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Investigation of pluronic and PEG-PE micelles as carriers of meso-tetraphenyl porphine for oral administration

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Abstract

Meso-tetraphenyl porphine (mTPP) is a highly lipophilic, florescent porphyrin derivate and it is used as photosensitizer on the treatment of malign neoplasms. The aim of this study was to prepare mTPP loaded pluronic F127 and polyethylene glycol-distearoyl phosphatidylethanolamine (PEG_{2000}-DSPE) micelles to evaluate polymeric micelles potential for the transport of drugs through intestinal mucosa. Transport and bioadhesion behaviors of polymeric micelles was investigated using Caco-2 cell monolayer and everted rat intestine models. In order to show that Caco-2 cells can be used as a transport model cytotoxicity of formulations was tested. Cell viability was more than 80%, showing that Caco-2 cells will keep their viability during the transport studies demonstrating that prepared formulations can be securely used as oral drug carrier systems. Plain micelles were labeled with a fluorescent agent rhodamine-phosphatidylethanolamine (Rh-PE) and their transport through Caco-2 cells was investigated beside mTPP loaded micelles. At the end of 4 h transport study through Caco-2 cells, cumulative transport (%) of fluorescent agents were around 14% and 1% in Rh-PE labeled and mTPP loaded micelles This difference was attributed to the different placement of mTPP and Rh-PE in the micellar core. Drug transport was not estimated in everted rat intestine model but the bioadhesion was 79% and 70% for mTPP loaded pluronic F127 and PEG_{2000}-DSPE micelles. These good bioadhesion rates are promising for oral drug delivery.

Keywords: Polymeric micelles; Oral drug delivery; Intestinal absorption; Pluronics; Polyethylene glycol-phosphatidylethanolamine (PEG-DSPE)

1. Introduction

Drug delivery via oral route is the most common and preferred way for the administration of therapeutic agents especially when long-term or daily use is required. Intestinal epithelium is sort of a guard that controls the transport of drugs. Since bioavailability of a drug is its fraction in the systemic circulation, it is important to know the absorption and metabolism of drugs in intestinal mucosa (Ferrec et al., 2001). Mechanisms that take place in intestinal drug absorption may differ depending on parameters like, low solubility of active agent in gastro intestinal (GI) tract, low permeability through GI epithelium, low stability and first pass effect.

In pharmaceutical technology drug investigation studies depending on receptor based elimination methods result in the synthesis of many water insoluble drugs. In order to have a specific affinity to a target receptor a drug candidate usually carries a hydrophobic group. The solubility of such drugs in GI tract is the limiting factor affecting their oral bioavailability (Francis et al., 2003). In oral drug administration, following the oral application, a dosage form has to first dissolve in a complex media formed from bile salts, ions, lipids, cholesterol and enzymes. After the release of active agent it has to be absorbed. For this reason there are many studies to improve the solubility and dissolution rate of poorly soluble drugs thus enhance their bioavailability (Crison and Amidon, 1997). It is known that particular uptake takes place in GI system via the M cells of Payer's patches, normal enterocytes and isolated follicles of gut-associated lymphoid tissue (GALT) (Chen and Langer, 1998). Existence of these particular absorption sites lead researchers to prepare lipid and polymeric colloidal particular systems with high affinity to specific absorption sites (Chen and Langer, 1998; Florence, 1997a,b). Polymerized liposomes (Florence, 1997a,b; Chen et al., 1996) nanoparticles (Florence, 1997a,b; Lowe and Temple, 1994; Allemann et al., 1998; Sakuma et al., 2001), dendrimers (Florence, 1997b; Aulenta et al., 2003; Liu and

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Frechet, 1999; Florence and Hussain, 2001) and mucoadhesive systems (Florence, 1997a; Lehr et al., 1992; Rao and Buri, 1989; Takeuchi et al., 2001; Borchard et al., 1996) are among these systems. Besides poorly soluble drugs, possible oral administration of peptide protein drugs with these particular systems is also investigated. Among these systems polymeric micelles are very promising particulate drug carrier systems, which received growing scientific attention in recent years (Torchilin, 2001; Sezgin et al., 2003). Oral application of polymeric micelles is not a commonly studied subject. The advantageous properties of polymeric micelles such as the ability of incorporating and protecting poorly soluble drugs, improving drugs bioavailability, small particle size (<100 nm), targeting ability, long circulation and easy production encouraged us to study their oral application.

