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Polyethylene glycol–phosphatidylethanolamine conjugate (PEG–PE)-based mixed micelles: Some properties, loading with paclitaxel, and modulation of P-glycoprotein-mediated efflux

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Abstract

Mixed micelles prepared of poly(ethylene glycol)2000–phosphatidyl ethanolamine conjugate (PEG_{2000}–PE) and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) in 1:1 molar ratio have been investigated. Micelle formation was confirmed by NMR spectroscopy. CMC of the micelles was found to be 1.5×10^{-5} M. Poorly soluble anti-cancer drug paclitaxel (PCL) was efficiently solubilized in 15 nm non-toxic PEG–PE/TPGS micelles. PCL entrapment was quite stable with only about 20% of the incorporated drug released from micelles after 48 h at 37 °C. In addition, PCL-containing PEG_{2000}–PE/TPGS micelles were stable in vitro under various conditions modeling the physiological ones, in particular, at low pH values and in the presence of bile acids, which is especially important for their possible oral administration. Fluorescently labeled micelles demonstrated time-dependent internalization by human colon adenocarcinoma cell line, Caco-2. The internalization of PEG_{2000}–PE/TPGS micelles loaded with P-glycoprotein (P-gp) substrate, rhodamine-123 (RH-123), opposite to the internalization of the free RH-123, was not influenced by the inhibition of the P-gp pump with verapamil hydrochloride, which assumes a P-gp-independent micelle internalization.

Keywords: Paclitaxel; Mixed micelles; PEG–PE; TPGS; Caco-2 cells; P-glycoprotein

1. Introduction

Micelles made from conjugates of polyethylene glycol (PEG) and phosphatidylethanolamine (PE) (Lukyanov and Torchilin, 2004; Torchilin, 2005) or mixed micelles made from combination of PEG–PE and certain surfactants, such as D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) (Mu et al., 2005), are of particular interest because of their solubilization efficiency towards various poorly soluble drugs and stability.

Here, continuing our systematic studies of polymeric micelles as drug carriers, we have investigated some properties of PEG–PE/TPGS mixed micelles, their efficiency in solubilizing and retaining the popular anti-cancer drug paclitaxel (PCL), and their ability to bypass the P-glycoprotein (P-gp)-mediated drug efflux. The choice of these issues for in-depth investigation has several important justifications.

Paclitaxel (PCL), a diterpenoid derived from *Taxus brevifolia*, is a potent inhibitor of cell replication, used in the treatment of various cancers including breast and ovarian cancer (Adler et al., 1994; Guastalla et al., 1994; Kubota et al., 1997). PCL possesses a high molecular weight (863 Da) and a very low aqueous solubility (<1 μg/mL) (Mathew et al., 1992). Due to PCL's low aqueous solubility, it is currently formulated in 1:1 cremophor EL and ethanol mixture that has to be further diluted with saline before intravenous (i.v.) administration. This cremophor/ethanol mixture has number of associated side effects including hypersensitivity, nephrotoxicity and neurotoxicity (Weiss et al., 1990; Szebeni et al., 1998; Singla et al., 2002). Another concern with the formulation is the precipitation of drug after dilution in an aqueous solution. To address these concerns several other delivery systems have been tried. This includes nanospheres, liposomes, cyclodextrin complexes, emulsions, lipid-based nanospheres,

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water-soluble prodrugs and micelles (Singla et al., 2002; Gao et al., 2002).

Another important complication in application of many anti-cancer drugs is the phenomenon of drug resistance in many cancer cells. Thus, for example, although oral administration is the commonly preferred route of administration of most of the pharmaceuticals, the oral bioavailability of various anti-cancer drugs including PCL is extremely low in animals and humans (Malingre et al., 2001a). Several investigators explain the poor bioavailability of PCL due to the multidrug efflux pump, P-gp that is abundantly present in the gastrointestinal tract (Monsarrat et al., 1990; Sparreboom et al., 1997; Walle and Walle, 1998). One may hypothesize that the oral bioavailability of anti-cancer drugs can be improved if the drug is administered with P-gp inhibitors such as cyclosporine and verapamil analogs (Hofsli and Nissen-Meyer, 1990; Fisher and Sikic, 1995; Malingre et al., 2001b; Woo et al., 2003).

Some non-ionic surfactants such as Pluronic or TPGS that can inhibit both presystemic drug metabolism and intestinal efflux mediated by P-gp resulting in an increased oral absorption of anti-cancer drugs including PCL, are reported (Dintaman and Silverman, 1999; Yu et al., 1999; Rege et al., 2001, 2002). The mechanism, by which these surfactants inhibit P-gp-mediated efflux, appears to involve the interaction with the lipid bilayer leading to increased membrane permeability and thus enhancing the transmembrane diffusion of P-gp substrates. Recently, some low molecular weight methoxypolyethylene glycol-block-polycaprolactone amphiphillic diblock copolymers were reported to enhance the Caco-2 cellular accumulation of the P-gp substrate, rhodamine 123 (RH-123) at concentrations above their critical micelle concentration (CMC) with little or no activity below the CMC (Zastre et al., 2002, 2004).

Although, it was reported that PCL could be successfully incorporated in PEG–PE-based micelles and mixed micelles and the potential of such formulation has already been shown by i.v. administration in tumor-bearing mice (Alkan-Onyuksel et al., 1994; Gao et al., 2003), more systematic studies on the properties of such micelles as well as on their ability to bypass P-gp-mediated drug efflux are needed. In this paper, we continued this line of research using the human colon adenocarcinoma cell line, Caco-2. We have also used the fluorescent dye RH-123 as a marker of the P-gp-mediated transport in tissue culture models.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG_{2000}–PE) and phosphatidylethanolamine lissamine rhodamine B (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. TPGS was a gift sample from Eastman Chemicals USA. PCL (>99%), pancreatin USP (from porcine pancreas), pepsin (from porcine gastric mucosa), bile salts (mixture of sodium cholate and sodium deoxycholate), verapamil hydrochloride, and RH-123 were purchased from Sigma Chem., Inc. (St. Louis, MO, USA). Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture media and supplements were from CellGro (Kansas City, MO, USA). Hoechst 33342, a nucleic acid stain was purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other reagents and buffer solution components were analytical grade preparations. Distilled and deionized water was used in all experiments.

