

Preparation and Characterization of Spray-Dried Inhalable Powders Containing Polymeric Micelles for Pulmonary Delivery of Paclitaxel in Lung Cancer

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ABSTRACT - Purpose: Local delivery of chemotherapeutic drugs to the lungs offers many advantages for lung cancer treatment compared to conventional systemic chemotherapy. In the present study, novel mixed polymeric micelles based on tocopheryl succinate-polyethylene glycol 1000 and 5000 Da (TPGS_{1K} and TPGS_{5K}) were synthesized and loaded with paclitaxel (PTX). Then, the optimized micelles were incorporated as colloidal drug delivery system into lactose carrier particles using a spray drying technique. Methods: The mixed micelles of TPGS_{5K} and TPGS_{1K} in different molar ratios (10:0, 7:3, 5:5, 3:7, 0:10) were prepared and physicochemical properties including: particle size, zeta potential, critical micelle concentration (CMC), drug loading, drug release rate, and *in vitro* cytotoxicity were investigated in details. The optimized nanoparticles were co-spray dried with lactose carriers to produce the spherical particle morphology of the inhalable particles. Results: Particle sizes and zeta potentials of the different formulations varied in the range of 102 to 196 nm and -9.4 to -13.8 mV, respectively. The lowest CMC values were calculated for 5:5 and 7:3 combinations (16.33 and 17.89 μ M, respectively). The drug release rate from different formulations were very slow and only 30% of the drug was released during 72 h. Cytotoxicity assay demonstrated increased cytotoxic activity of PTX-loaded mixed micelles compared to the free drug. The *in vitro* deposition data indicated that spray drying of PTX-loaded micelles with lactose resulted in the production of inhalable powders with the high fine particle fraction (60%). Conclusion: These results demonstrate that this novel PTX-loaded micelles embedded in dry powder inhalation aerosol platform has a great potential to be used in lung cancer treatment.

INTRODUCTION

Local delivery of drugs to the lungs is highly desirable for the treatment of pulmonary diseases such as lung cancer, tuberculosis, chronic obstructive pulmonary diseases, asthma, and other respiratory infections [1]. The main advantages of the local treatment of pulmonary disorders include rapid onset of action, higher drug concentrations at the site of action, lower dose requirements, and fewer systemic side effects [1,2]. In addition, if local pulmonary administration is accompanied with the sustained release property, the therapeutic outcomes of a drug could be efficiently enhanced. Sustained release of drugs in the lungs might result in favorable therapeutic index by prolonging residence time at the target site, reducing its side effects, and enhancing patient compliance [3]. Regional delivery of cytotoxic drugs has been recently proposed as a promising treatment strategy for lung cancer.

Inhalation of chemotherapeutic agents in primary or metastatic lung cancer, could enhance exposure of tumor cells to the drug while minimize systemic side effects [3]. Some animal studies have demonstrated the pharmacokinetic advantages and anti-tumor effect of aerosol delivery of chemotherapeutic solutions such as paclitaxel (PTX) and doxorubicin [4, 5]. However, regional toxicity is the main obstacle for clinical application of inhaled solution of cytotoxic agents. In recent years, there is an increasing interest in the development of inhalable nanocarriers for lung cancer treatment. Nanocarriers such as liposomes, nanoparticles, and polymeric micelles are able to encapsulate lipophilic cytotoxic drugs resulting in reduction of drug-associated

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toxicity and accumulation of their cargo in cancer tissue via enhanced permeation and retention (EPR) effect [6-8]. Moreover, mucociliary clearance and macrophages phagocytosis can be overcome by using nano-size systems [4]. However, one limitation of nano-size carriers for pulmonary delivery of drugs is that their sizes are not suitable for deep lung deposition and they are easily exhaled from the respiratory tract [9]. The most common approach to proper inhalable powders is using a carrier system such as lactose or manitol microparticles. These carbohydrates are non-toxic, readily degradable and approved by Food and Drug Administration (FDA) as excipients for inhalation purposes [9]. The carrier particles should ensure an appropriate mass median aerodynamic diameter (MMAD) and a suitable fine particle fraction (FPF) for a maximum alveolar deposition of the embedded drugs at normal inhalation rates. Carrier particles are dissolved after coming in contact with the aqueous medium and release the nanoparticles. Azarmi *et al.* developed doxorubicin-loaded nanoparticles and incorporated into lactose carriers using a spray-freeze-drying technique [10]. They demonstrated the incorporation of nanoparticles did not affect the FPF or MMAD of the microparticles. In another study hematoporphyrine was encapsulated into polymeric micelles and subsequently incorporated into lactose carriers by spray drying [11]. The results revealed that the micelles remained stable after dissolution of the lactose microparticles [11]. Armita and co-workers investigated the in-vitro deposition pattern of cisplatin from dry powder containing the drug-loaded gelatin nanoparticles [12]. They demonstrated the possibility of delivering the nanoparticles to the lungs by incorporating them into carrier particles by simple freeze drying technique [12]. PTX is extensively used for the treatment of a broad spectrum of cancers specially breast, ovarian, and non-small lung cancers [13]. Thus, PTX represents one of the best candidates to be delivered into the lungs. Alipour *et al.* fabricated alginate microparticles for pulmonary delivery of PTX [14]. The *in vitro* release profile demonstrated slow release rate of PTX from microparticles and the drug-loaded particles inhibited the growth of human non-small cell lung cancer cell line A549 [14]. However, these microparticles due to their large particle sizes would be rapidly taken up by alveolar macrophages and that is the major practical challenge in clinical application of polymeric microparticles for pulmonary delivery. *In vitro*

studies have demonstrated that using nano-size carriers could prevent macrophages phagocytosis and enhance efficient drug uptake by epithelial and cancer cells [15-17]. For instances, in the work conducted by Gill *et al.*, [17], intratracheally administered PTX-loaded polymeric micelles showed higher accumulation of PTX in the lungs and lower content of the drug to non-targeted organs compared to intravenously administered formulation. In other similar studies, solid lipid nanoparticles, liposomes and polymeric nanoparticles were investigated for pulmonary delivery of PTX [18-20]. In all aforementioned studies, the nanoparticles were used intratracheally which could not be applied clinically. In the current study, we aimed to formulate a dry powder particulate system embedding PTX-loaded polymeric micelles suitable for pulmonary delivery. Here we fabricated polymeric micelles from the mixture of two different molecular weights, 1000 and 5000 Da, of α -tocopheryl polyethylene glycol succinate (TPGS_{1K} and TPGS_{5K}). TPGS_{1K}, a water-soluble derivative of natural vitamin E, is synthesized by esterification of α -tocopheryl succinate (α -TS) with polyethylene glycol 1000 (PEG) and has been approved by FDA as a safe pharmaceutical adjuvant used in drug formulation. TPGS_{1K} has an amphiphilic structure which forms micelles in aqueous media. PTX has a high solubility in α -TS (11 mg/mL) which enhances polymer-drug miscibility thereby improving drug loading into the micelles [21]. It has also been found that TPGS_{1K} could inhibit drug efflux pumps or p-glycoproteins (p-gp) mediated multi-drug resistance, thus facilitates permeation of the drugs to the cells and greatly enhances the therapeutic effects of antitumor agents. In addition, TPGS could effectively inhibit the growth of cancer cells by its ability to induce apoptosis [21,22]. Despite the unique advantages, TPGS_{1K} has a high CMC value (0.12 mg/mL), which decreases the stability of micelles in physiological environment. Thus, till now, TPGS_{1K} has been usually used together with other lipids or synthetic copolymers to form micelles and the presence of TPGS_{1K} in the micelles has greatly improved the drug encapsulation efficiency and decreased the release rate of loaded drugs [23,24]. In this work, we synthesized a new TPGS_{5K} amphiphilic polymer via reaction between α -TS and methoxy PEG of molecular weight 5000 (MePEG5000). The new synthesized TPGS_{5K} showed much lower CMC value compared with the traditional TPGS_{1K}. This change