The main purpose of the study was to enhance the solubility of a lypohilic model drug in polymeric micelles and to investigate their behaviors as oral drug carrier systems. A highly lypophilic drug, meso-tetraphenyl porphine (mTPP) was chosen as drug model to be loaded in polymeric micelles. mTPP is a derivate of porphyrins which is used as photosensitizer on the treatment of malign neoplasms (Martindale, 1993). When combined with a fixed-frequency laser light porphyrins destroy tumor cells. This type of cancer treatment is called photodynamic therapy (PDT). PDT with porphyrins is especially effective in the treatment of gastric, esophageal, colorectal and bronchial tumors. Since porphyrins are effective in gastric tumors their oral application would be beneficial in terms of direct administration of the drug to the site of action and patient comfort. Polymeric micelles were preferred to reduce drug cytotoxicity towards healthy cells in the GI tract, reduce GI irritation, increase drug concentration at absorption sites thus improve drug bioavailability, ensure drug stability in GI tract and test the particular absorption.

In our previous study mTPP was loaded in to various types of pluronic and PEG-PE micelles. Micelles were evaluated in terms of size, zeta potential, loading efficiency and stability (Sezgin et al., 2006). Two formulations with the highest drug loading efficiency were chosen for this study. *In vitro* drug release was estimated for formulations. Two different models, Caco-2 transport model and everted rat intestine model were used to investigate the transport and bioadhesion behaviors of polymeric micelles. Before using cells as transport model, cytotoxicity of formulations against Caco-2 cells was tested to ensure that cell model was usable. Fluorescent agents are usually used to quantitatively and qualitatively assess intestinal uptake because it allows sensitive assay. Transport of mTPP loaded and rhodamine-PE labeled micelles was studied from apical to basolateral site in Caco-2 cells. By using two different florescent agents, we purposed to compare the effect of the agents location in micellar shape on the transport.

2. Materials and methods

2.1. Materials

l,2-Distearyl-*sn*-glysero-3-phosphoethanolamine-*N*-[metoksi(polyethyleneglycol)-2000] (PEG_{2000}-DSPE), and lissamine rhodamine B sulfonyl (Rh-PE) were purchased from, Avanti Polar-Lipids (Alabaster, AL, USA). Pluronic F127 was kindly provided by BASF (Mount Olive, NJ, USA). Meso-tetraphenyl porphine (mTPP) was purchased from Frontier Scientific (Logan, UT, USA). 96 Cell titer aqueous one solution was purchased from Promega (Madison, USA). All cell culture media were obtained from CellGro (Kansas City, MO, USA). Hepes free acid was obtained from ICN Biomedicals Inc. All other chemicals and components for buffer solutions were analytical grade preparations.

2.2. Incorporation of active agents into polymeric micelles

mTPP was loaded into micelles by film formation method as described previously (Sezgin et al., 2006). Briefly, mTPP solution in chloroform was added to the solution of polymers in chloroform to obtain 10:0.5 and 10:2 polymer/drug (w/w) ratios. Organic solvents were removed under vacuum and a drug–polymer film was obtained. Micelles were formed by extensive vortexing of this film in Hepes buffer solution (HBS), pH 7.4. Nonincorporated drug was separated by filtration of micelle suspension through a 0.2 μm filter. The mTPP concentration in filtrates was estimated by measuring its fluorescence at an excitation wavelength (λ_{ex}) of 514 nm and an emission wavelength (λ_{em}) of 650 nm (Hitachi F2000 fluorescence spectrometer/Japan) after extracting drug into toluene. The drug loading efficiency was expressed as the percentage of the extracted drug amount from micelles with respect to the initially added drug amount. Micelles were labeled with Rh-PE, by adding the solution of polymers in chloroform to the Rh-PE solution in chloroform. Organic solvents were removed under vacuum and a film was obtained. HBS, pH 7.4 was added to this film to get 10 mg/ml polymer concentration. After extensive vortexing of this film, micelle suspension was filtrated through a 0.2 μm filter. Before transport studies samples were diluted with Hank's balanced salt solution (HBSS).

