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Polymeric Micelles for Delivery of Poorly Soluble Drugs: Preparation and Anticancer Activity *In Vitro* of Paclitaxel Incorporated into Mixed Micelles Based on Poly(ethylene Glycol)-Lipid Conjugate and Positively Charged Lipids

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Abstract

Paclitaxel-loaded mixed polymeric micelles consisting of poly(ethylene glycol)-distearoyl phosphoethanolamine conjugates (PEG-PE), solid triglycerides (ST), and cationic Lipofectin® lipids (LL) have been prepared. Micelles with the optimized composition (PEG-PE/ST/LL/paclitaxel = 12/12/2/1 by weight) had an average micelle size of about 100 nm, and zeta-potential of about 26 mV. Micelles were stable and did not release paclitaxel when stored at 4°C in the darkness (just 2.9% of paclitaxel have been lost after 4 months with the particle size remaining unchanged). The release of paclitaxel from such micelles at room temperature was also insignificant. However, at 37°C, approx. 16% of paclitaxel was released from PEG-PE/ST/LL/paclitaxel micelles in 72 h, probably, because of phase transition in the ST-containing micelle core. *In vitro* anticancer effects of PEG-PE/ST/LL/paclitaxel and control micelles were evaluated using human mammary adenocarcinoma (BT-20) and human ovarian carcinoma (A2780) cell lines. Paclitaxel in PEG-PE/ST/LL micelles demonstrated the maximum anti-cancer activity. Cellular uptake of fluorescently-labeled paclitaxel-containing micelles by BT-20 cells was investigated using a fluorescence microscopy. It seems that PEG-PE/ST/LL micelles, unlike micelles without the LL component, could escape from endosomes and enter the cytoplasm of BT-20 cancer cells thus increasing the anticancer efficiency of the micellar paclitaxel.

Keywords

Polymeric micelles; mixed micelles; PEG-PE; cationic lipids; paclitaxel; *in vitro* anticancer effect

Introduction

Paclitaxel (Taxol), a diterpenoid derived from the needles and bark of the Pacific Yew tree (*Taxus brevifolia*), is an anticancer agent for the treatment of various cancers including ovarian and breast cancers (Baselga et al. 1998, Ling et al. 1998). The anticancer mechanism of paclitaxel as a potent inhibitor of cancer cell replication is related to its ability to block cancer cells in the late G2-mitotic phase of the cell cycle by stimulating microtubule polymerization and suppressing their dynamics (Horwitz, 1994, Jordan, 2002). Paclitaxel has very low water solubility, and clinically it is used as a solution in Cremophor EL/ethanol (1/1, w/w) (Fjallskog et al. 1993). However, Cremophor (polyethoxylated castor oil) can provoke a number of side effects, such as hypersensitivity, nephrotoxicity and neurotoxicity (Nassberger et al. 1991,

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Windebank et al. 1994, He et al. 2003). Although the incidence of serious hypersensitivity reactions has been reduced by a premedication regimen with corticosteroids and antihistamine agents, side effects have still been found to occur in 5-30% of treated patients (Weiss et al. 1990). High toxicity of paclitaxel itself represents an additional issue, and the maintenance of therapeutically significant systemic concentration of the drug was reported to cause severe reactions (Rowinsky et al. 1993, Sarosy and Reed, 1993).