2.2. Micelle formation and drug loading

PCL solubilized in "plain" micelles or mixed micelles was prepared by the following method. To obtain drug-loaded plain micelles, PCL dissolved in methanol was added to PEG_{2000}–PE solution in chloroform; whereas in order to prepare mixed micelles, different amount of PCL (1–2 mg) in methanol was added to 45 mg of PEG_{2000}–PE and TPGS (1:1 molar ratio) in chloroform. Whenever fluorescent tagging was desired, 0.5 mol% of the fluorescent Rh-PE or 4.0 mol% of RH-123 was added to the micelle composition. The organic solvents were removed by the rotary evaporation to form a thin film of drug/micelle material mixture. This film was further dried under high vacuum overnight to remove any traces of remaining solvent. The dried film was then hydrated in 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered saline (HBS), pH 7.4 at PEG_{2000}–PE concentration of 5 mM. The mixture was incubated in water bath at 50 °C for 20 min. Non-incorporated PCL was separated by filtration of the micelle suspension through a 0.2 μm polycarbonate membrane (Millipore Co., Bedford, MA). The filtrate was stored into vials under argon until further use. Alternatively, the micelle samples can be freeze-dried by putting the aliquots of the filtrate in 5 mL glass vials, freezing them in liquid nitrogen and vacuum-drying (Labconco, USA, $p < 133 \times 10^{-3}$ mbar, condenser temperature < −40 °C). Following lyophilization, the samples were sealed under argon and stored at 4 °C until use.

2.3. Characterization of micelles

2.3.1. Micelle size determination

The micelle size (hydrodynamic diameter) was measured by dynamic light scattering (DLS) using N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL). The micelle suspensions/freeze-dried powders were diluted with the deionized distilled water until the concentration providing light scattering intensity of 5×10^4 to 1×10^6 counts/s was achieved.

2.3.2. Zeta-potential measurement

Zeta-potential of micelle formulations was measured by Zeta Phase Analysis Light scattering (PALS) with an ultra sensitive Zeta Potential Analyzer instrument (Brookhaven Instruments, Holtsville, NY). The micelle suspensions were diluted with 5 mM HEPES buffer pH 7.4 to have a significant intensity within the limits required by the instrument. For each sample, zeta-potential measurement was repeated eight times.

2.3.3. NMR characterization

^{1}H NMR spectra were recorded on Varian 200 MHz spectrometer in deuterated water (D_2O) or deuterated chloroform ($CDCl_3$) at RT.

2.3.4. Critical micelle concentration (CMC) determination

CMC was estimated by the pyrene method as in (La et al., 1996). Briefly, tubes containing 1 mg crystals of pyrene were prepared. To these crystals 10^{-4} to 10^{-7} M micellar solution of PEG_{2000}–PE, TPGS and PEG_{2000}–PE/TPGS in HBS was added. The mixtures were incubated for 24 h with shaking at RT. Free pyrene was removed by filtration through 0.2 μm polycarbonate membranes. The fluorescence of filtered samples was measured at the excitation wavelength of 339 nm and emission wavelength of 390 nm using F-2000 fluorescence spectrometer (Hitachi, Japan). CMC values correspond to the concentration of the polymer at which the sharp increase in fluorescence is observed.

2.3.5. Drug solubilization efficiency

The amount of PCL in the micellar phase was measured by the reversed phase-HPLC. The clear aqueous dispersion was diluted with the mobile phase prior to applying onto the HPLC column (since the mobile phase contains acetonitrile, micelles are disrupted and free PCL is determined). The D-7000 HPLC system equipped with a diode array and fluorescence detector (Hitachi, Japan) and Spherisorb ODS2 column, 4.6 mm × 250 mm (Waters, Milford, MA, USA) was used. The column was eluted with acetonitrile/water (52:48%, v/v) at 1.0 mL/min. PCL was detected at 227 nm. Injection volume was 50 μL; all samples were analyzed in triplicate.

2.3.6. In vitro drug release

The in vitro release of PCL from micelles was monitored by the membrane dialysis at 37 °C. Micelle suspension containing 1 mg of PCL were placed into a Spectra/Pro® regenerated cellulose dialysis membrane with the molecular weight cut off size of 1000 Da, and dialyzed against a large excess of phosphate buffered saline (PBS) pH 7.4. The release medium was stirred at 100 rpm. At specific time intervals, samples (1 mL) of the release medium were withdrawn and replaced with an equal volume of fresh medium. The concentration of PCL in samples was determined by HPLC as described above.

2.3.7. Micelle stability

2.3.7.1. Storage stability. The drug-loaded micelles were stored at 4 °C for 3 months. The physical stability of micelles was evaluated by monitoring the time-dependent changes in the physical characteristics (drug precipitation, change in micelle size) of the formulation. The chemical stability of the drug in micelles was evaluated by the HPLC as described above.

2.3.7.2. Stability in media modeling physiological conditions and effect of dilution. To test the stability of PEG_{2000}–PE/TPGS micelles in the conditions modeling the physiological ones, micelles were incubated with HBS, pH 7.4; simulated gastric fluid (SGF), pH 1.2; simulated intestinal fluid (SIF) with and without bile salts (5 mM), pH 7.4, at the PEG_{2000}–PE concentration of 5 mM for 12 h at RT. Following the incubation, at specific time intervals the micellar solutions were filtered through 0.2 μm membrane filter and analyzed for the drug content by the HPLC as described above. The presence of micelles was evaluated by measuring their size by the dynamic light scattering (DLS).