2.3. In vitro release studies

The release experiment was carried out as followed: 3 ml of mTPP-loaded micelles were put into a dialysis tube (MWCO: 12–14,000) and then dialyze tube was introduced into a vial with 100 ml HBS and media was shaken at 37 °C. At specific time intervals, 0.5 ml of medium was taken and replaced with fresh HBS pH 7.4. *In vitro* release of samples was studied for 24 h. The concentration of released mTPP was determined spectrophotometrically at 419 nm (Schimadzu 1202 UV Visible/Japon). Each experiment was carried out in triplicate.

2.4. Cell culture

All cell culture studies were done under laminar flow and sterile conditions. Caco-2 cells were maintained from ATCC (American Type Culture Collection) and grown in ATCC complete growth medium containing Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS (balanced salt solution) adjusted to contain 1.5 g/L sodium bicarbonate,

0.1 mM non-essential amino acids, and 1.0 mM sodium private, 80%; fetal bovine serum, 20%.

Cell culture medium was removed and rinsed with 0.25% trypsin, 0.53 mM EDTA (ethylenediaminetetraacetic acid) solution. After removal of the solution and an additional 1–2 ml of trypsin–EDTA solution was added. Flask was allowed to sit at room temperature (or at 37 °C) until the cells detach. Fresh culture medium was added, aspirated and dispensed into new culture flasks. Cultures were maintained at a cell concentration between 10^4 and 10^5 cell/cm^2.

2.5. Cytotoxicity assay

In vitro cytotoxicity of polymers and mTPP-loaded micelles was evaluated by using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assay. Caco-2 cells were seeded using 96 well culture plates (Corning, Inc., Corning, NY, USA) at a seeding density of 5×10^4 cells/well in DMEM (Dulbecco's Modified Eagle Medium) culture medium. The cells were cultured in an atmosphere of 95% air and 5% CO_2 at 37 °C and 90% humidity for 48 h. Subsequently, culture medium was removed. Medium containing polymers or mTPP loaded micellar solutions and positive control Vitamin K (a known toxin for cells) was added to the wells. Cells were further incubated at 37 °C, 5% CO_2 for 24 or 4 h. Thereafter media was removed. One hundred microliters of HBSS and 20 μl 96 CellTiter solution was added to each well and cells were incubated for another 4 h at 37 °C. The absorbance at 492 nm was measured using an ELISA reader (Labsystem Multiskan MCC/340, Finland).

2.6. Transport studies through Caco-2 cells

The cells were seeded at a density of 8×10^4 cells/cm^2, on polycarbonate transwell inserts (12 mm, 0.4 μm pore size inserts, 1.1 cm^2 growth area). Inserts were fed every 2 days for 21 days. Before transport studies we indicated the integrity of each cell monolayer by measuring transepithelial electric resistance (TEER) using a Millicell-ERS voltohmeter (Milipore, Bedford, MA). The TEER readings for each cell monolayer exceeded 300 Ω/cm^2, showing the formation of tight junctions. Transport of the test compounds in the apical to basolateral direction across cell monolayer was performed by adding 0.5 ml mTPP loaded or Rh-PE labeled micelles to the apical side and 1.5 ml cell culture medium to the basolateral side of the monolayer. The inserts were moved to a well with 1.5 ml fresh transport medium in predetermined time intervals. A minimum of three different membranes was studied for each micellar formulation. As a control same transport study was repeated by using empty transwell inserts. Micellar solutions and transport medium was sterilized by filtration through 0.2 μm membrane filter before transport studies. After the completion of transport study TEER values were measured again. Florescence intensity of samples before and after transport study was measured by using florescence spectrophotometer. The wavelengths used were λ_{ex} 514 nm, λ_{em} 650 nm for mTPP-loaded samples and λ_{ex} 558 nm, λ_{em} 574 nm for rhodamine-PE labeled samples. 0.5% Triton was added to rhodamine-PE labeled micelles before performing the fluorescence measurements. Results were given in a graph where the I_t/I_0 (%) versus time was plotted. (I_0: initial florescence intensity measured in apical side and I_t: florescence intensity measured at time t in basolateral side.)