makes it possible to form stable micelles by TPGS alone without any other polymers or lipids. Therefore, this work focuses on synthesis of TPGS_{5K} which are mixed with TPGS_{1K} to form micelles and solubilize PTX. The drug-loaded optimized formulation are subsequently entrapped in lactose microparticles to render them suitable for pulmonary delivery. The physicochemical properties of the micelles and carriers are investigated in details. Moreover, the *in vitro* cytotoxicities of the different formulations are evaluated and compared with Stragen[®] (commercial formulation of PTX in Cremophor[®] EL) against human lung cancer cell line, A549.

METHODS AND MATERIALS

MATERIALS

α -Tocopheryl polyethylene glycol 1000 succinate (TPGS_{1K}) was purchased from Eastman Co. (USA). Paclitaxel (PTX), methoxypolyethylene glycol (MePEG, $M_w = 5000$ Da), dicyclohexylcarbodiimide (DCC), 4-(dimethylamino) pyridine (DMAP), pyrene, anhydride dimethyl sulfoxide (DMSO), and anhydrous dichloromethane (DCM) were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade methanol and acetonitrile were supplied by Caledon (Ontario, Canada). Paclitaxel Stragen[®] (30 mg/5 mL) was provided by Sobhan Oncology (Rasht, Iran). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), fetal bovine serum (FBS) and antibiotics for cell culture were provided by Sigma–Aldrich (St. Louis, MO). Human non-small cell lung cancer cell line (A549) was obtained from Pasteur institute of Iran (Tehran, Iran). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied by Sigma–Aldrich.

METHODS

Synthesis of TPGS_{5K}

TPGS_{5K} was synthesized through the reaction of activated carboxyl group of D- α -tocopheryl succinate (α -TS) and hydroxyl group of MePEG in the presence of DCC and DMAP. Briefly, α -TS (265 mg, 0.5 mmol), DCC (206 mg, 1 mmol), and DMAP (30.5 mg, 0.25 mmol) were dissolved in anhydrous DCM and stirred under nitrogen for 24 h in dark to activate the carboxyl group of α -TS. The solution of

MePEG (500 mg, 0.1 mmol) in DCM was added into the activated α -TS solution and the reaction mixture was stirred for 48 h under nitrogen. The mixture was then dialyzed against ethanol for 48 h to remove unreacted α -TS, followed by dialysis against water for 24 h using dialysis membrane (MWCO: 2 kDa, Viskase Companies Inc., Darien, IL) and lyophilized to get pure TPGS_{5K} powder (Freeze Dryer Model ALPHA 2-4 LD plus, Christ Company, Stuttgart, Germany). The chemical structure of TPGS_{5K} was identified and calculated by ¹H-NMR (Bruker, Biospin, AC-400, Mannheim, Germany) and Fourier transform infrared (FT-IR) (WQS-510/520, Raileigh, China) spectra.

Preparation of the blank and PTX-loaded micelles

To prepare empty mixed micelles, TPGS_{5K} alone or in mixture with TPGS_{1K} at different molar ratios (10:0 to 0:10) was dissolved in DCM and the organic solvent was then removed by rotary vacuum evaporation. The resultant film was hydrated with a suitable volume of deionized water, incubated at 37 °C for 30 min, and then sonicated for a few minutes. The drug-loaded polymeric micelles were prepared by dissolving 3 mg PTX in DCM which was then added to 10 mg polymer mixture already dissolved in DCM [21]. The micelle preparation steps described above were exactly repeated. The PTX-loaded micellar solution was then centrifuged at 7000 rpm for 10 min, and filtered through a 0.45 μ m pore size microfiltration membrane to remove unloaded drug. Drug-loaded micelles were lyophilized and kept at 4°C until further evaluations.

Characterization of the micelles

Measurement of particle size and zeta potential

The average hydrodynamic diameters and zeta potentials of the mixed micelle solutions (1 mg/mL) in phosphate buffer saline (PBS, pH 7.4) were measured with dynamic light scattering (DLS) using a ZetaSizer (3000HS, Malvern Instruments Ltd., Malvern, UK).

As outlined in the next section, the micelles were spray-dried with lactose to produce respirable micron size particles with a narrow particle distribution. Therefore, micelle size was also determined after spray drying of the micelles with lactose. A 50 mg aliquot of the micelles embedded in lactose powder was dissolved in 4 mL of distilled water and the particle size was determined directly

without any further dilution. All measurements were carried out at 25 °C and performed in triplicate.

Determination of critical micelle concentrations

Critical micelle concentration (CMC) was determined by a fluorescence probe technique using pyrene as a fluorescence probe [21]. Briefly, 2 mg/mL of the polymer mixtures at different molar ratios were prepared in DCM. Different volumes of these solutions were added to 15-mL empty vials. Then 1 mL of 6×10^{-6} M solution of pyrene in DCM was added in every vial and mixed well. The DCM was left to evaporate for 24 h in order to form a pyrene film in the vials. Finally, 10 mL deionized water was added to the vials to obtain a final pyrene concentration of 6×10^{-7} M and the polymer solutions with concentrations ranging from 0.001 mg/mL to 1 mg/mL at different weight ratios. The solutions were kept on a shaker at 37 °C for 24 h to reach equilibrium before fluorescence measurement. Fluorescence spectra were recorded using a spectrofluorometer (Jasco FP 750, Tokyo, Japan) with the excitation wavelength at 336 nm. From the pyrene emission spectra, the intensity ratio of the first peak (I_1 , 388 nm) to the third peak (I_3 , 391 nm) was plotted against the logarithm of polymer concentrations. Two tangents were then drawn and the CMC values were taken from the intersection between the two tangents.

Determination of drug loading

Two mg of the lyophilized sample was dissolved in 25 mL of acetonitrile and the solution was ultrasonicated for 10 min to entirely dissolve PTX-loaded micelles. The resultant solution was filtered through 0.45 μ m filter unit and PTX concentration was determined by HPLC method developed and validated in our laboratory [25]. Chromatographic separation was performed using a reversed-phase C₁₈- μ Bondapak (3.9 mm \times 250 mm) HPLC column. The mobile phase consisted of potassium dihydrogen phosphate (0.01 M)/acetonitrile (52:48) with final pH adjusted to 4 ± 0.1 with orthophosphoric acid. The mobile phase eluted at 1.5 mL/min and the effluent was monitored at 227 nm using a UV detector. Column temperature was kept at 40°C and 30 μ L of the sample was injected into the HPLC column. The calibration curve constructed in the range of 2.5 – 12 μ g/mL of PTX was linear ($r^2 > 0.998$). The inter- and intra-day precision and accuracy of the assay were less than 10%. Drug entrapment efficiency (EE) and drug loading (DL) were calculated by following equations:

$$EE\% = \frac{\text{weight of the drug in the micelles}}{\text{weight of the feeding drug}} \times 100$$

$$DL\% = \frac{\text{weight of the drug in the micelles}}{\text{weight of the micelles}} \times 100$$