To test the stability of micelles in the blood serum, Rh-PE labeled micelles were incubated with the fetal bovine serum (FBS) at the PEG_{2000}–PE concentration of 1.3 mM for 24 h at RT. The samples were diluted in HBS by 1000-fold and analyzed for the presence of micelles by size exclusion HPLC as described below.

The effect of the dilution on micelles was studied by incubating the micelles in various buffers mentioned above at 10-fold diluted PEG_{2000}–PE concentration of 0.5 mM for 12 h at RT. Following the incubation, the samples were filtered through 0.2 μm membrane filter and analyzed for presence of micelles by the HPLC as described below.

HPLC of micelles was performed using a D-7000-based HPLC system equipped with a diode array and fluorescence detectors (Hitachi, Japan). The separation was done using a size exclusion Shodex® KW-804 column (Shoko Co., LTD, Japan) with phosphate buffer (100 mM phosphate, 150 mM Na_2SO_4), pH 7, as a mobile phase at RT and flow rate of 1 mL/min. Peaks were detected at 220 nm.

2.3.8. Cell culture

Caco-2 cell line (HTB 37) was maintained in EMEM cell culture medium at 37 °C, 5% CO_2. EMEM media were supplemented with 20% FBS, 1 mM Na-pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin.

2.3.9. Cytotoxicity assay

The cytotoxicity of PEG–PE/TPGS micelles was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, Caco-2 cells were plated at 2×10^4 cells per well density in 96-well plates (Corning, Inc., Corning, NY). After 24 h incubation at 37 °C, 5% CO_2, the medium was replaced with medium containing empty PEG–PE/TPGS micelles at equal molar concentration ranging from 0.2 to 20 μM. After an additional 96 h incubation at 37 °C, 5% CO_2, each well was washed twice with the Hank's buffer followed by addition of 20 μL of Cell Titer 96® A_{queous} One solution (Promega, Madison, WI). After 3 h incubation at 37 °C, 5% CO_2, the cell viability was determined by measuring the absorbance of the degraded MTT at 492 nm using an ELISA reader (Labsystems Multiscan MCC/340, Finland).

2.3.10. Endocytosis of micelles by Caco-2 cells estimated by fluorescence microscopy

Micelle–cell interaction was studied as in (Gao et al., 2003). Briefly, Caco-2 cells were grown on glass cover slips placed in six-well tissue culture plates. After the cells reached a confluence of 70–80%, they were washed twice with the complete serum-free medium and incubated with Rh-PE-labeled PCL-containing micelles with PEG_{2000}–PE and TPGS at equal molar concentration of 70 μM. After 1, 3, 6, and 12 h incubation at 37 °C, 5%

CO_2, the cover slips were washed thrice with sterile PBS, cells were stained with the Hoechst 33342 and mounted individually cell-side down on fresh glass slides using a fluorescence-free glycerol-based Trevigen® mounting medium (Trevigen, Gaithburg, MD). Mounted slides were observed with a Nikon Eclipse E400 microscope (Nikon, Japan) under Hoechst/UV-2B filter, or Rhodamine/TRITC filter. For better clarity, cell observation was performed at 1000× magnification, which resulted in only few cells in a single field even at the confluency of 80%, and the typical fields were photographed.

2.3.11. Endocytosis of micelles by Caco-2 cells as estimated by fluorescence spectroscopy

Caco-2 cells were grown six-well tissue culture plates. After the cells reached a confluence of 70–80%, the cells were washed twice with the complete serum-free medium and incubated with Rh-PE-labeled PCL-loaded micelles with PEG_{2000}–PE and TPGS at equal molar concentration of 70 μM. After 1, 3, 6, and 12 h of incubation at 37 °C, 5% CO_2, the cells were washed thrice with sterile PBS and lysed with 400 μL of DMSO. The 0.2 mL aliquots were then diluted with 2.8 mL of water. The fluorescence of samples was measured at the excitation wavelength of 550 nm and emission wavelength of 590 nm using Hitachi F-2000 fluorescence spectrometer (Hitachi, Japan).

2.3.12. Caco-2 accumulation of RH-123 studied by fluorescence microscopy

Caco-2 cells were grown on glass cover slips placed in six-well tissue culture plates. After the cells reached a confluence of 70–80%, they were washed with Hank's buffer twice. RH-123 solution was added to each well so that they contained 5.0 μM of RH-123 in 1% methanol in Hank's buffer or 5.0 μM of RH-123 encapsulated in PEG_{2000}–PE/TPGS mixed micelles in Hank's buffer with or without 100 μM of verapamil hydrochloride. After the incubation for 120 min at 37 °C, 5% CO_2, cells were washed with the Hank's buffer and further incubated with or without 100 μM verapamil hydrochloride for another 45 min at 37 °C, 5% CO_2. The cover slips were washed thrice with sterile PBS, and mounted individually cell-side down on fresh glass slides using a fluorescence-free glycerol-based Trevigen® mounting medium (Trevigen, Gaithburg, MD). Mounted slides were observed with Nikon Eclipse E400 microscope (Nikon, Japan) under Rhodamine/FITC filter.

