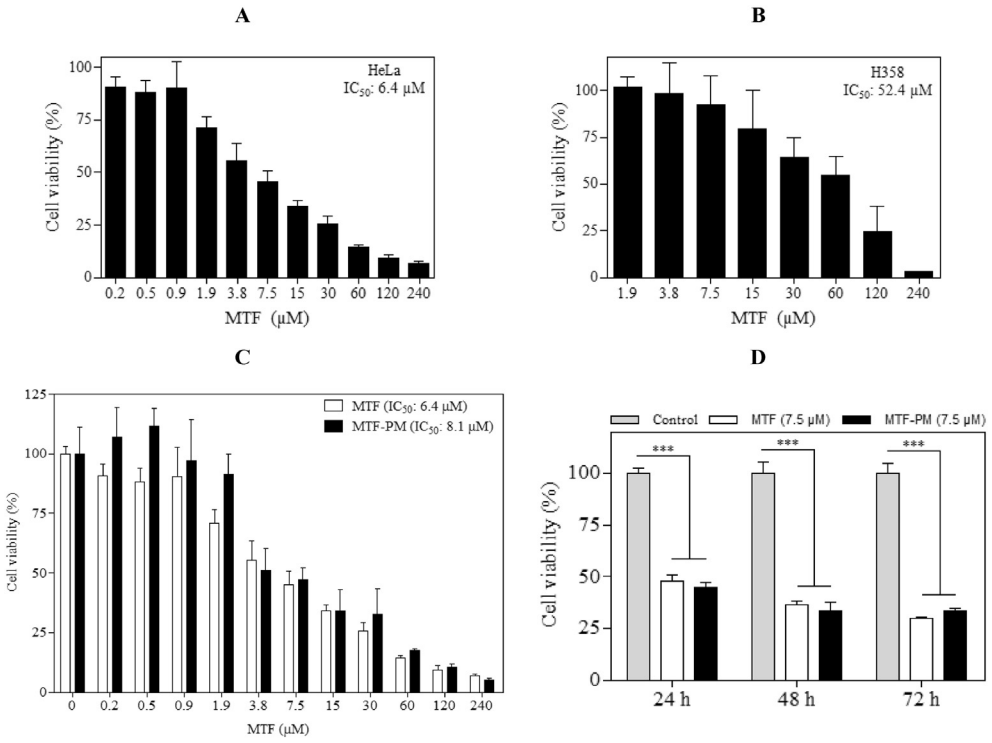

Fig. 5. Cytotoxicity results by MMT assay: (A) cytotoxicity against HeLa epithelioid cervix carcinoma cell line of free miltefosine (MTF) after 24 h; (B) cytotoxicity against H358 bronchioalveolar carcinoma cell line of free MTF after 24 h; (C) comparison between free MTF and MTF-loaded polymeric micelles (PM) cytotoxicity against HeLa cell line at several MTF concentrations after 24 h; (D) comparison between free MTF and MTF-loaded PM cytotoxicity against HeLa cell line at a fixed MTF concentration (7.5 μM) and different times (24, 48 and 72 h). Asterisks indicate a significant difference between the groups relative to the control (***$p < 0.001$).

also showed miscibility between the polymer and drug, since a decrease in melting point was observed [43,44]. Pluronic F127 has a lower melting temperature than MTF and, therefore, the decrease in melting temperature suggests miscibility of MTF and the polymer chains [45].

The TGA of Pluronic F127, free MTF, physical mixture and MTF-loaded PM were performed, and the curves obtained for the pure polymer, and free MTF showed a weight loss of 94% and 25%, respectively, with a decomposition temperature above 254 °C for MTF [46]. Physical mixture showed thermal degradation at 245 °C with a weight loss of 95.5%. The decomposition temperature of MTF-loaded PM (359–392 °C) was higher than the one for the drug alone (254 °C), indicating an increase in MTF stability upon co-aggregation with Pluronic F127.