Drug release studies

The lyophilized powder of PTX-loaded micelles (2 mg) was dissolved in 2 mL of phosphate buffer solution (0.1 M, pH 7.4). The resulting solution was placed in a dialysis bag (MWCO: 10 kDa) which was immersed in a glass beaker with 100 mL phosphate buffer (0.1 M, pH: 7.4) containing 0.2 % tween 80 to provide sink condition with agitation of 100 rpm at 37 °C [21]. Sample aliquots were withdrawn from the release medium at pre-determined time intervals and replaced with fresh buffer. The content of PTX was determined by the above described HPLC method. Dissolution efficiency (DE) was used to compare the release rates amongst formulations. DE of release profiles were calculated from the area under the curve at time t_j (measured using the trapezoidal rule) and expressed as the percentage of the area of the rectangle described by 100% dissolution in the same time [21]

Cell culture

Human lung cancer cells, A549, were maintained in DMEM/F12 supplemented with 10% (v/v) FBS and 1% penicillin–streptomycin at 37 °C and 5% CO₂. Cells were subcultured regularly using trypsin/EDTA.

Cytotoxicity assay

The cytotoxicities of the free drug in DMSO, PTX-loaded $\frac{\text{TPGS}_{5K}}{\text{TPGS}_{1K}}$ (7:3), blank $\frac{\text{TPGS}_{5K}}{\text{TPGS}_{1K}}$ (7:3), TPGS_{5K} micelles and Stragen[®] were assessed against A549 cell line using MTT assay. The cells were seeded at density of 7×10^4 cells/mL in a 96-well culture plate (SPL Lifescience, Gyeonggi-Do, Korea), and following an overnight cultivation for the cells to be attached to the wells, the cells were treated with samples for 72 h at the equivalent PTX concentrations of 0.03–3 μ g/mL.

After incubation, 20 μ L of MTT solution (5 mg/mL in 0.02 M phosphate buffer) was added to each well and the plate was incubated for another 4 h. Subsequently, unreacted MTT and medium was removed and the formazan crystals in cells were dissolved in 150 μ L of DMSO. The absorbance was

measured at 570 nm using enzyme-linked immunosorbent assay plate reader (Stat Fax-2100; Awareness Technology Inc., Palm City, FL). Untreated cells were taken as the negative control

with 100% viability and the blank culture medium was used as a blank control [25]. Cell viability for each sample was calculated using following equation.

$$\text{Cell viability}\% = \frac{\text{mean absorbance of each group} - \text{mean absorbance of blank}}{\text{mean absorbance of negative control} - \text{mean absorbance of blank}} \times 100$$

Spray drying procedure

Five grams of lactose was dissolved in 75 mL of distilled water and heated up to 40 °C to completely solubilize the lactose. Then the solution was mixed with 25 mL of colloidal solution of PTX-loaded micelles equivalent to 10 mg of PTX. The dispersion was spray dried using a Buchi mini spray dryer B-290 (Flawil, Switzerland) with pneumatic atomizer through a 1.4 mm nozzle. The spray drying was performed using pump flow rate of 2 mL/min, 85% aspiration, inlet temperature of 50 ± 5 °C with an

outlet temperature of 60–65 °C [26]. The resultant powder was blown through the cyclone separator and collected in a container. Exhaust air extracted out of the cyclone by a vacuum pump and filtered through a fiber filter.

The process yield and drug content uniformity of the spray dried powders

The spray-drying process yield was calculated by gravimetric method, comparing the initial total solids amount with the resultant powder amount after spray drying as follows [27]:

$$\text{process yield}\% = \frac{\text{weight of resultant powder}}{\text{total solid (lactose + PTX - loaded micelles)}} \times 100$$

In order to study drug content uniformity in the spray dried samples, 5 mL of acetonitrile was added to 5 mg of spray dried powder and the suspension was sonicated for 10 min using a bath sonicator to dissolve PTX. The resultant solution was filtered through 0.45 µm filter unit and PTX concentration was determined by the HPLC method. The experiment was performed on three spray-dried samples.

Scanning electron microscopic analysis

Surface morphology of the lactose-micellar particles was examined using a scanning electron microscope (SEM; Hitachi, S-2400, Japan). The microparticles were vacuum dried, coated with gold palladium and observed microscopically.

Deposition studies spray-dried powder

The powders were aerosolized using a dry powder inhalation device (spinhaler). The *in vitro* deposition of the aerosolized drug was investigated using Andersen cascade impactor (ACI, Thermo Andersen,

Smyrna, GA). A total of 10 mg of each spray-dried powder was loaded into hard gelatin capsules (size 3). An air stream of 60 L/min was produced throughout the system by attaching the outlet of the using ACI to a vacuum pump for 4 s. The powder deposited in stages 0 to 7, the mouthpiece and the stages were collected and the drug content in each stage was extracted by acetonitrile. The drug contents were then determined using HPLC method. From drug deposition data, the fine particle fraction (FPF_{ED < 5.5 µm}), mass median aerodynamic diameter (MMAD), and percentage of emitted dose (ED%) were calculated. FPF is defined as the fraction of the total dose of the powder less than or equal to an effective cut-off aerodynamic diameter of 5.5 µm which is calculated by dividing the powder mass recovered from stage 1-7 of the impactor by the total particle mass in the capsule. MMAD was calculated by linear interpolation of the cumulative mass distribution to obtain the particle size for which the cumulative mass under this size was 50%. Emitted dose was calculated as follow:

$$ED = \frac{\text{initial mass in capsule} - \text{final mass remaining in capsule}}{\text{initial mass in capsule}} \times 100$$

STATISTICAL ANALYSIS

Data are expressed as means of three separate experiments and were compared by independent sample t-test for two groups, and one-way ANOVA following LSD Post Hoc test for multiple groups. A p -value < 0.05 was considered statistically significant in all cases. All statistical analyses were performed using SPSS 17.