To overcome these problems and increase paclitaxel bioavailability, many types of drug delivery systems, such as nanoparticles (Damascelli et al. 2003, Mitra and Lin, 2003, Potineni et al. 2003), liposomes (Kunstfeld et al. 2003), emulsions (Kan et al. 1999, Constantinides et al. 2000, Rodrigues et al. 2002) and various micelles (Liggins and Burt, 2002 Krishnadas et al. 2003 Lukyanov et al. 2003a), have been tried as pharmaceutical carriers for paclitaxel. Recently published data suggest that polymeric micelles may be of particular interest for delivery of sparingly soluble drugs including anticancer drugs. Micelles are spherical nanoparticles of a colloidal size, into which many amphiphilic molecules self-assemble. In water, hydrophilic parts of such molecules form the micelle corona, while hydrophobic fragments form the core of a micelle that may serve as a cargo space for poorly soluble pharmaceuticals (Muranishi 1990, Lasic 1992). Because of their small size (approx. 5-50 nm), micelles are able to spontaneously accumulate in pathological areas with the damaged ("leaky") vasculature, such as infarcts (Palmer et al. 1984) and tumors (Gabizon 1995, Yuan et al. 1995), via the enhanced permeability and retention (EPR) effect (Maeda et al. 2000; 2001).

Polymeric micelles formed by amphiphilic synthetic copolymers demonstrate a whole set of attractive properties as drug carriers (Kwon and Kataoka 1995, Jones and Leroux 1999, Torchilin 2001). Because of their low critical micelle concentration, polymeric micelles are stable *in vitro*. In addition, the micelle corona formed by hydrophilic polymer blocks provides longevity to micelles *in vitro* by preventing their opsonization and capture by the cells of the reticuloendothelial system (Torchilin and Trubetskoy 1995). Micelles made from conjugates of poly(ethylene glycol) (PEG) and diacyllipids, such as phosphatidylethanolamine (PE), are of particular interest (Trubetskoy and Torchilin 1995) because the use of lipid moieties as hydrophobic blocks allows for an efficient incorporation (solubilization) of poorly soluble drugs and provides additional stability to the micelles, since the existence of two hydrocarbon chains in the lipid moiety strongly increases the hydrophobic interactions in the micelle's core. We have reported some data on stable and long-circulating polymeric micelles formed by PEG-PE conjugates (Trubetskoy and Torchilin 1996). Such micelles can be loaded with a variety of poorly soluble drugs including paclitaxel (Weissig et al. 1998a, Gao et al. 2002) and are capable of delivering their load even into poorly permeable tumors in mice with a higher efficiency than other long-circulating carriers (Weissig et al. 1998b, Lukyanov et al. 2002).

There may be several ways to still further enhance the drug delivery potential of polymeric micelles, PEG-PE-based micelles among them. Thus, targeting ligands, including antibodies, could be attached to the micelle surface to increase the accumulation of micelles and micelle-incorporated drugs in appropriate targets including tumors (Torchilin 2001). We have recently described the preparation of PEG-PE-based immunomicelles modified with monoclonal 2C5 antibody with nucleosome-restricted specificity [2C5; reactive towards a variety of different cancer cells (Iakoubov and Torchilin 1997)], and have demonstrated that paclitaxel-loaded 2C5-immunomicelles specifically recognize various cancer cells, possess increased cytotoxicity *in vitro*, deliver increased quantities of the drug into experimental tumors, and provide improved tumor growth inhibition *in vitro* (Gao et al. 2003, Torchilin et al. 2003).

On the other hand, it is known that the net positive charge usually enhances the endocytosis of various nanoparticles, and positively charged lipid mixtures, such as Lipofectin® (an equimolar mixture of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride,

DOTMA, and dioleoyl phosphatidylethanolamine, DOPE) noticeably improves the endocytosismediated intracellular delivery of various drugs and DNA entrapped within liposomes and other lipid constructs made of these compositions (Chawla and Amiji, 2002, Ota et al. 2002, Kaiser and Toborek, 2003, Almofti et al. 2003). PEG-PE micelles have been found to carry a net negative charge (Lukyanov et al. 2003b), which might hinder their internalization by cells. The alteration of this negative charge by the addition of positively charged lipids to PEG-PE could improve the uptake of paclitaxel-loaded mixed PEG-PE/positively charged lipid micelles by cancer cells thus increasing the efficiency of this drug delivery system. With this in mind, we attempted to increase intracellular delivery and, thus, the anticancer activity of the micellar paclitaxel by preparing paclitaxel-containing micelles from the mixture of PEG-PE and Lipofectin® lipids. In addition, since it was shown that triglycerides could form paclitaxel-loaded emulsions (Lundberg 1997, Kan et al. 1999, Constantinides et al. 2000, Rodrigues et al. 2002), we introduced solid triglycerides into the micelle core to provide a higher load of paclitaxel and to minimize its release in the circulation. Here, we present the results of our *in vitro* studies with these systems.