2.3.13. Caco-2 accumulation of RH-123 studied by fluorescence spectroscopy

Caco-2 cells were grown in 12-well tissue culture plates. After the cells reached a confluence of 60–70%, they were washed twice with the Hank's buffer. The cells were exposed to 5.0 μM RH-123 in 1% methanol in the Hank's buffer or encapsulated in PEG_{2000}–PE/TPGS mixed micelles in Hank's buffer with or without 100 μM of verapamil hydrochloride. After the incubation for 120 min at 37 °C, 5% CO_2, cells were washed with the Hank's buffer and incubated with or without 100 μM verapamil hydrochloride for 2 h at 37 °C, 5% CO_2. The cells were washed twice with sterile PBS, lysed with 100 μL of DMSO, and diluted to 1 mL with PBS. The fluorescence of samples was measured at the excitation wavelength of 485 nm and emission wavelength of 530 nm using a F-2000 fluorescence spectrometer (Hitachi, Japan).

3. Results and discussion

3.1. Characterization of micelles

3.1.1. Micelle size and zeta potential

The average micelle size, and micelle size distribution of both empty and PCL loaded PEG_{2000}–PE and PEG_{2000}–PE/TPGS micelles are in Fig. 1. The average size of plain PEG_{2000}–PE micelles as well as mixed micelles composed of PEG_{2000}–PE/TPGS was about 14 nm with rather narrow size distribution patterns. Loading micelles of both compositions

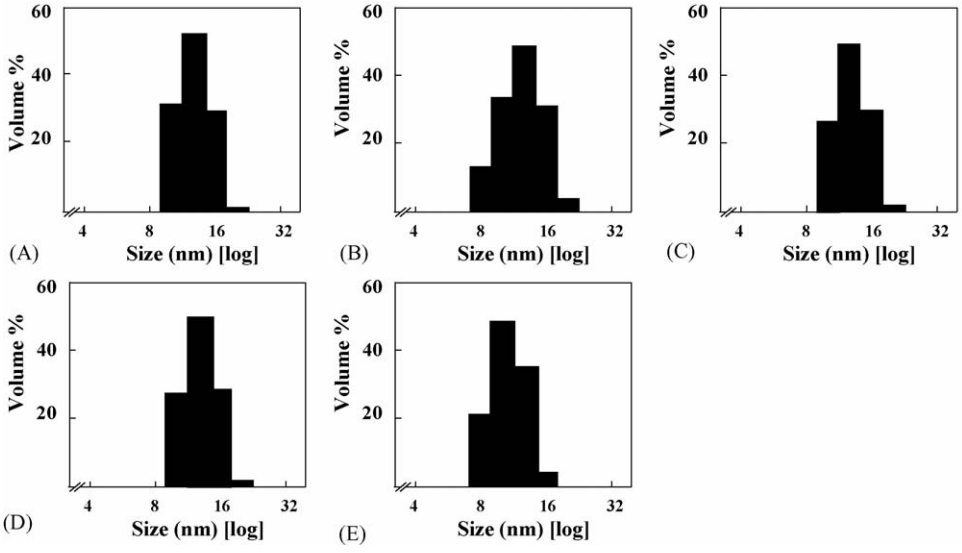

Fig. 1. Micelle size and size distribution of empty PEG_{2000}–PE micelles (A); PCL-loaded PEG_{2000}–PE micelles (B); empty PEG_{2000}–PE/TPGS micelles (C); PCL-loaded PEG_{2000}–PE/TPGS micelles (D); PCL-loaded PEG_{2000}–PE/TPGS micelles reconstituted after freeze-drying (E).

with PCL did not visibly affect their size and size distribution. Very slight and statistically insignificant average size increase after PCL loading (from 14.1 to 14.7 and 14.2 to 15.3 nm for plain PEG–PE micelles and mixed PEG–PE/TPGS micelles, respectively) might nevertheless reflect a certain increase in the hydrophobic micelle core size because of PCL solubilized there.

Freeze-drying and subsequent reconstitution of PCL-loaded PEG_{2000}–PE/TPGS micelles did not result in any noticeable changes in micelle size and size distribution (see Fig. 1E). The result confirms that drug-loaded micelles can be stored as a freeze-dried powder and reconstituted before use.

Both PEG_{2000}–PE and PEG_{2000}–PE/TPGS empty micelles were negatively charged with zeta-potential of approx. −18 and −16 mV, respectively. Micelle loading with PCL slightly decreases the negativity of the micelles with the final zeta-potential being of −16.5 and −13.5 mV for PCL-loaded PEG–PE and PEG–PE/TPGS micelles, respectively.

3.1.2. NMR characterization

In $CDCl_3$, resonance peaks corresponding to hydrophilic and hydrophobic parts of both PEG_{2000}–PE and TPGS were clearly observed (Fig. 2): 3.56 and 3.6 ppm for PEG, 1.26 ppm for PE, and 1.29, 2.35 and 2.62 ppm for Vitamin E. In contrast, only PEG resonance peaks were detected in D_2O whereas both PE and Vitamin E peaks were hardly observed. This result clearly confirms the core-shell structure of micelles. Intact micelles are present D_2O, which does not destroy their integrity, and only the micelle shells consisting of PEG blocks could be well solvated in D_2O and therefore show clear NMR spectra, while the resonance peaks of PE and Vitamin E blocks, which constitute the core, were not observed due the lack of the solvent within the micelle core and insufficient motion of protons there in D_2O. $CDCl_3$, however, solubilizes and disintegrates micelles and thus able to solvate both blocks of the unimers allowing for the peaks corresponding to all groups to be recorded.

3.1.3. Critical micelle concentration

The in vitro and in vivo stability of micelles depend upon CMC values of micelles forming compounds. The CMC values PEG–PE micelles were repeatedly shown to be very low allowing for their high stability in solutions and upon dilution (Lukyanov and Torchilin, 2004). In our experiments, the CMC value for plain PEG_{2000}–PE micelles was also shown to be as low as 1.1×10^{-5} M. More important however, is the fact that the CMC of the mixed micelles composed of 1:1 molar mixture of PEG_{2000}–PE and TPGS was also rather low -1.5×10^{-5} M, which point on their high stability and ability to maintain the integrity even upon strong dilution in the body.