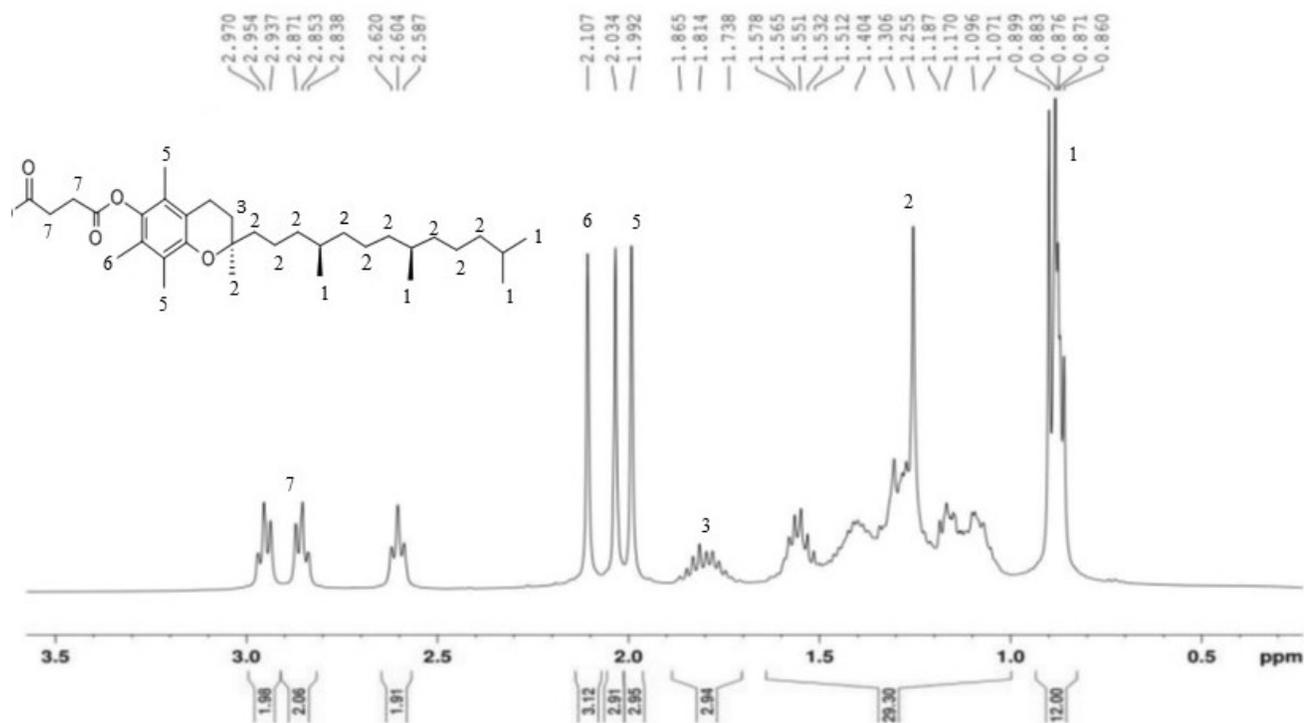
RESULTS

Synthesis of TPGS_{5K}

The formation of TPGS_{5K} was confirmed by ¹H-NMR and FT-IR spectra. As shown in Fig. 1, peaks around 0.86 and 1–1.5 ppm, which were attributed to the methyl and methylene protons of the long alkyl chain of α -TS, are present in the spectrum of TPGS_{5K} (Fig. 1c), while no such peaks exist at the same chemical shifts in the spectrum of MePEG (Fig. 1b). A strong signal around 3.5 ppm in Fig. 1b and Fig. 1c are assigned to hydrogens of the repeating units (CH₂CH₂O) of PEG. The hydrogens of methylene

group of PEG adjacent to the hydroxyl group, OH-CH₂-CH₂-O-(CH₂CH₂O)_n-, appeared at 3.77 ppm in the spectrum of MePEG which shifted to 3.73 ppm in the spectrum of TPGS indicating ester bond formation.

The coupling reaction between α -TS and MePEG was further confirmed by FT-IR analysis. The FT-IR spectra of α -TS, MePEG, and the final product are shown in Fig 2. In FT-IR spectrum of α -TS (Fig. 2a), the absorption band around 1720 cm⁻¹ related to the stretching vibration of the carbonyl group of succinyl disappeared in the spectrum of TPGS (Fig. 2c) and another band at 1752 cm⁻¹ due to the C=O stretching vibration of newly formed ester group is raised. MePEG (Fig. 2b) and TPGS_{5K} spectra had the same band at 1109 cm⁻¹ which corresponded to the presence of C-O stretch of PEG. Furthermore, the absorption bands at 2931 and 2860 cm⁻¹ representing asymmetric and symmetric stretching vibrations of the CH₂ and CH₃ of alkyl chain of α -TS were observed in the spectrum of TPGS_{5K}. These observations confirm the formation of ester bond between the carboxyl group of α -TS and the end hydroxyl group of MePEG.



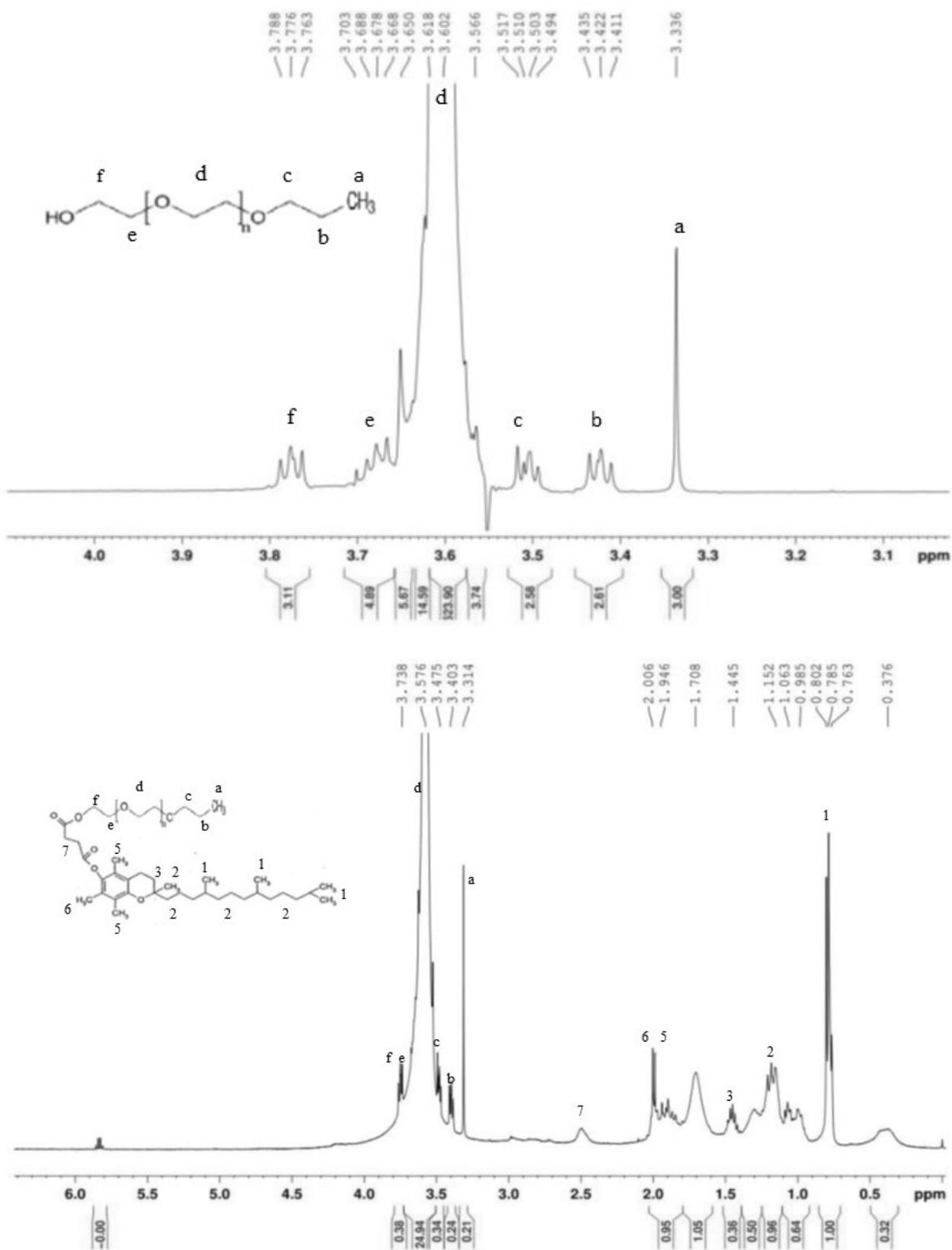


Figure 1. ¹H-NMR spectra of (a) α-TS in DMSO-d₆, (b) MePEG in D₂O, and (c) TPGS_{5K} in D₂O.