Blood compatibility of nanomaterials is an important feature for intravenous administration [47,48]. The hemolytic effect of MTF can be explained by the fact that this molecule presents a classic surfactant structure and aggregates in micelles in aqueous solutions. Micelles are related to cell lysis by a mechanism involving micellar dissolution and partition into the membrane followed by a phase transition between lamellae and micelles with membrane solubilization. In general, it is accepted that the amount of surfactant required for membrane solubilization increases with the ease of forming micelles, *i.e.*, with the CMC [24]. We found that MTF causes 100% hemolysis at 200 μM while MTF-loaded PM at 80 μM MTF presents a relatively low hemolytic potential, around 6.1% (the free drug exhibited 30% hemolysis at the same concentration). Unloaded PM of Pluronic F127 had no hemolytic effect at 7.2 mM, which is the concentration of polymer in the PM. Our results show that significant differences in hemolytic potential are found between the free drug and MTF-loaded PM at MTF concentrations up to 125 μM ($p < 0.001$). There is one previous report of MTF incorporation in emulsions, but for 100 μM of MTF, the hemolytic potential was reduced from 100% (free drug) to 80% (MTF in the emulsion) [49]. We found 60% of hemolysis reduction with 100 μM of MTF incorporated in Pluronic F127 PM. Therefore, MTF incorporation into PM may enable the drug transport in the bloodstream significantly reducing hemolysis and consequently improving drug safety.

Regarding MTF release, data from the literature shows that *in vitro* release of drugs from Pluronic F127 micelles is characterized by a first stage with relatively rapid release followed by a slow and sustained release according to Higuchi and Power law, indicating a diffusional kinetic mechanism [45]. Since Pluronic micelles are self-aggregated nanostructures and miltefosine does not present specific interactions with the Pluronic molecules, the same diffusional kinetic mechanism should take place for MTF-loaded PM.

The cytotoxicity of MTF was evaluated in human epithelioid cervix carcinoma (HeLa), and bronchioalveolar carcinoma (H358) cell lines after 24 h of incubation and HeLa cells were found to be eight times more sensitive to MTF, with IC_{50} of 6.4 μM. To confirm that MTF cytotoxicity would be retained when incorporated in PM, cytotoxicity assays were also performed with MTF-loaded PM and the same cytotoxic profile was observed, with an IC_{50} of 8.1 μM for the drug incorporated in the PM. Regarding the effect of Pluronic F127 on HeLa cells (Fig. S4) a slight decrease in cell viability (< 25%) is noticed, but no significant cytotoxicity was detected ($p > 0.05$). This effect might result from the surfactant nature of Pluronic F127 that may have limited cell adhesion to the surface of the culture plate. The MTF-loaded PM and free MTF at the same drug concentrations exhibited similar cytotoxicity in HeLa cells after 24, 48 and 72 h of incubation (Fig. 5D), indicating the potential of the developed formulation to treat human epithelioid cervix carcinoma.

Since our main concern here was to prove that MTF activity would be preserved in PM, we chose a sensitive cell line (HeLa) for proof of concept. Nonetheless, MTF is cytotoxic for several cancer cell lines, such as soft tissue sarcoma, metastatic colorectal cancer, and head and neck squamous cell carcinoma [2], suggesting that our MTF-loaded F127 formulation may be used as a therapeutic strategy to deliver MTF safely to other cancer types.

5. Conclusions

The MTF-loaded PM are a promising alternative to treat cancers by the intravenous administration since they preserve the drug antitumoral activity and lower hemolytic effect. In other words, the therapeutic formulation developed take advantage of MTF potential for

cancer treatment and improve the pharmacotherapy by reducing side effects. Additionally, our results show that PM are promising for the delivery of other surfactant-like drugs, bringing new possibilities for drugs once put aside due to surfactant-related toxicity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2017.07.040.