Materials and methods

Materials

Paclitaxel (Taxol) was purchased from Sigma Chem., Inc. (St. Louis, MO). (1,2-diacyl-SN-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-PE) and phosphatidylethanolamine lissamine rhodamine B (Rh-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The mixture of positively charged lipids (Lipofectin®) was obtained from Invitrogene™ (Carlsbad, CA). All other reagents and components of buffer solutions were analytical grade preparations.

Preparation of solid triglyceride

Solid triglyceride (ST, melting point of 31-37°C) was extracted from margarine (Land OàLakes, Inc., MN) as described in Kallio *et al*. (1989). Briefly, 5 g of margarine was dissolved in 100 ml of hexane, shaken, and let stand in the dark for 12 h. A clear solution was obtained after a small amount of insoluble materials settled down. The supernatant was paper filtered three times. The filtrate was washed with 60% ethanol (1/1, v/v) by shaking for 10 min and then centrifugating for 10 min at 2000 rpm. The hexane layer was separated and dried. The filtrate was washed in triplicate as above. The residual ST was weighed and dissolved in chloroform for the following experiments.

Preparation of paclitaxel-loaded micelles

To prepare paclitaxel-loaded micelles, various quantities of PEG-PE, ST, Lipofectin lipids (LL), and paclitaxel were dissolved in 30 ml of chloroform. To fluorescently label the micelles, 1 wt% of Rh-PE (λex568/λem590) was added to the composition for micelle preparation. Via its hydrophobic fragment, PE, Rh-PE firmly anchors into the micelle core and remains associated with the micelle as long as the micelle exists. Chloroform was evaporated using N_2 gas, and the film formed was additionally dried in a vacuum. A required volume of 0.9% NaCl to make a final paclitaxel concentration of 1 mg/ml was added to the dried film and the mixture was incubated in a water bath at 37°C and shaken 5 min. Then, the mixture was placed in the ice water, sonicated for 10 min, and subsequently filtered three times through each of 0.45, 0.2, and 0.1 mm polycarbonate membrane (Millipore Co., Bedford, MA). The filtrate was divided into ampoules and sealed under N_2.

Efficiency of paclitaxel incorporation

To find the quantity of the micelle-incorporated paclitaxel, each paclitaxel-containing micelle preparation was filtered through a 200 nm polycarbonate filter (Millipore Co., Bedford, MA), and the percentage of the paclitaxel in the filtrate (micellized paclitaxel) was determined using the HPLC method as described previously (Willey et al. 1993). The value obtained was named the micellization efficiency (calculated from calibration curves). The D-7000 HPLC system was used equipped with a diode array and fluorescence detector (Hitachi, Japan) and a 150 × 6.0 mm YMC-Pack ODS column (YMC Co., Ltd., Japan). The mobile phase was acetonitrile/water (60/40, v/v), and the flow rate was 1.0 ml/min. Paclitaxel was detected at 227 nm.

Micelle size measurement

The particle size measurement and size distribution analysis were performed using a Coulter® N4-Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL). Forty microlitres of mixed micelle dispersion was diluted with the deionized distilled water until a concentration providing a light scattering intensity of 5×10^4-1×10^6 counts per second was achieved. The particle size distribution of each sample was measured in triplicate.

Zeta-potential measurement

Micelle surface charge analysis was performed using a Zeta Phase Analysis Light Scattering (PALS) UltraSensitive Zeta Potential Analyzer instrument (Brookhaven Instruments, Holtsville, NY). Each sample of micelle suspension was diluted with deionized distilled water to have the signal intensity within the limits required by the instrument. The zeta-potential of each sample was determined from five-to-eight independent measurements.