3.1.4. PCL solubilization efficiency

The amount of PCL in solubilized/micellar form was determined by the reversed phase HPLC. The calibration curve for the quantification of PCL was linear over standard PCL concentration of 0.2–40 μg/mL with the correlation coefficient of $R^2 = 0.999$.

To confirm that the reversed phase HPLC method is suitable for determining the chemical stability of PCL, the drug was exposed to acid/base-catalyzed or peroxide-catalyzed oxidative degradation and the products of the degradation were analyzed using the HPLC. Fig. 3 clearly demonstrates that the HPLC protocol used allows for good separation of the PCL degradation products from the intact drug, and the HPLC method can be used to evaluate the stability of drug in micelle formulation.

Earlier, it was reported that the use of mixed micelles made up of PEG_{2000}–PE and phosphatidyl choline almost doubled the

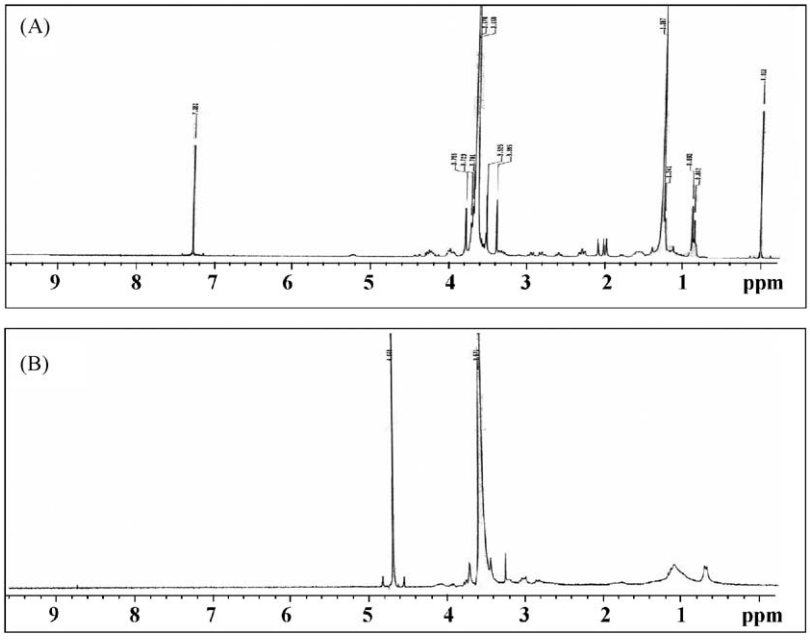

Fig. 2. ^1H NMR spectra of PEG_{2000}–PE/TPGS micelles in $CDCl_3$ (A) and D_2O (B).

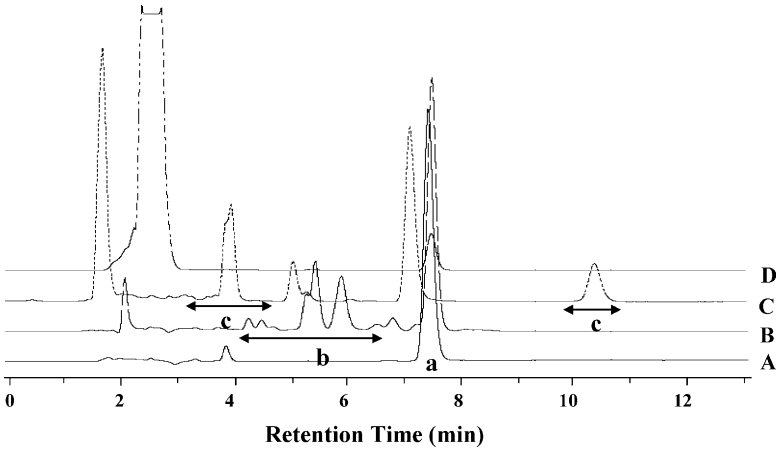

Fig. 3. Reverse phase HPLC profile of degradation study of PCL: (A) PCL standard; (B) PCL and products of acid degradation; (C) PCL and products of alkaline degradation; (D) PCL and products of oxidative degradation. The peaks correspond to (a) standard PCL, (b) acid degradation products of PCL, (c) alkaline degradation products of PCL.

quantity of the solubilized PCL (Lukyanov and Torchilin, 2004), probably because of some "loosening" of the micelle core allowing for more drug incorporation. TPGS was also reported as a potential solubilizer for PCL and what is even more important it also acts as P-gp inhibitor (Rege et al., 2002). One can hypothesize that the addition of TPGS to PEG–PE micelles might increase the solubilization efficiency of micelles towards PCL. The drug load of mixed micelles made of PEG_{2000}–PE/TPGS (1:1 molar ratio) was found to be 3.5% w/w compared to only 1.2% w/w as was found for the micelles prepared of PEG_{2000}–PE alone, i.e. the addition of TPGS actually improves PCL solubilization by mixed micelles. This 3.5 wt.% load was found to work within a broad range of concentrations of micelle-forming materials and the total quantity of the solubilized PCL per ml of the micelle suspension linearly depended on the concentration of the PEG_{2000}–PE/TPGS mixture (Fig. 4; $R^2 = 0.997$, $y = 0.0335x + 0.0237$), which corresponds well to a general solubilization pattern of poorly soluble drugs by micelles (Yokoyama et al., 1998). Thus, micelle formulations can be prepared containing up to 3–5 mg of PCL per ml at still reasonable PEG_{2000}–PE/TPGS concentrations.