References

[1] S. Sundar, P.L. Olliaro, Miltefosine in the treatment of leishmaniasis: clinical evidence for informed clinical risk management, Ther. Clin. Risk Manag. 3 (2007) 733.
[2] J.A. Pachioni-Vasconcelos, A.M. Lopes, A.C. Apolinário, J.K. Valenzuela-Oses, J. Costa, L.O. Nascimento, A. Pessoa, L. Barbosa, C.O. Rangel-Yagui, Nanostructures for protein drug delivery, Biomater. Sci. 4 (2016) 205.
[3] M. Malta de Sá, V. Sresht, C.O. Rangel-Yagui, D. Blankschtein, Understanding miltefosine–membrane interactions using molecular dynamics simulations, Langmuir 31 (2015) 4503–4512.
[4] P. Hilgard, T. Klenner, J. Stekar, C. Unger, Alkylphosphocholines: a new class of membrane-active anticancer agents, Cancer Chemother. Pharmacol. 32 (1993) 90–95.
[5] C. Munoz, K. Alzoubi, J. Jacobi, M. Abed, F. Lang, Effect of miltefosine on erythrocytes, Toxicol. in Vitro 27 (2013) 1913–1919.
[6] H. Sindermann, J. Engel, Development of miltefosine as an oral treatment for leishmaniasis, Trans. R. Soc. Trop. Med. Hyg. 100 (2006) S17–S20.
[7] M. Zhukova, O. Romanenko, V. Nikolaevich, Hemolytic properties of miltefosine in liposomes of various lipid compositions, Pharm. Chem. J. 44 (2010) 507–509.
[8] F. Danhier, O. Feron, V. Préat, To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery, J. Control. Release 148 (2010) 135–146.
[9] O.C. Farokhzad, R. Langer, Nanomedicine: developing smarter therapeutic and diagnostic modalities, Adv. Drug Deliv. Rev. 58 (2006) 1456–1459.
[10] D.A. Groneberg, M. Giersig, T. Welte, U. Pison, Nanoparticle-based diagnosis and therapy, Curr. Drug Targets 7 (2006) 643–648.
[11] A.S. Thakor, S.S. Gambhir, Nanooncology: the future of cancer diagnosis and therapy, CA Cancer J. Clin. 63 (2013) 395–418.
[12] R. Zeisig, I. Fichtner, D. Arndt, S. Jungmann, Antitumor effects of alkylphosphocholines in different murine tumor models: use of liposomal preparations, Anti-Cancer Drugs 2 (1991) 411–418.
[13] L.H. Lindner, M. Hossann, M. Vogeser, N. Teichert, K. Wachholz, H. Eibl, W. Hiddemann, R.D. Issels, Dual role of hexadecylphosphocholine (miltefosine) in thermosensitive liposomes: active ingredient and mediator of drug release, J. Control. Release 125 (2008) 112–120.
[14] A. Papagiannaros, S. Hatziantoniou, K. Dimas, G.T. Papaioannou, C. Demetzos, A liposomal formulation of doxorubicin, composed of hexadecylphosphocholine (HePC): physicochemical characterization and cytotoxic activity against human cancer cell lines, Biomed Pharmacother 60 (2006) 36–42.
[15] M. Teymouri, H. Farzaneh, A. Badiee, S. Golmohammadzadeh, K. Sadri, M.R. Jaafari, Investigation of hexadecylphosphocholine (miltefosine) usage in pegylated liposomal doxorubicin as a synergistic ingredient: in vitro and in vivo evaluation in mice bearing C26 colon carcinoma and B16F0 melanoma, Eur. J. Pharm. Sci. 80 (2015) 66–73.
[16] Z. Luo, J. Jiang, pH-sensitive drug loading/releasing in amphiphilic copolymer PAE–PEG: integrating molecular dynamics and dissipative particle dynamics simulations, J. Control. Release 162 (2012) 185–193.