In vitro paclitaxel release

The *in vitro* paclitaxel release was investigated at 4, 25, and 37°C. At each temperature, 1 ml of drug-loaded micelles was placed into a SpectraPor® molecular porous regenerated cellulose dialysis membrane with a molecular weight cut off size of 3500 Da, and dialyzed against 4 L of PBS (pH 7.4). After 12 h, the dialysis medium was replaced with 4 L of fresh medium, and 10 μl of micelle suspension was taken out and diluted 40 times with methanol/chloroform (1/2, v/v) for determination of paclitaxel using HPLC method (Willey et al. 1993). The concentration of paclitaxel in each sample was calculated using a calibration curve.

Storage stability

Drug-loaded micelles were stored in a dark place at 4°C for 6 months. The stability was monitored by the changes in particle size and drug concentration during the storage period. The particle size distribution and paclitaxel concentration were determined as above.

Cell cultures

Human mammary adenocarcinoma (BT-20) and human ovarian carcinoma (A2780) cell lines were purchased from the American Type Culture Collection (Manassas, VA). BT-20 and A2780 cells were maintained in RPMI 1640 and EMEM cell culture medium, respectively. Cell culture media were supplemented with FBS to 10%, Na pyruvate to 1mM, L-glutamine to 1 mM and penicillin and streptomycin to 50 U/ml and 50 mg/ml, respectively.

In vitro anticancer effects

The *in vitro* anticancer effects of drug-loaded micelles were evaluated using the MTT [3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl-tetrazolium bromide] method (Ni et al. 1998). Briefly, 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 a medium containing

different paclitaxel formulations. After an additional 24 h incubation at 37°C, 5% CO_2, the media were replaced with PBS containing 0.1 mg/ml MTT, and the cells were incubated for 3 h at 37°C, 5% CO_2. The method is based on the fact that living cells reduce MTT to formazan. The cytotoxicity was measured following the absorbance of the degraded MTT (formazan) at 492 nm using a MCC/340 ELIZA Reader (Lab Systems, Finland). The IC50 values, or the concentrations of various preparations at which the cell growth inhibition was 50% compared to untreated control cells, were estimated (or extrapolated) from the dose-response curves.

Cellular uptake of micelles by BT-20 cancer cells

The cellular uptake of various micelles was studied using micelles labeled with 1% Rh-PE (w/w). Adherent BT-20 cells were grown on glass cover slips placed into six-well tissue culture plates. When the cells reached a confluency of 60-70%, the cells were washed twice with Hank's buffer and treated with a 1% solution of bovine serum albumin (Hank's/BSA) in EMEM medium. After 1 h incubation at 37°C, 5% CO_2, the BSA-containing EMEM was replaced with Rh-PE labeled micelles in the medium to a PEG-PE concentration of 3.5×10^{-3} mg/ml. After additional incubation for 0.5, 2, and 4 h, at 37°C, 5% CO_2, the cover slips were washed three times with cold saline, and mounted individually cell-side down on clean glass slides using a fluorescence-free glycerol-based Trevigen® mounting medium (Trevigen, Gaitherburg, MD). Mounted slides were studied with a Nikon Eclipse E400 microscope under fluorescence using a Rh/TRITC filter.

Results and discussion

Formulation study

To find a ST/paclitaxel optimum ratio, which allows for the best paclitaxel solubilization, a series of paclitaxel-loaded mixed micelles were prepared with different ST/paclitaxel weight ratios (34.0, 17.0, 8.5, 4.25, and 2.125) at the same quantity of PEG-PE and LL, and the paclitaxel micellization efficiency by each of the micelle dispersions was determined. The effect of the ST on paclitaxel micellization efficiency is shown in Figure 1, which demonstrates that to acquire a paclitaxel micellization of 100% when PEG-PE/LL/paclitaxel ratio was fixed at 12/2/1 (w/w/w), the ST/paclitaxel weight ratio should be greater than 8.5.