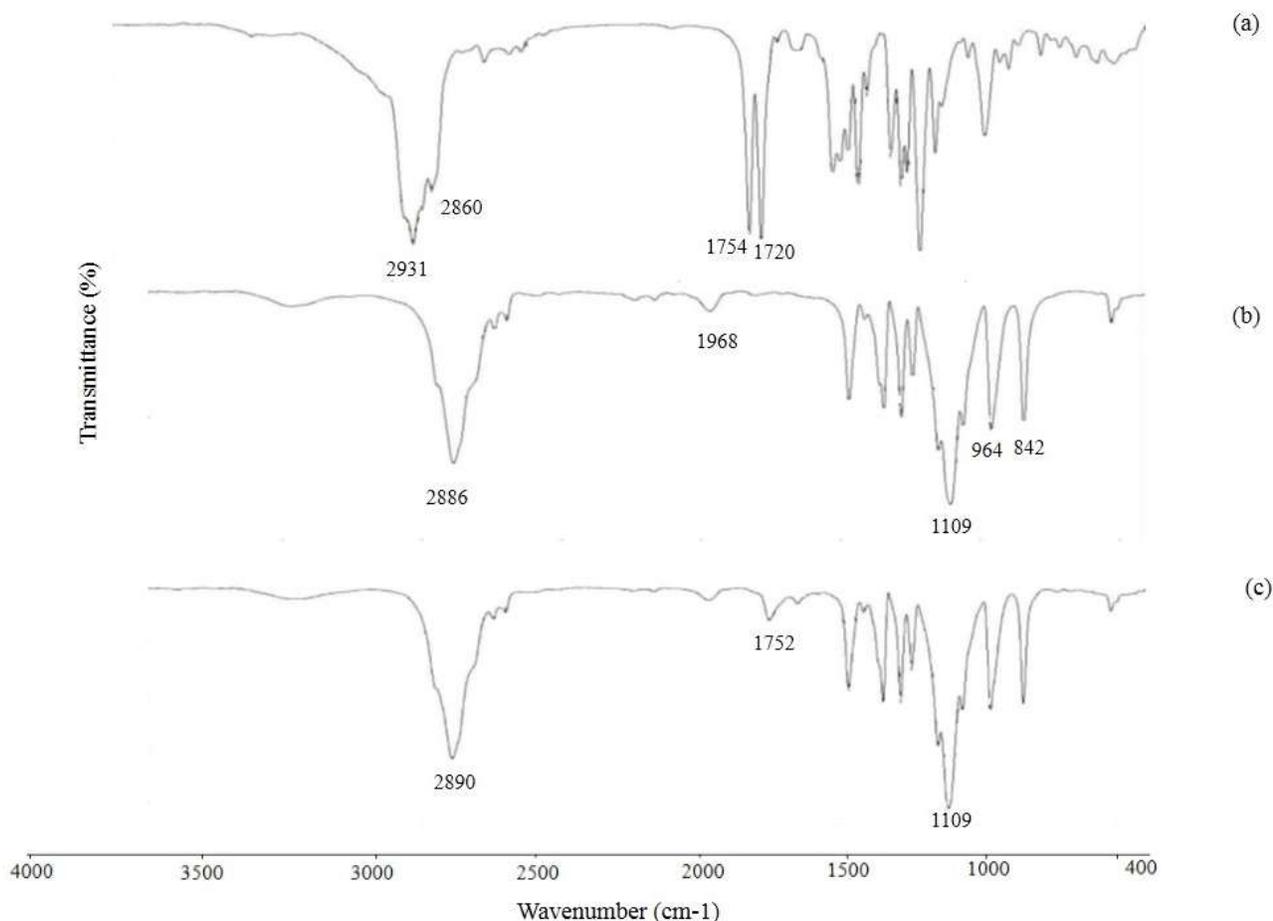


Figure 2. FT-IR spectra of (a) α -TS, (b) MePEG, and (c) TPGS_{5K}.

Characterization of blank and PTX-loaded micelles

Fig. 3 shows the changes of fluorescence intensity ratios (I_1/I_3) against the logarithm of TPGS_{5K}/TPGS_{1K} in different molar ratios. The CMC of different mixtures in deionized water are also presented in Table 1. The CMC values decreased from 360 μ M for TPGS_{1K} to 8.4 μ M for TPGS_{5K}. As revealed, increasing the content of TPGS_{5K} in the mixtures has significantly decreased the CMC value of the micelles. The CMC values for TPGS_{5K}/TPGS_{1K} (7:3) and TPGS_{5K}/TPGS_{1K} (5:5) were found to be 17.89 and 16.33 μ M, respectively, which are much lower than those of low molecular weight surfactants in water and indicate the micellar stability in biological environment. Table. 1 also listed the average sizes and zeta potentials of the lyophilized mixed micelles. The micellar sizes significantly increased from 102.6 ± 7.2 nm for TPGS_{1K} to 195.6 ± 18.6 nm for TPGS_{5K} micelles and

the mixtures containing different quantity of TPGS_{5K} did not show significant changes in their sizes ($P > 0.05$). The zeta potentials were varied from -13.8 to -9.4 mV and did not significantly change ($P > 0.05$) between different formulations. The EE varied from 30% to 41% in the different combinations. The loading capacity of the micelles prepared by TPGS_{1K} alone was about 10% which reflected the low stability of TPGS_{1K} micelles to incorporate the drug. In the current study, incorporation of TPGS_{5K} as a part of micelle structure increased the EE to 40%.

In vitro drug release studies

In vitro PTX release profiles from different micellar formulations and Stragen[®] are shown in Fig. 4. The release rates of PTX from the micellar formulations were very slow and between 30-40% of the loaded drug was released after 72 h. In comparison, PTX was rapidly released from Stragen[®] and 70% of the drug was released during 48 h. the DE%₇₂ for 10:0,

7:3, and 5:5 micellar combinations were calculated $22.25\% \pm 3.7$, $24.93\% \pm 2.2$, and $19.73\% \pm 2.3$ respectively and significant differences were not observed ($P > 0.05$). While, the $DE\%_{72}$ of Stragen[®] ($58.87\% \pm 7.2$) was significantly greater ($P < 0.05$) than micellar formulations indicating the slower drug release pattern.

In vitro cytotoxicity studies

Fig. 5 compares cytotoxic effects of free PTX in DMSO, Stragen[®], blank, and the drug-loaded micelles against A549 cells. Stragen[®] and free PTX showed similar cytotoxicity in the range of 0.03 to 1.5 $\mu\text{g}/\text{mL}$ of PTX concentrations. However, the cytotoxicity of the free drug did not significantly

change in concentrations higher than 1 $\mu\text{g}/\text{mL}$ which might be attributed to the precipitation of the drug at high concentrations. The higher cytotoxic effect ($p < 0.05$) of Stragen[®] compared to the free drug at concentrations of 2 and 3 $\mu\text{g}/\text{mL}$ could be due to the cytotoxicity of its vehicle, Cremophor[®] EL. PTX-loaded mixed micelles exhibited the highest cytotoxicity among studied groups which was significant at concentrations higher than 1 $\mu\text{g}/\text{mL}$. The empty micelles did not show any measurable toxicity at low concentrations ($< 1 \mu\text{g}/\text{mL}$), however, the viability of the cells was significantly decreased to about 75% once the micelle concentrations increased which imply the cytotoxic effect of TPGS.