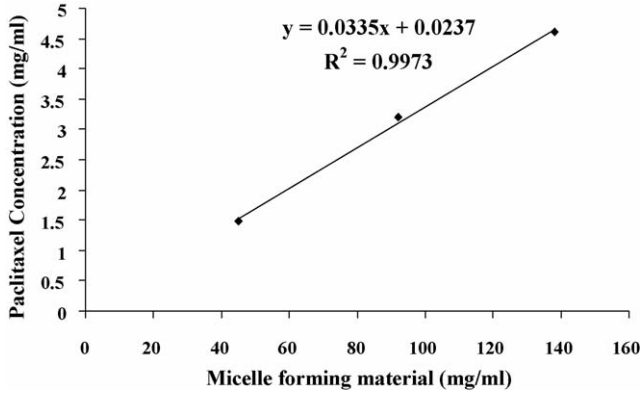

Fig. 4. Effect of total micelle-forming material concentration on solubilized PCL concentration in micelle suspension.

3.1.5. In vitro release

The in vitro release of PCL from mixed micelles in PBS pH 7.4 under sink conditions was very slow with not more than 20% of the drug being released even after 48 h.

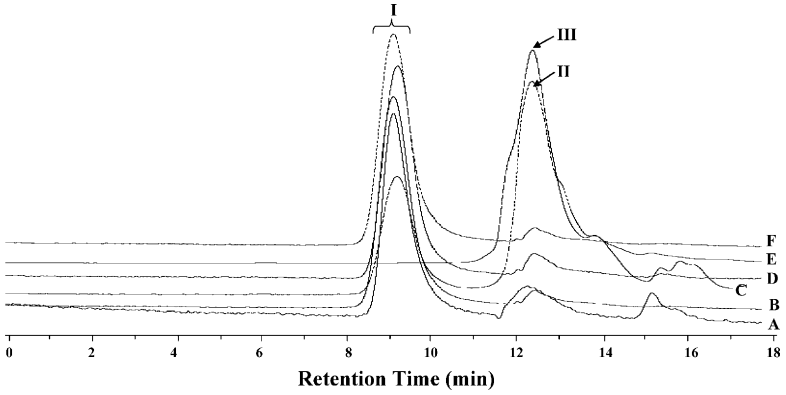

Fig. 5. Size exclusion chromatography of PEG_{2000}–PE/TPGS mixed micelles incubated with: (A) HBS after 0 h; (B) HBS after 12 h; (C) SGF after 12 h; (D) SIF without bile salts after 12 h; (E) SIF with bile salts after 12 h; (F) FBS after 24 h. The peaks correspond to (I) PEG_{2000}–PE/TPGS micelles, (II) components from SGF, (III) PEG_{2000}–PE/TPGS and bile salts mixed micelles.

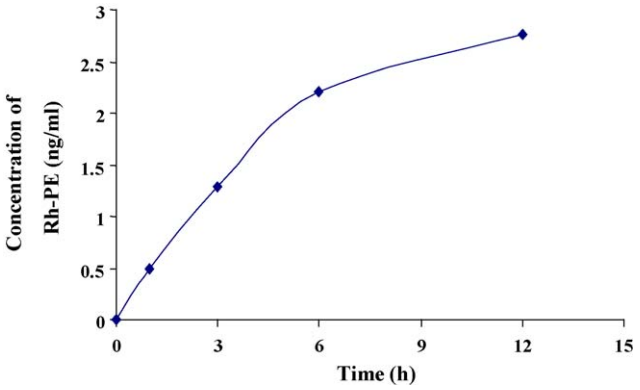

Fig. 6. Time-dependent internalization of Rh-PE-labeled PCL-loaded PEG_{2000}–PE/TPGS micelles by Caco-2 cells as determined by fluorospectrometry.

3.1.6. Micelle stability

3.1.6.1. Storage stability. The drug-loaded micelles were stable during storage at 4 °C for 3 months. No precipitation of drug or micelle size/size distribution changes was noted during this period.

3.1.6.2. Micelle stability in conditions mimicking the physiological ones. Since micellar preparations can, theoretically, be administered orally, the easiest and most convenient drug administration route, we have investigated the stability of PCL-loaded micelles in acidic conditions characteristic of the stomach and in gastrointestinal medium. The principal micelle-destabilizing factors along the oral route are pH, bile salts and pancreatic enzymes. Thus, micelles administered orally should be able to resist solubilization by approx. 5 mM bile salts.

Micelle stability was investigated at 37 °C in both SGF pH 1.2, and SIF (with and without 5 mM bile salts, pH 7.4) for 12 h. It was observed that in both media, the drug content and size of micelles did not change significantly within this time. In the case of micelles incubated with SIF/bile salts mixture, certain decrease in micelle size was observed, probably because of formation of smaller mixed micelles with bile acids. No drug precipitation occurred further indicating the stability of the drug-loaded micelles. Size exclusion chromatography data are presented in Fig. 5 for the micelle concentration of 0.5 mM (notice a certain increase in micelle retention time for the sample