The effects of the addition of ST, LL and paclitaxel on the size and zeta-potential of the initial PEG-PE micelles are shown in Table I. The particle size of the plain PEG-PE micelles was 12.8 ± 2.6nm. When additionally loaded with 1:1 weight ratio of ST, their average size was enlarged to 66.8 ± 6.6 nm due to the efficient solubilization of ST by the micelle core. Further addition of LL to the final PEG-PE/ST/LL weight ratio of 12/12/2 resulted in an additional increase in the micelle size to 95.7 ± 3.5 nm. When paclitaxel was also loaded into the micelles yielding the final preparation with PEG-PE/ST/LL/paclitaxel weight ratio of 12/12/2/1, the micelle size remained virtually unchanged at 98.2 ± 7.2 nm. The plain PEG-PE micelles were negatively charged with a zeta-potential of -31.1 ± 1.7 mV. The addition of the uncharged ST to PEG-PE micelles did not noticeably change the micelle zeta-potential. A slight increase in the zeta-potential of the resulting micelles to -27.9 ± 4.5 mV might be explained by the increase in the micelle size because of ST solubilization in the micelle core (Table I) and resulting drop in the micelle surface charge density. When positively charged Lipofectin® lipids were added to PEG-PE micelles with coresolubilized ST, the zeta-potential of such micelles was dramatically increased to -8.0 ± 0.4mV. Additional loading of paclitaxel into mixed micelles that yielded the final PEG-PE/ST/LL/paclitaxel micelles did not cause any further change in micelle zeta-potential (it was -6.2 ± 0.7 mV).

Thus, our final optimized preparation of paclitaxelloaded micelles had a composition as PEG-PE/ST/LL/paclitaxel of 12/12/2/1 by weight, average micelle size of slightly under 100 nm,

and zeta-potential of about 26 mV. The content of paclitaxel in the preparation was about 4 wt %, and paclitaxel concentration was approx. 1 mg/ml.

The *in vitro* release of paclitaxel from these mixed micelles in PBS, pH = 7.4, at different temperatures is presented in Figure 2, which shows that after the incubation for 72 h at 4°C, the release of paclitaxel was 2.3 ± 0.2%, and only slightly higher (4.1 ± 0.3%) after the same incubation time at room temperature. However, when kept for 72 h at 37°C, paclitaxel release was 16.3 ± 1.2%. Such a significant difference in the release rate between room temperature and 37°C may be explained by the fact that in the micelles prepared, paclitaxel was loaded in the hydrophobic micelle core with high ST content. The triglyceride is in its solid form at room temperature and below, while at a temperature around 37°C it undergoes a phase transition into its liquid form thus facilitating paclitaxel release.

The preparation of paclitaxel in mixed micelles, after storage for 4 months at 4°C in the dark, showed a loss of only 2.9% of paclitaxel, probably because of the partial paclitaxel decomposition. The micelle size remains practically unchanged (98.2 ± 7.2 nm vs 96.8 ± 16.4 nm before and after storage, respectively). The long-term stability of paclitaxel-containing mixed micelles is currently being evaluated.

In vitro anticancer effects

The *in vitro* anticancer effects of paclitaxel-containing micelles are shown in Figures 3 and 4. Clearly, the addition of LL facilitates the intracellular uptake of paclitaxel-containing micelles with the compensated negative charge, since the anticancer effect of paclitaxel in PEG-PE/ST/LL micelles was significantly greater than that of free paclitaxel and of paclitaxel in PEG-PE/ST micelles in both cancer cell lines. In A2780 cancer cells, the IC50 values of free paclitaxel, paclitaxel in PEG-PE/ST micelles, and paclitaxel in PEG-PE/ST/LL micelles were 12.2, 3.9, and 0.7 μM, respectively (Figure 3). In BT-20 cancer cells, the IC50 values of the same preparations were 13.0, 8.5 and 4.7 μM, respectively (Figure 4).