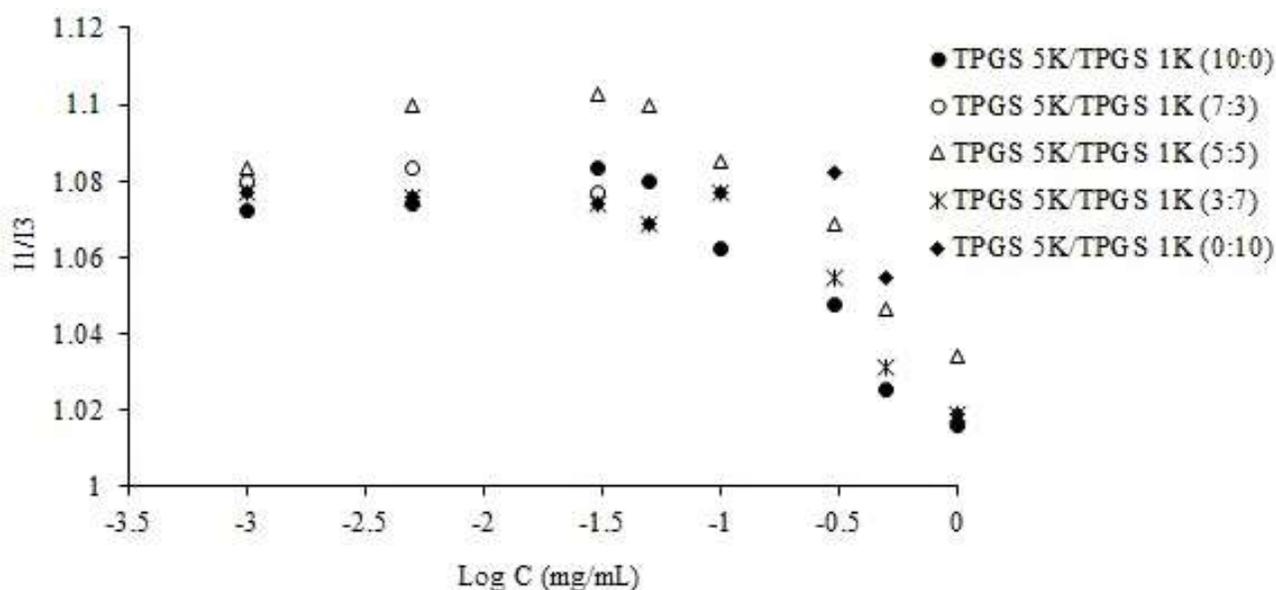


Figure 3. Fluorescence intensity variations of I_1/I_3 for pyrene emission against the logarithm of TPGS_{5K}/TPGS_{1K} concentrations

Table 1. Characteristics of blank and drug-loaded TPGS_{5K}/TPGS_{1K} mixed micelles.

formulations	CMC (μM)	Micellar size (nm)	Zeta potential (mV)
TPGS _{5K} /TPGS _{1K} (10:0)	8.4	195.6 \pm 18.6	-13.8 \pm 1.6
TPGS _{5K} /TPGS _{1K} (7:3)	17.89	175.1 \pm 19.2	-12.7 \pm 1.9
TPGS _{5K} /TPGS _{1K} (5:5)	16.33	145.2 \pm 12.3*	-11.9 \pm 2.1
TPGS _{5K} /TPGS _{1K} (3:7)	65	115.8 \pm 9.5*	-10.6 \pm 0.8
TPGS _{5K} /TPGS _{1K} (0:10)	360	102.6 \pm 7.2*	-9.4 \pm 1.2
	EE % (DL%)	Micellar size (nm)	Zeta potential (mV)
PTX/TPGS _{5K} /TPGS _{1K} (10:0)	41.22 \pm 5.3 (11.8 \pm 1.4)	255.2 \pm 21.2	-7.86 \pm 1.1
PTX/TPGS _{5K} /TPGS _{1K} (7:3)	41.36 \pm 6.1 (12.4 \pm 1.6)	244 \pm 19.5	-7.68 \pm 0.8
PTX/TPGS _{5K} /TPGS _{1K} (5:5)	37.7 \pm 3.9 (11.3 \pm 1.1)	261.9 \pm 26.3	-7.62 \pm 0.9
PTX/TPGS _{5K} /TPGS _{1K} (3:7)	30.9 \pm 2.8 (9.27 \pm 0.94)*	222.8 \pm 25.1*	-5.19 \pm 0.8
PTX/TPGS _{5K} /TPGS _{1K} (0:10)	10.1 \pm 1.1 (2.2 \pm 0.2)*	118.6 \pm 12.5*	-5.3 \pm 0.5

EE: entrapment efficiency

DL: drug loading

*P value ≤ 0.05 compared to TPGS_{5K}/TPGS_{1K} (10:0)

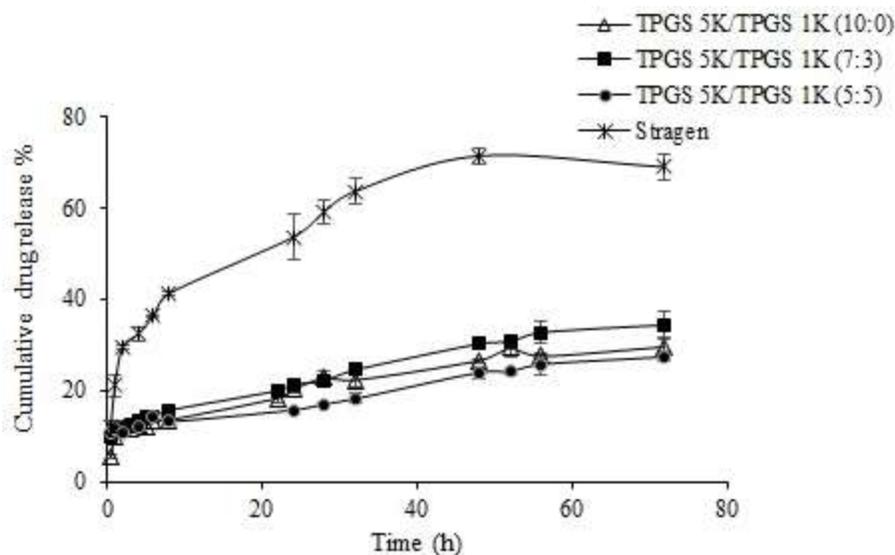


Figure 4. *In vitro* drug-release profiles from Stragen® and the mixed micelles with different molar ratios of the structural composition. Data represent the mean ± standard deviation (n = 3).