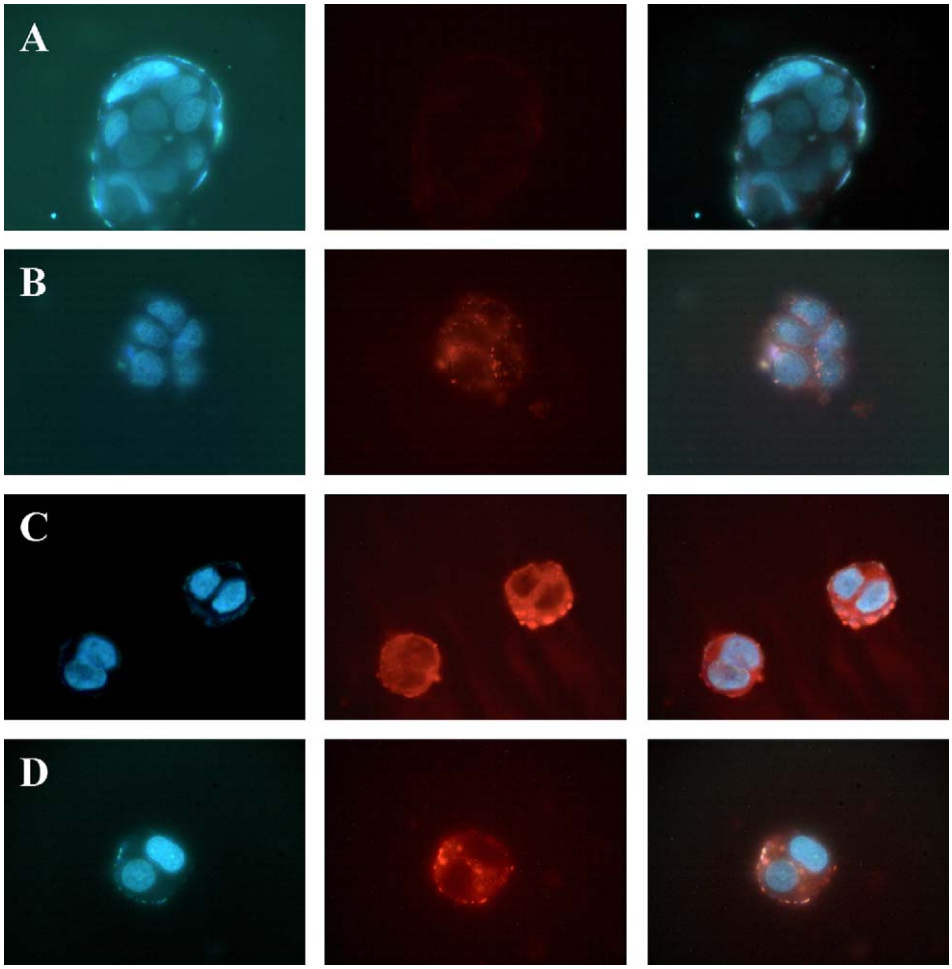

Fig. 7. Internalization of Rh-PE-labeled PCL-loaded PEG_{2000}–PE/TPGS micelles by Caco-2 cells following fluorescence microscopy. Left panel (blue fluorescence) represents nuclear staining of Caco-2 cells using Hoechst 33342, middle panel (red fluorescence) represents the fluorescence from Rh-PE-labeled PCL-loaded mixed micelles associated with Caco-2 cells; the right panel represents the composite of the two: 1 h incubation (A); 3 h incubation (B); 6 h incubation (C); 12 h incubation (D).

Table 1
Viability of Caco-2 cells after incubation with PEG_{2000}–PE/TPGS mixed micelles (per cent cell viability ± S.D., $n = 4$).

PEG–PE/TPGS molar concentration, μM (in parenthesis, PCL concentration, μg/mL, that can be achieved with each given PEG–PE/TPGS concentration)	Cell viability in the presence of various concentrations of empty PEG–PE/TPGS micelles
0.2 (0.01)	113.50 ± 6.67
2.0 (0.10)	101.04 ± 1.37
20.0 (1.00)	95.72 ± 1.37

incubated with SIF/bile salts pointing at some decrease in the micelle size).

It is also important that the micelles were not affected by the blood plasma components as follows from the absence of any changes in the shape, and retention time of the micelle peaks after the micelle incubation with the blood serum for 24 h at RT (Fig. 5).

3.1.7. Cytotoxicity assay

Caco-2 cell line was chosen for this study because this cell line is known to express P-gp (Perloff et al., 2003). The data presented in Table 1, clearly show the lack of cytotoxicity of mixed PEG–PE/TPGS (i.e. of micelle-forming materials) against Caco-2 cells even at PEG–PE/TPGS concentration, which can provide PCL concentration in the micelle suspension as high as 1 μg/mL.

3.1.8. Fluorescence microscopy of micelle endocytosis by Caco-2 cells

It is reported that micelles can be internalized by an endocytic pathway, and can enhance drug uptake by inhibiting P-gp drug efflux systems in the cells (Zastre et al., 2004). To investigate the ability of the micelles to bypass the P-gp drug efflux system, we have studied the interaction of micelles with Caco-2 cells using rhodamine as a fluorescent probe. With fluorescence spectroscopy, it was found that the amount of the internalized micelles (the quantity of rhodamine fluorescence associated with the cells) increased rapidly till 6 h and slowed down after that (Fig. 6). Time-dependent internalization of micelles into Caco-2 cells was also confirmed by the direct fluorescence microscopy (Fig. 7). Cell-associated fluorescence of Rh-labeled PCL-loaded mixed micelles was observed already after 1 h of the exposure, and the fluorescence increased rapidly within the first 6 h.

3.1.9. Accumulation of RH-123 in Caco-2 cells

RH-123, a fluorescent probe, is a substrate for P-gp and can, therefore, be used as a marker for P-gp activity in cells, including Caco-2 cells. On the other hand, verapamil is known to be an inhibitor of P-gp and thus prevents the efflux of P-gp substrates from the cells. We have found that significantly higher accumulation of RH-123 in Caco-2 cells was observed in cells treated with verapamil hydrochloride as compared to non-treated cells (Fig. 8), evidently, due to the inhibition of P-gp efflux by verapamil. Although the panel I of Fig. 8 (left panels)