It is now believed that the endocytosis of the DNA-loaded Lipofectin® particles is the major mechanism of cellular delivery of DNA by the lipofection (Legendre and Szoka, 1992, Felgner et al. 1994, Zabner et al. 1995, Sakurai et al. 2000). After endocytosis, the DNA-loaded particles can escape from the endosomes and enter the cytoplasm of most cells because of the interaction between cationic lipid and endosome membranes (Hafez et al. 2001). Therefore, it is possible that after the enhanced endocytosis, paclitaxel-loaded mixed PEG-PE/ST/LL micelles escaped from the endosomes and entered the cytoplasm of cancer cells, where paclitaxel could slowly release from the micelles and kill cancer cells with higher efficiency than free drug or drug in strongly negatively charged PEG-PE micelles. (This hypothesis was further verified by the set of experiments described in the next paragraph.)

In addition, it is known that Lipofectin® particles with high positive charge could induce a significant non-specific toxicity towards cells as was shown with Lipofectin-based gene delivery systems (Filion and Phillips, 1997). At the same time, mixed PEG-PE/positively charged lipid micelles with the net charge close to neutral were not toxic (paclitaxel-free control micelles did not affect the normal morphology and attachment of experimental cells), while they still increased the anticancer efficiency of the micelle-incorporated paclitaxel.

Micelle interaction with BT-20 cells by fluorescence microscopy

The interaction with cells and the intracellular fate of paclitaxel-containing PEG-PE/ST/LL micelles and similar micelles prepared without the addition of the LL was investigated by fluorescence microscopy using the BT-20 cell culture. As follows from the data presented in Figure 5, both PEG-PE/ST and PEG-PE/ST/LL micelles underwent endocytosis by BT-20

cells, which was confirmed by the presence of fluorescent endosomes in cells after co-incubation with fluorescently-labeled micelles. When the co-incubation time was 30 min, only LL-containing micelles were found in endosomes of some BT-20 cells. In 2 h, both LL-free and LL-containing micelles were found to be endocytosed by BT-20 cells. In the case of PEG-PE/ST/LL micelles, however, endosomes look partially degraded, which may serve as evidence of a destabilizing effect of the LL component on the endosomal membranes. This hypothesis is still further confirmed by the pattern observed after 4 h: if one can clearly see late (fused) fluorescent endosomes in the case of LL-free PEG-PE/ST micelles, cells with PEG-PE/ST/LL micelles demonstrate the presence of small fluorescent structures in the cytoplasm, but no "normal" endosomes could be seen. This observation supports the possibility of the enhanced cytoplasmic delivery of drugs, when endosome-destabilizing PEG-PE/ST/LL micelles are used as drug carriers.

In conclusion, the *in vitro* anticancer effects of paclitaxel were significantly improved when the drug was carried by mixed micelles made of PEG-PE and positively charged lipids. Such micelles may become a promising intracellular delivery system for clinical applications of paclitaxel.

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Figure 1.
The effect of the ST content in mixed micelles on paclitaxel micellization efficiency.

Figure 2.
The *in vitro* release of paclitaxel from mixed PEG-PE/ST/LL/paclitaxel (12/12/2/1 by weight) micelles in PBS, pH =7.4 at different temperatures.

Figure 3.
The *in vitro* viability of A2780 cancer cells after the incubation with different paclitaxel preparations. See “Materials and methods section” for details.

Figure 4.
The *in vitro* viability of BT-20 cancer cells after the incubation with different paclitaxel preparations. See “Materials and methods section” for details.