2.7. Transport and bioadhesion studies through everted rat intestine

Uptake and transport studies of mTPP loaded polymeric micelles were investigated in everted rat intestine. This study was approved by the Ankara University Animal Care and Use Committee. For 1 day before experiments male Wistar rats weighing about 300 mg were fasted and provided with only distilled water. Rats were anesthetized with ether and abdominal incision was done. Mesentery was removed carefully and a 15 cm long part was taken from jejunum. Immediately rats were euthanized by aortic dissection. Excised intestines were rinsed by ice cold Krebs bicarbonate solution (pH 7.4) aerated by 95% O_2 and 5% CO_2 and everted by using a 5 cm long glass stick. One end of the everted segment was ligated and a 4 cm long tygon tube was inserted to other end and a syringe was attached to end of the tygon tube. 3.5 ml Krebs bicarbonate solution was injected into rat sac and suspended in cells containing 15 ml, 37 °C, Krebs bicarbonate solution (pH 7.4) gassed continuously with 95% O_2 and 5% CO_2 and incubated for 15 min. At $t = 0$ micellar solutions at a concentration of 4.09×10^{-3} mg/ml was added to mucosal side. In predetermined time intervals 500 μl samples were taken from serosal side via syringe and replaced with Krebs bicarbonate solution. In order to see mucosal adhesion 500 μl samples were taken from mucosal side at $t = 0$ and $t = 120$ min and same volume of buffer solution was added to maintain volume. Each experiment was repeated three times by using different subjects. Collected samples were lyophilized and drug content was measured spectrophotometrically at 419 nm after extracting into toluene.

3. Results and discussion

3.1. Loading of drugs in polymeric micelles

By using the film formation method, different loading efficiencies of mTPP was obtained among different polymer:drug ratios. At an initial mTPP/PEG_{2000}-DSPE (w/w) ratio 10:1 and mTPP/pluronic F127 (w/w) ratio 10:0.5, mTPP incorporates into micelles with an efficiency of 95.4% and 90.1%, respectively (Sezgin et al., 2006). For this reason these two formulations were chosen for the investigation of intestinal absorption (Santh et al., 2005).

The hydrodynamic diameters of polymeric micelles were measured by dynamic light scattering method. Mean diameters for mTPP loaded PEG-PE and pluronic micelles were 6 and 30 nm. These particle size measurements were within the characteristic micelle size (<100 nm) ensuring the formation of polymeric micelles (Sezgin et al., 2006). In intestinal absorption, it is previously shown that decreasing particle size and increasing specific surface area lead improvement in particular

uptake and drug absorption (Francis et al., 2003). Because of this reason small sizes of PEG-PE and pluronic micelles would be beneficial for oral drug delivery.

3.2. In vitro drug release studies

At the end of 24 h *in vitro* drug release study no mTPP was detected on the release medium. mTPP is a highly lipophilic drug and probably the amount of released drug was even below detection limits of spectrophotometer. Strong hydrophobic interactions consist between polymer and mTPP because of highly hydrophobic phenyl groups in mTPP structure molecule and this blocks drug release from hydrophobic micelle core. This result might be advantageous in stability aspect (Gao et al., 2002). Since micelles retain all of the incorporated mTPP, the formulations have high stability and drug in micelle core would be protected from biological degradation in GI before reaching absorption site.