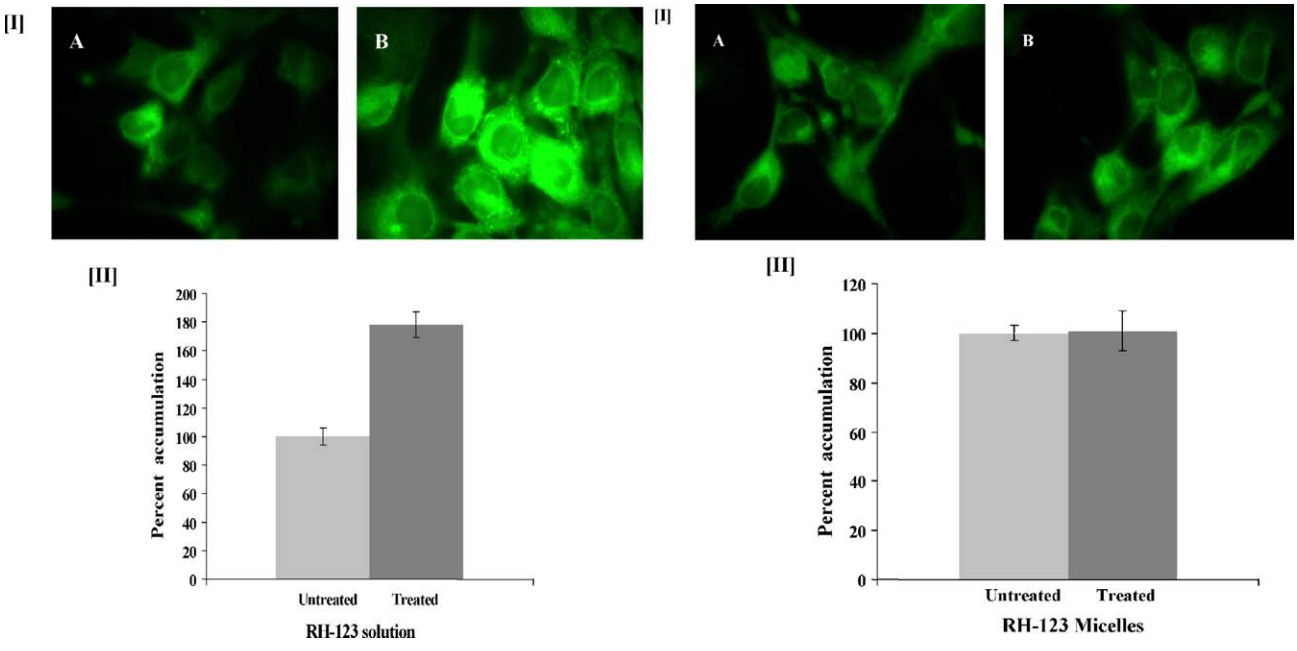

Fig. 8. The effect of micelle incorporation on P-gp-mediated efflux of RH-123. Left panels—Free RH-123: [I] Fluorescence microscopy of RH-123 accumulation in Caco-2 cells after incubation with RH-123 solution in the absence (A) and in the presence (B) of verapamil hydrochloride. [II] Fluorospectrometric quantitation of RH-123 accumulated in Caco-2 cells after incubation with RH-123 solution in absence of verapamil (untreated) (A) and in presence of verapamil (treated) (B) (mean ± S.D., $n = 3$). Right panels—Micellar RH-123: [I] Fluorescence microscopy of RH-123 accumulation in Caco-2 cells after incubation with RH-123-loaded PEG_{2000}–PE/TPGS mixed micelles in the absence (A) and in the presence (B) of verapamil hydrochloride. [II] Fluorospectrometric quantitation of RH-123 accumulated in Caco-2 cells after incubation with RH-123-loaded micelles in the absence of verapamil (untreated) (A) and in the presence of verapamil (treated) (B) (mean ± S.D., $n = 3$).

suggests a more pronounced increase in RH-123 accumulation after the verapamil treatment compared to the quantitative measurement presented in the part II, the data from the part II should be considered as more representative, since it provides the total effect of the fluorescence accumulation for the whole cell population.

However, when the same cells were incubated with RH-123 encapsulated into PEG–PE/TPGS micelles, the presence or absence of verapamil did not influence RH-123 accumulation in the cells (Fig. 8, right panels). The most evident explanation for this phenomenon is that RH-123-bearing PEG–PE/TGPS micelles are capable of bypassing the drug efflux by P-gp. This property might be very important for the development of micellar forms of poorly soluble drugs including those for oral administration.

One can notice that the uptake of RH-123 in the free form in the presence of verapamil HCl as almost twice as high as the uptake of the micellar RH-123, which remains on the same level as in the absence of verapamil. The reason for that phenomenon is the inhibition of the P-gp-mediated efflux of the free drug in the presence of verapamil and P-gp-independence of the endocytotic uptake of the micellar RH-123. These two phenomena proceed via different mechanisms and should not be directly compared. Our main goal here was not to increase the uptake of RH-123 by its incorporation into micelles, but rather to demonstrate the possibility of the P-gp-independent uptake of the P-gp substrate when the latter is inside micelles. From this point of view, the most important for us is the fact established here that the administration of the P-gp substrate (RH-123 or, eventually, a real drug) in a micellar form eliminates the need for co-administration of P-gp inhibitors.

4. Conclusion

Non-toxic mixed micelles prepared of PEG_{2000}–PE and TPGS are stable under various conditions and demonstrate good solubilization efficiency towards PCL. PCL-containing PEG_{2000}–PE/TPGS micelles are stable in vitro under conditions modeling the physiological ones, in particular, at low pH values and in the presence of bile acids, which is especially important for their possible oral administration. Fluorescently labeled micelles demonstrated time-dependent internalization by Caco-2 cells. Moreover, this internalization, opposite to the internalization of a model fluorescent dye in the free form, was not influenced by the inhibition of the P-gp pump, which assumes P-gp-independent micelle internalization. Since oral delivery of PCL is limited because of the multidrug P-gp efflux pump, which is abundantly present in the gastrointestinal tract, PEG_{2000}–PE/TPGS micelles may represent a potential oral delivery system for PCL.

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