[17] G.P. Mishra, D. Nguyen, A.W. Alani, Inhibitory effect of paclitaxel and rapamycin individual and dual drug-loaded polymeric micelles in the angiogenic cascade, Mol. Pharm. 10 (2013) 2071–2078.
[18] T. Wang, V.A. Petrenko, V.P. Torchilin, Paclitaxel-loaded polymeric micelles modified with MCF-7 cell-specific phage protein: enhanced binding to target cancer cells and increased cytotoxicity, Mol. Pharm. 7 (2010) 1007–1014.
[19] X.J. Loh, J.S. del Barrio, P.P.C. Toh, T.-C. Lee, D. Jiao, U. Rauwald, E.A. Appel, O.A. Scherman, Triply triggered doxorubicin release from supramolecular nanocontainers, Biomacromolecules 13 (2011) 84–91.
[20] X.J. Loh, Z.-X. Zhang, Y.-L. Wu, T.S. Lee, J. Li, Synthesis of novel biodegradable thermoresponsive triblock copolymers based on poly[(R)-3-hydroxybutyrate] and poly(*N*-isopropylacrylamide) and their formation of thermoresponsive micelles, Macromolecules 42 (2008) 194–202.
[21] X.J. Loh, S.J. Ong, Y.T. Tung, H.T. Choo, Dual responsive micelles based on poly[(R)-3-hydroxybutyrate] and poly(2-(di-methylamino) ethyl methacrylate) for effective doxorubicin delivery, Polym. Chem. 4 (2013) 2564–2574.
[22] C.K. Liu, Q. Dou, S.S. Liow, J.N. Kumar, X.J. Loh, Cationic micelles based on polyhedral oligomeric silsesquioxanes for enhanced gene transfection, Aust. J. Chem. 69 (2016) 363–371.
[23] X. Fan, Z. Li, X.J. Loh, Recent development of unimolecular micelles as functional materials and applications, Polym. Chem. 7 (2016) 5898–5919.
[24] C.O. Rangel-Yagui, A. Pessoa Jr., L.C. Tavares, Micellar solubilization of drugs, J. Pharm. Pharm. Sci. 8 (2005) 147–163.
[25] X.J. Loh, S.H. Goh, J. Li, Biodegradable thermogelling poly[(R)-3-hydroxybutyrate]-based block copolymers: micellization, gelation, and cytotoxicity and cell culture studies, J. Phys. Chem. B 113 (2009) 11822–11830.
[26] N.A.K. Meznarich, Effect of Ternary Solutes on the Evolution of Structure and Gel Formation in Amphiphilic Copolymer Solutions, The University of Michigan, 2012.
[27] S.R.M. Grallert, C.D.O. Rangel-Yagui, K.F.M. Pasqualoto, L.C. Tavares, Polymeric micelles and molecular modeling applied to the development of radiopharmaceuticals, Braz. J. Pharm. Sci. 48 (2012) 1–16.
[28] X. He, X. Wu, C. Gao, K. Wang, S. Lin, W. Huang, M. Xie, D. Yan, Synthesis and self-assembly of a hydrophilic, thermo-responsive poly(ethylene oxide) monomethyl ether-block-poly(acrylic acid)-block-poly(*N*-isopropylacrylamide) copolymer to form micelles for drug delivery, React. Funct. Polym. 71 (2011) 544–552.
[29] A.V. Kabanov, V.Y. Alakhov, Pluronic® block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002).
[30] W. Zhu, A. Yu, W. Wang, R. Dong, J. Wu, G. Zhai, Formulation design of microemulsion for dermal delivery of penciclovir, Int. J. Pharm. 360 (2008) 184–190.
[31] C.O. Rangel-Yagui, H.W. Hsu, L.R. Barbosa, W. Caetano, A. Pessoa Jr., L.C. Tavares, R. Itri, Novel potential drug against *T. cruzi* and its interaction with surfactant micelles, Pharm. Dev. Technol. 12 (2007) 183–192.
[32] G. Ciapetti, E. Cenni, L. Pratelli, A. Pizzoferrato, In vitro evaluation of cell/biomaterial interaction by MTT assay, Biomaterials 14 (1993) 359–364.