3.3. Cytotoxicity assay

The transepithelial transport of potential drug candidates is widely tested by using Caco-2 cells as *in vitro* model. Caco-2 cells are human colon adenocarcinoma cells which form a barrier with morphological and biochemical similarity to the small intestinal epithelium when cultured on semi permeable membranes. Before transport studies Caco-2 cell viability in the presence of anticancer drug loaded micelles was determined. This test was important because of several reasons including the investigation of formulations safety for oral drug delivery and usability of Caco-2 as a transport model for mTPP.

Cytotoxicity tests were done using MTS assay. The cytotoxicity of the polymers used in micelle preparation was evaluated by incubating Caco-2 cells and polymers with various concentration (0.1–555 mg/ml), over a 24 h time course. At these concentrations a viability of at least 80% was observed (Fig. 1A). No significant change was detected in cell viability with the increasing polymer concentration. Thus the safety of the polymers used in micelle preparation was shown.

The cytotoxicity of mTPP loaded PEG-PE and pluronic micelles against Caco-2 cells was also determined for 4 and 24 h (Figs. 1 and 2Figs. 1B and 2B). Within 4 h cell viability was more than 80% whereas the cells incubated with the negative control, Vitamin K (40 μg/ml), demonstrated a viability of 37% (Fig. 2A). Increasing mTPP concentration did not affect the cell viability (Fig. 2B). Similar results were obtained for mTPP-loaded micelles in 24 h test (Fig. 1B). This results show that Caco-2 cells will keep their viability during the transport study period and they can be used to investigate *in vitro* mTPP loaded micelle permeation. Thus the benign nature of micelles was demonstrated (Bromberg and Alakhov, 2003). Since mTPP is an anticancer drug its nontoxic effect on tumor cells may be a questioned at this point. Studies showed that a light activation is necessary for the cell destroying effect of porphyrins (Alvarez et al., 2000). So in our case no cytotoxicity was observed due to lack of light, which activates mTPP. Cytotoxicity results also

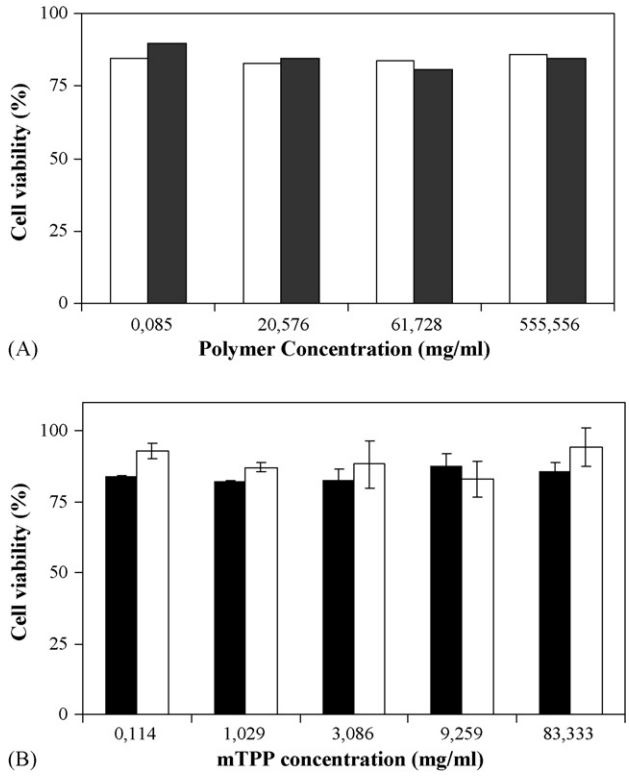

Fig. 1. Twenty-four-hour cytotoxicity test results for: (A) PEG_{2000}-DSPE (■) and pluronic F127 (□) copolymers and (B) mTPP loaded PEG_{2000}-DSPE (■) and pluronic F127 micelles (□). Data shown as mean ± S.E.