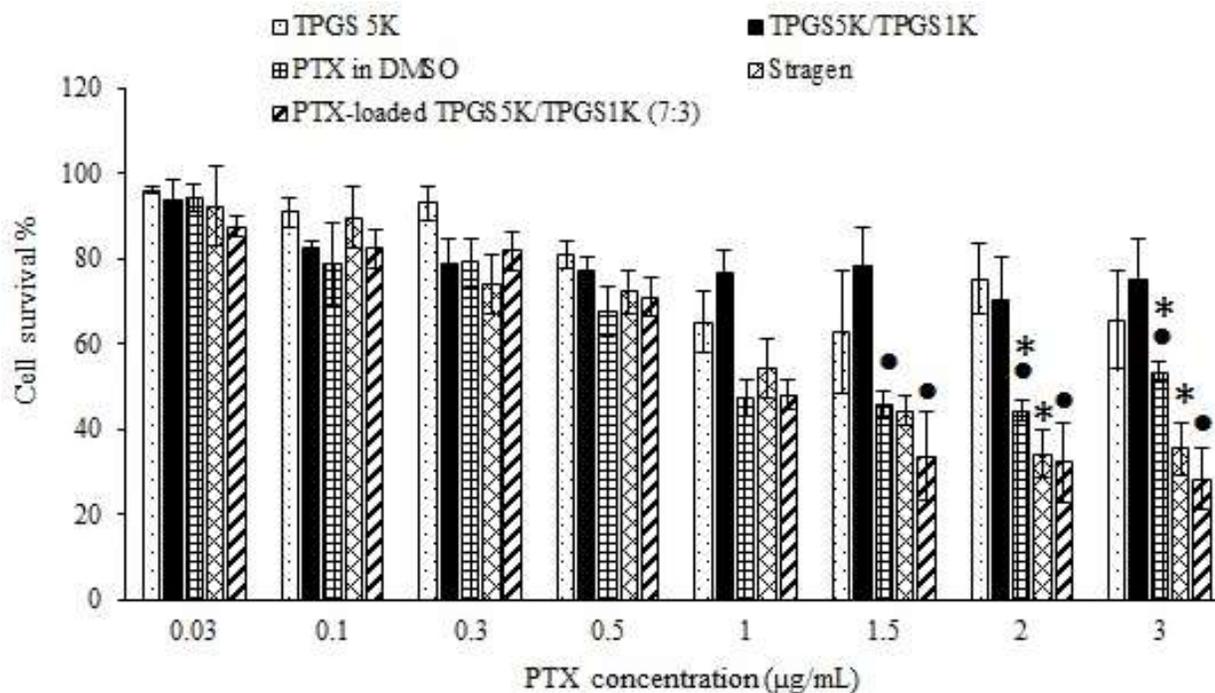


Figure 5. *In vitro* cytotoxic activities of various PTX formulations and blank micelles against A549 cell line after 72 h incubation. Data are plotted as the mean ± standard deviation (n = 3) *• P< 0.05 *Higher cytotoxic effect ($p < 0.05$) of Stragen® compared to the free drug at concentrations of 2 and 3 µg/mL •Significant greater cytotoxicity of PTX-loaded TPGS5K/TPGS1K micelles compared to the free drug at concentrations higher than 1 µg/mL

Characterization of spray-dried powders

The mean particle sizes of the drug-loaded micelles were measured before spray-drying and after re-

dissolving of the spray-dried powders. The mean particle sizes of the micelles increased from 250 nm to 310 nm during spray drying procedure. Although after spray-drying the size of the micelles were

significantly increased, they were still in the nano-range sizes. The percentages of content uniformity for powders were between 95–105% and the process yields was calculated about 30–45 %.

Size and morphology of PTX-loaded embedded in lactose microparticles

The particle dimensions and surface morphology of the spray-dried powders were visualized using scanning electron microscopy (Fig. 6). Spray-dried lactose particles containing PTX-loaded micelles were perfectly spherical with a smooth surface. The mean geometric particle size of spray-dried powders was 2.5 μm , within the respirable particle size range ideal for maximizing pulmonary deposition of dry powders.

In vitro deposition studies using ACI

Fig. 7 shows the percent of drug deposited on various stages of ACI. Each bar represents the powder of certain sizes collected on a defined stage of the ACI. The fraction of all particles smaller than 5.5 μm represents the inhalable $\text{FPF}_{\text{ED}<5.6\mu\text{m}}$. The percentage of $\text{FPF}_{\text{ED}<5.6\mu\text{m}}$ and $\text{ED}\%$ were $60.1\% \pm 10.23$ and $89.8\% \pm 4$, respectively. The MMAD of pure lactose and PTX-loaded micelles embedded in lactose were

3.2 ± 0.52 and 3.8 ± 0.98 μm , respectively. A *t*-test analysis showed that the incorporation of nanoparticles into lactose by spray-drying had no significant effect on the fine particle fraction and MMAD of the powders.

DISCUSSION

Pulmonary delivery of drugs to treat respiratory diseases offers several advantages such as rapid onset of therapeutic action, high drug concentration within the lung, and minimum systemic side effects [28]. In addition, many drugs delivered via intravenous or oral route often result in high systemic concentrations of drugs while relatively low amount of drugs in the lung. Specifically, for lung cancer, pharmacokinetic data has demonstrated low drug concentrations in the lung after systemic administration, which could consequently cause the failure of treatment and development of the high proportion of chemotherapeutic drug resistant cancer cells. Nebulizers, pressurized metered dose inhalers (PMDI), and dry powder inhalers (DPI) are the various types of pulmonary drug delivery systems which are commercially used for the treatment of lung disorders.

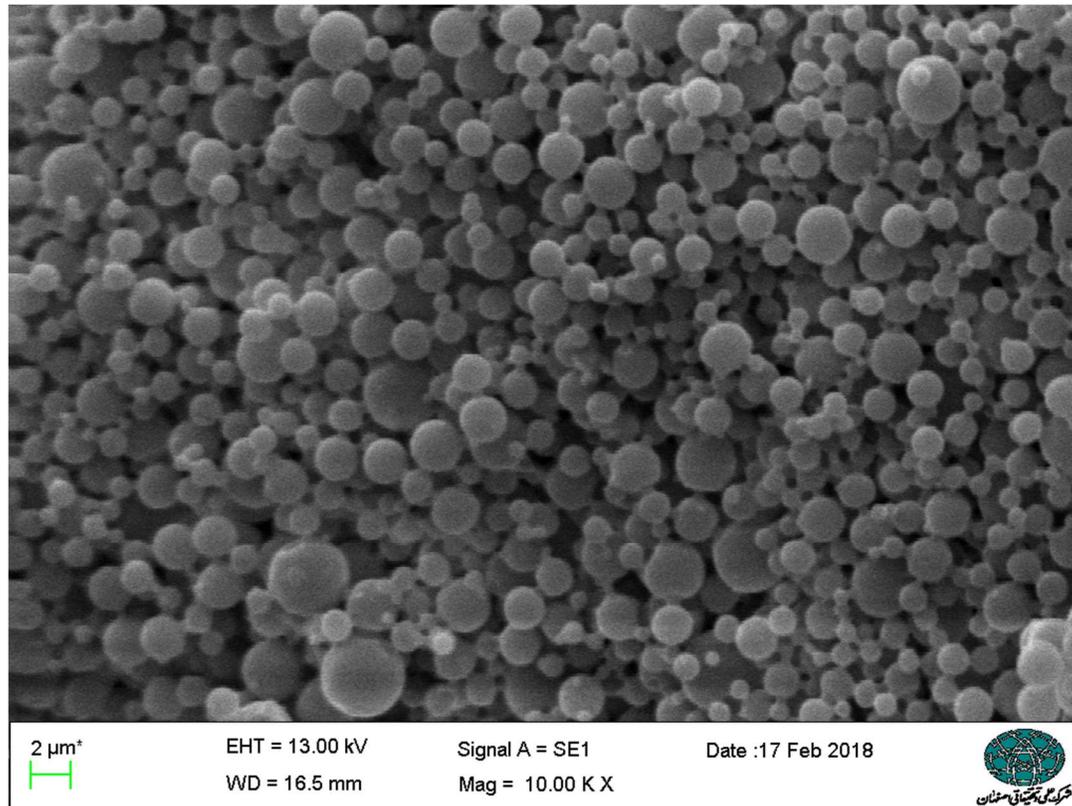


Figure 6. Spray-dried powder morphology visualized by scanning electron microscopy