[33] B. de Castro, P. Gameiro, J.L. Lima, C. Matos, S. Reis, Interaction of drugs with hexadecylphosphocholine micelles. Derivative spectroscopy, acid–base and solubility studies, Mater. Sci. Eng. C 18 (2001) 71–78.
[34] R.A. Fule, T.S. Meer, A.R. Sav, P.D. Amin, Artemether-soluplus hot-melt extrudate solid dispersion systems for solubility and dissolution rate enhancement with amorphous state characteristics, J. Pharm. 2013 (2013).
[35] R.C. Rowe, P.J. Sheskey, M.E. Quinn, Handbook of Pharmaceutical Excipients, 6° ed., Pharmaceutical Press, 2009.
[36] M.E. Davis, D.M. Shin, Nanoparticle therapeutics: an emerging treatment modality for cancer, Nat. Rev. Drug Discov. 7 (2008) 771–782.
[37] T. Calogeropoulou, P. Angelou, A. Detsi, I. Fragiadaki, E. Scoulica, Design and synthesis of potent antileishmanial cycloalkylidene-substituted ether phospholipid derivatives, J. Med. Chem. 51 (2008) 897–908.
[38] 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.
[39] Y. Wang, L. Yu, L. Han, X. Sha, X. Fang, Difunctional pluronic copolymer micelles for paclitaxel delivery: synergistic effect of folate-mediated targeting and Pluronic-mediated overcoming multidrug resistance in tumor cell lines, Int. J. Pharm. 337 (2007) 63–73.
[40] M. Nilsson, B. Håkansson, O. Söderman, D. Topgaard, Influence of polydispersity on the micellization of triblock copolymers investigated by pulsed field gradient nuclear magnetic resonance, Macromolecules 40 (2007) 8250–8258.
[41] A. Albanese, E.A. Sykes, W.C. Chan, Rough around the edges: the inflammatory response of microglial cells to spiky nanoparticles, ACS Nano 4 (2010) 2490–2493.
[42] G.S. Kwon, K. Kataoka, Block copolymer micelles as long-circulating drug vehicles, Adv. Drug Deliv. Rev. 16 (1995) 295–309.
[43] M. Bragagni, C. Beneitez, C. Martín, D.H.P. de la Ossa, P.A. Mura, M.E. Gil-Alegre, Selection of PLA polymers for the development of injectable prilocaine controlled release microparticles: usefulness of thermal analysis, Int. J. Pharm. 441 (2013) 468–475.
[44] C. Rouzes, M. Leonard, A. Durand, E. Dellacherie, Influence of polymeric surfactants on the properties of drug-loaded PLA nanospheres, Colloids Surf. B: Biointerfaces 32 (2003) 125–135.
[45] A. Sahu, N. Kasoju, P. Goswami, U. Bora, Encapsulation of curcumin in Pluronic block copolymer micelles for drug delivery applications, J. Biomater. Appl. 25 (2011) 619–639.
[46] P. Mishra, D. Javia, Development and Performance Evaluation of Miltefosine Loaded Nanostructured Lipid Carriers for Managements of Viceral Leishmaniasis, National Institute of Pharmaceutical Education and Research, India, 2015.
[47] A. Yildirim, G.B. Demirel, R. Erdem, B. Senturk, T. Tekinay, M. Bayindir, Pluronic polymer capped biocompatible mesoporous silica nanocarriers, Chem. Commun. 49 (2013) 9782–9784.
[48] T. Yu, A. Malugin, H. Ghandehari, Impact of silica nanoparticle design on cellular toxicity and hemolytic activity, ACS Nano 5 (2011) 5717–5728.
[49] M.M. Eissa, R.M. El-Moslemany, A.A. Ramadan, E.I. Amer, M.Z. El-Azzouni, L.K. El-Khordagui, Miltefosine lipid nanocapsules for single dose oral treatment of *Schistosomiasis mansoni*: a preclinical study, PLoS One 10 (2015) e0141788.