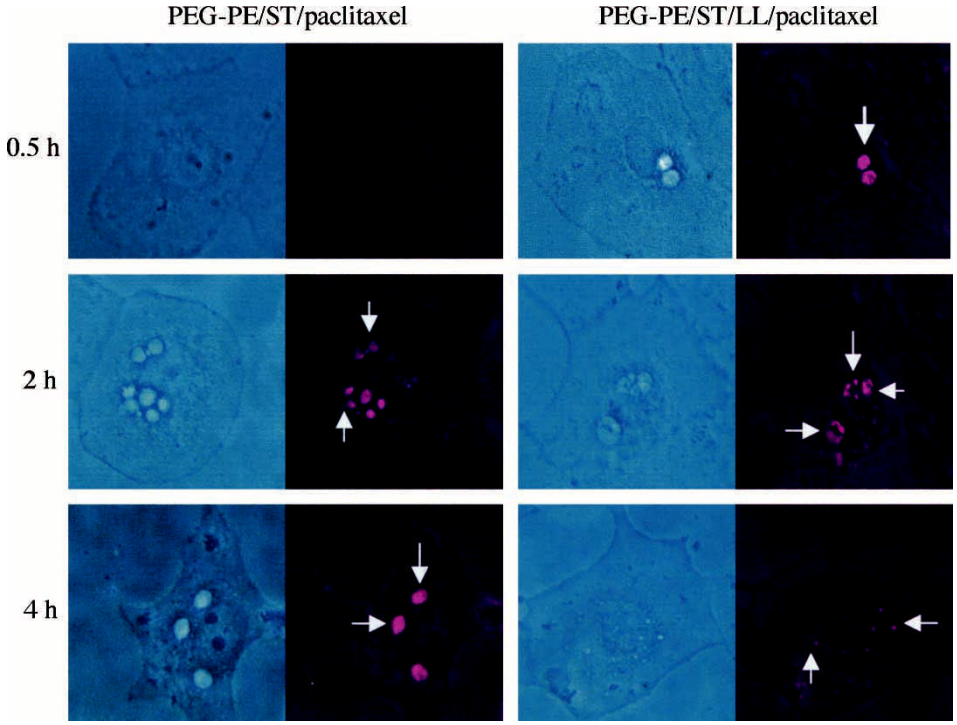

Figure 5.

Light (left images in each pair) and fluorescent (right images in each pair) microscopy of BT-20 cells incubated with Rh-PE-labeled PEG-PE/ST/paclitaxel micelles and PEG-PE/ST/LL/paclitaxel micelles for 0.5, 2, and 4 h. Arrows on fluorescent microscopy images show: fluorescent endosomes in cells incubated with PEG-PEG-PE/ST/LL/paclitaxel micelles for 0.5 h and with PEG-PE/ST/paclitaxel micelles for 2 h; partially degraded endosomes in cells incubated with PEG-PE/ST/LL/paclitaxel micelles for 2 h; fused late endosomes in cells incubated with PEG-PE/ST/paclitaxel micelles for 4 h; small fluorescent structures in cells incubated with PEG-PE/ST/LL/paclitaxel micelles for 4 h. See “Materials and methods section” for details.

Table I

Effect of solid triglyceride (ST), Lipofectin® lipids (LL), and paclitaxel on the particle size and zeta-potential of the micelles.

Micelle components (w/w)	Average diameter (nm)*	Zeta-potential (mV)†
PEG-PE	12.8 ± 2.6	-31.1 ± 1.7
PEG-PE/ST (12/12)	66.8 ± 6.6	-27.9 ± 4.5
PEG-PE/ST/paclitaxel (12/12/1)	71.2 ± 7.8	-27.0 ± 4.8
PEG-PE/ST/LL (12/12/2)	95.7 ± 3.5	-8.0 ± 0.4
PEG-PE/ST/LL/paclitaxel (12/12/2/1)	98.2 ± 7.2	-6.2 ± 0.7

*Average diameter is presented as mean±SD (n = 3).

†Zeta-potential is presented as mean ±SD ($n = 5 \sim 8$).