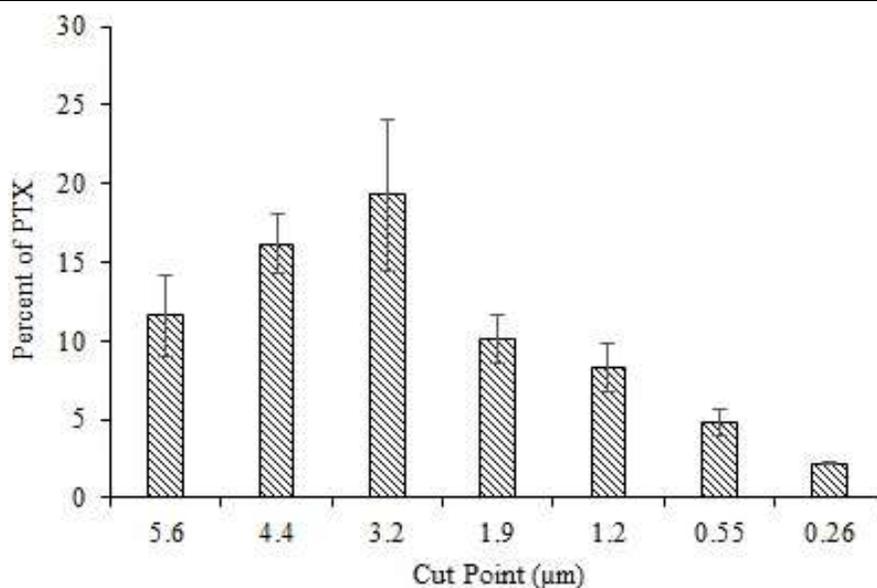


Figure 7. Drug deposition patterns of spray-dried PTX-loaded micelles embedded in lactose powder in the cascade impactor.

Nebulizers and PMDI are liquid-based aerosols while DPI are in a solid form. DPI offers some advantages compared to other types of pulmonary delivery systems [29]. This includes designing appropriate particle size and exact amount of drug loaded into the system, improving chemical stability relative to liquid aerosols and long-term stability during storage and ease of use for patients. Because of the local toxicity of anticancer agents they cannot be directly aerosolized. In this study, PTX was entrapped in polymeric micelles and then incorporated into lactose microparticles as a DPI pulmonary delivery system for lung cancer. Polymeric micelles have a core-shell structure where lipophilic drugs can be solubilized in the cores of micelles. Here we fabricated micelles with the mixture of TPGS_{1K} and TPGS_{5K} at different molar ratios to achieve the optimized formulation with the lowest CMC values and the highest drug loading. Despite the unique advantages of TPGS_{1K} such as anticancer activity and inhibition of p-gp, it has a high CMC value (360 μM), which decreases the stability of micelles in physiological environment. Collnot et al [30] synthesized a homologous group of TPGS analogs which varied in PEG chain length (TPGS 200, 238, 400, 600, 1000, 2000, 3400, 3500, 4000, and 6000) and evaluated the effect of PEG chain length on inhibition of p-gp efflux pump. The results revealed that commercial TPGS_{1K} was the most potent analogs in inhibition of p-gp. Thus in the

current study, the micelles were formulated from TPGS_{1K} and TPGS_{5K} in different molar ratios to improve the stability of the micelles and achieve higher cytotoxic effect.. TPGS_{5K} was synthesized through the reaction of α -TS with MePEG 5kDa with much lower CMC value (8.4 μM) compared to TPGS_{1K} alone. In agreement with our results, Mi and co-workers synthesized TPGS_{2K} which was conjugated to folic acid for targeted delivery of docetaxel [31]. The CMC value of TPGS_{2K} was significantly much lower than that of TPGS_{1K}. In another study the effects of degree of substitution and molecular weight of MePEG on CMC value of MePEG-conjugated octyl-chitosan micelles were evaluated [32]. The results revealed that by increasing the molecular weight of MePEG and its degree of substitution, the CMC value was significantly decreased.

As shown in Table 1, incorporation of TPGS_{5K} as a part of micellar structure significantly increased the micellar size, which might result from the stretch of PEG 5kDa with long chains on the micelle surface toward water phase. In other studies, incorporation of PEG with long chain in micellar structure increased the size and decreased the zeta potentials of the nanoparticles [25,33]. As listed in Table 1, the size of PTX-loaded micelles was larger than those of the blank micelles which is attributed to the drug loaded in the micellar core. The reduction in zeta potential after the drug loading could be due to the

change of size and surface charge density of the micelles. In our previous studies using polymeric micelles for delivery of PTX, loading the drug in the core of micelles increased the size and decreased the zeta potentials of the micelles [21, 25, 34].

The sustained release behaviors observed in all of the formulations could be explained by high solubility of PTX in the micelles' core and the strong hydrophobic interactions between PTX and the bulk lipophilic portion of TPGS molecules that prevent the drug release in the medium. In other published studies, α -TS as a part of micelle formation decreased the drug release rate which is in accordance with our results [21,22, 33]. The cytotoxic effects of different formulation was evaluated against human lung cancer cells and demonstrated the highest cytotoxicity for the micellar formulation. The increased cytotoxicity of PTX-loaded micelles could be explained by solubilization of PTX in the core of micelles and unique apoptosis-inducing properties of TPGS. . Recent studies revealed that TPGS_{1K} possesses potent antitumor activity even higher than that of α -TS [34,35]. The cellular results indicated that antitumor effects of TPGS_{1K} is due to its ability to induce reactive oxygen species (ROS) generation and apoptosis [35]. In addition, TPGS_{1K} through the inhibition of drug efflux pumps or P-gps, could facilitate delivery of PTX to the site of action, which is located in the nucleus. Recently, we developed a novel mixed polymeric micelles containing TPGS_{1K} for co-delivery of PTX and retinoic acid to the cancer cells [21]. The results of cell culture studies on human breast cancer cells, MCF-7, demonstrated the cytotoxic effect of TPGS_{1K}. The decrease in cell viability for the empty micelles at higher concentrations also implies the cytotoxic effect of TPGS. Based on the physicochemical evaluation and cytotoxicity studies, the 7:3 micellar formulation was adopted as an optimized formulation. The micelles were spray-dried together with lactose carriers and produced powder particle diameters of several micrometers with a narrow particle distribution. Through MMAD and FPF obtained by ACI it could be predicted that maximum deposition of the embedded drugs in the trachea-bronchial and deep alveoli regions for normal inhalation rate. After deposition in the lung, the carrier matrix dissolves and releases the nanoparticles where the micelle sizes are still in the nano-size scale after spray drying indicating drug accumulation in cancer cells.

CONCLUSION

In the current study polymeric micelles containing PTX was peppered and further co-spray-dried with lactose carrier. The physicochemical characterization and excellent aerosol dispersion performance of these particles indicates that they would be suitable to deliver PTX to the middle and deep regions of the lungs. This study supports the approach of local lung cancer treatment using nanoparticles as a drug delivery vector. The development of inhalable nanoparticles loaded with bioactive molecules is a new delivery platform which can allow targeting of lung specific diseases in the future.

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