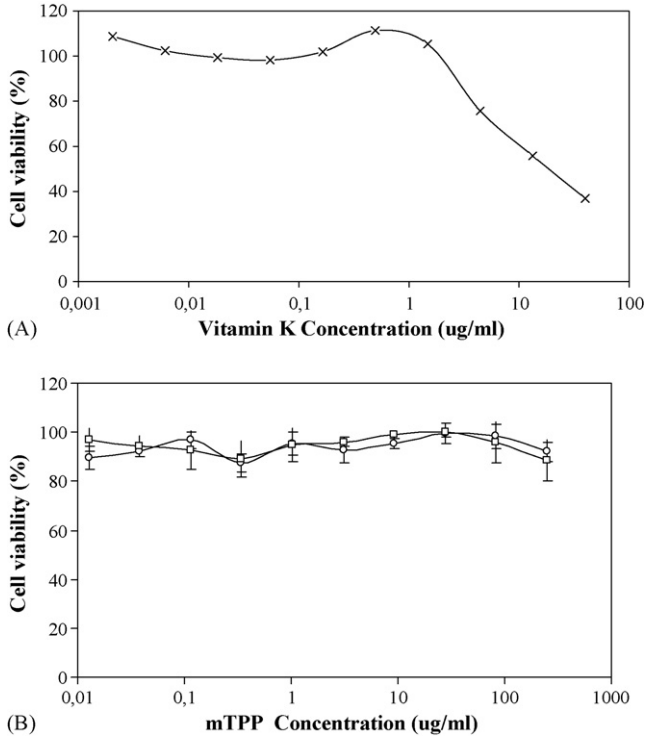

Fig. 2. Four-hour cytotoxicity test results for: (A) Vitamin K (×) and (B) mTPP loaded PEG_{2000}-DSPE (□) and pluronic F127 micelles (◯). Data shown as mean ± S.E.

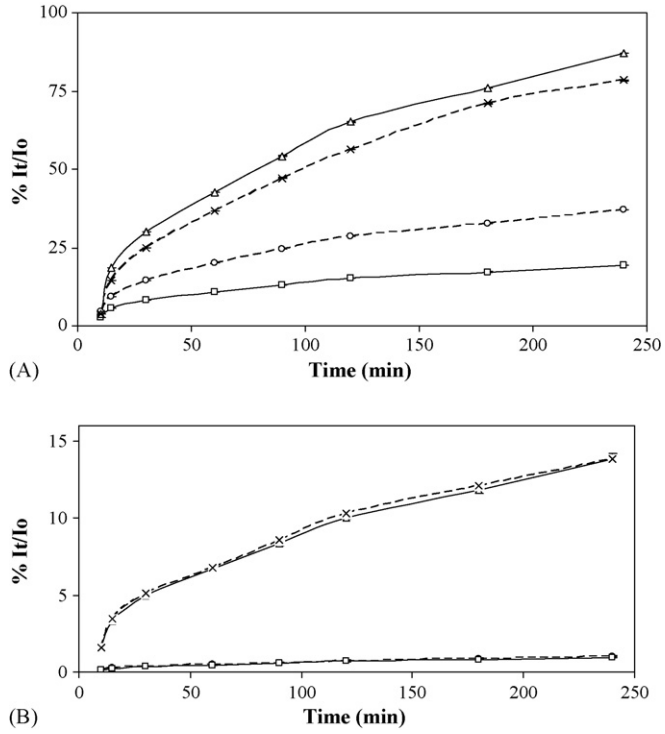

Fig. 3. Transport of florescent agents through: (A) empty Transwell inserts and (B) Caco-2 cell monolayer. Each point represents mean ± S.E. (mTPP loaded PEG_{2000}-DSPE micelles (◯), mTPP loaded pluronic micelles (□), Rh labeled PEG_{2000}-DSPE micelles (△) and Rh labeled pluronic micelles (×).)

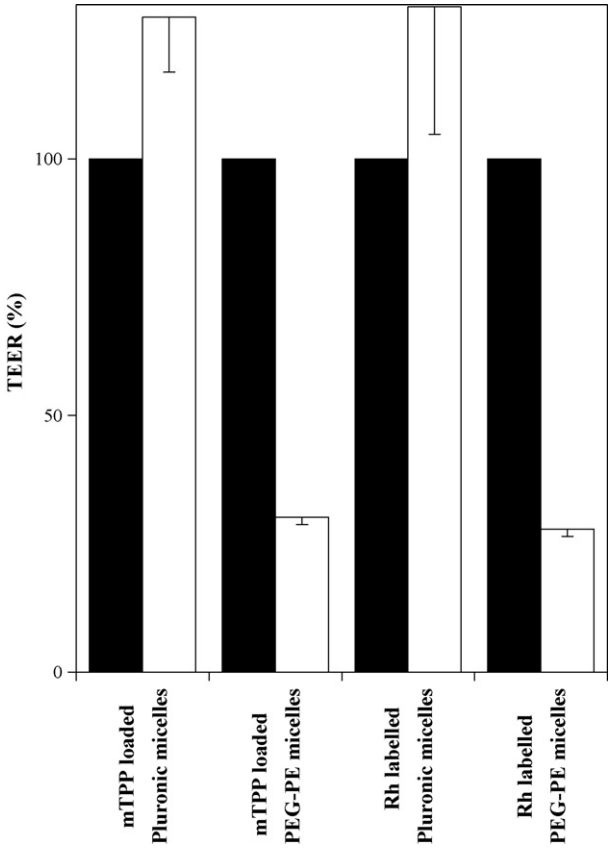

Fig. 4. Comparison of TEER values of Caco-2 cell monolayer before (■) and after (□) transport study. Data shown as mean ± S.E.

demonstrated that prepared formulations can be securely used as oral drug carrier systems.

3.4. Transport studies through Caco-2 cells

There are many different test models, which can be used to investigate drug absorption in GI system. Although the most reliable results can be obtained by *in vivo* experiments in humans, there are different *in vitro*, ex vivo and *in vivo* techniques such as cell cultures, isolated tissues different animals, etc. All this methods bring advantages and disadvantages that influence the transport results. In this study two models, Caco-2 and everted rat intestine transport models were used.

In Caco-2 cell monolayer transport model transport of mTPP itself could not be investigated because in transport medium, it was not dissolved and formed aggregates with no florescence. Theoretically it is known that undissolved drugs cannot pass through intestine. By solubilizing mTPP in micellar solution, it was possible to get florescence and investigate its epithelial transport in Caco-2 cells. Transepithelial transportations of formulations through cell seeded and empty polycarbonate transwell inserts were as shown in Fig. 3. When the profiles were compared the barrier function of Caco-2 cells was clearly recognized. In empty inserts, the mTPP amount determined at the end of the transport was only 20–35% of the initial doses. It seems that some mTPP was bound to polycarbonate membrane. Thus polycarbonate membrane itself came out to be a transport-limiting factor. In case of Rh-PE this value was around 75% indicating a less binding to the membrane. Cumulative transport of Rh-PE labeled micelles through Caco-2 monolayer was around 14%. When compared to transport of mTPP loaded micelles which is around 1%, this higher transport may be due to higher binding of mTPP to polycarbonate membrane or different location of Rh-PE in micellar structure than mTPP because of its more hydrophilic nature. While mTPP entraps in micelle core, water soluble "Rhodamine B Sulfonoil" part of Rh-PE locates between hydrophilic PEG chains and PE part in hydrophobic micelle core. Release of Rh-PE from micelles due to decreasing hydrophobic interrelation in the micelle core might also lead high % Rh-PE transportation. High Rh-PE transport made us think that peptide and proteins active agents may be hydrophobized and loaded into polymeric micelles since its positioning in micelle would be the same as Rh-PE. By this way drug would be preserved from degradation and their oral absorption will be increased.

Before performing transport studies TEER values were measured in empty and cell seeded polycarbonate transwell inserts. The results were 125 and 890 Ω/cm^2 respectively. This difference and high TEER (>300 Ω/cm^2) in cell seeded inserts showed that tight junctions were formed and the cells were ready for transport study. At the end of 4 h % changes in TEER were calculated as in Fig. 4. Increase in TEER values was seen in cell monolayers after transport study with pluronic micelles. But TEER values were decreased 30% and 28%, in mTPP and Rh-PE loaded PEG-PE micelles. These types of reductions on TEER values after transport is usually explained by possible opening

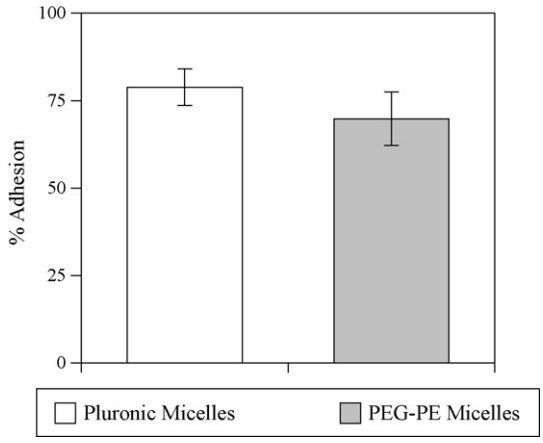

Fig. 5. Mucosal bioadhesion results of polymeric micelles in everted rat intestine model. Data shown as mean ± S.E.

affect on tight junctions thus improved paracellular transport or cell death. In our case it was previously shown that formulations have no apparent cytotoxicity on Caco-2 cells so this TEER reduction might be due to opening tight junctions. If this is the issue, opening tight junctions did not lead an increase in Rh labeled PEG-PE micelles transport showing that paracellular transport is not a transport pathway for these micelles.

It is thought that collagen coating in transwell inserts and absence of physiological factors (mucous, salts, cholesterol) might limit the transport of micelles. These factors may primarily affect the adhesion and dispersion of the micelles and solubilization of the drug. Being a static model also brings disadvantages to this method (Ferrec et al., 2001).

3.5. Transport and bioadhesion studies through everted rat intestine

In the transport study with everted rat intestine model mTPP was not estimated in the serosal side. This result contributed to several reasons such as lack of perfusion in this model. Normally in intestinal absorptions drug in lumen reaches to blood and lymphatic vessels in lamina propia. But in this transport model drug has to pass through the whole intestine wall to reach the serosal side thus transported amount of drug will decrease (Ferrec et al., 2001). High volume of medium in serosal site (15 ml) might also dilute transported drug under the detection limit in spectrophotometer.

This study revealed the high mucoadhesion of drug as shown in Fig. 5. Bioadhesion was 79% and 70% for mTPP loaded pluronic F127 and PEG_{2000}-DSPE micelles. It was statistically shown that polymer types did not affect the bioadhesion rate significantly ($p < 0.05$). Good bioadhesion rates seem to be promising for oral drug delivery. It is known that prolonged residence time due to increased duration of contact with mucosal surface in GI system can improve drug bioavailability (Pimienta et al., 1992). Behaviors of the formulations should be tested with *in vivo* models to eliminate possible transport limiting factors of Caco-2 cells and everted rat intestine models. Owing to good bioadhesion results better transport results is expected in *in vivo* bioavailability test.

In vitro drug release studies showed that micelles retain all of the incorporated mTPP; lack of transport through the intestinal wall in both models may also be due to unreleased drug from micellar core.

4. Conclusions

mTPP, a poorly water-soluble anticancer drug was introduced into polymeric micelle core successfully by film formation method. *In vitro* drug release test showed that micelles retain all of the incorporated mTPP, thus the formulations were stable and drug would be protected from GI degradation. With the cytotoxicity test in Caco-2 cells it was demonstrated that prepared formulations can be securely used as oral drug carrier systems and Caco-2 cells were suitable to be used as *in vitro* transport model. In the transport studies with mTPP loaded polymeric micelles, no significant transport of mTPP was observed in Caco-2 cells and everted rat intestine. This result could be associated with the disadvantages of transport models. However micellar formulations showed good bioadhesive property in everted rat intestine. This seems to be promising for oral drug delivery because increased duration of contact with mucosal surface in GI system can improve drug bioavailability. *In vivo* effectiveness of mTPP loaded polymeric micelles should be further evaluated considering the disadvantages of models used in the study.

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