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Vitamin E TPGS coated liposomes enhanced cellular uptake and cytotoxicity of docetaxel in brain cancer cells

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ABSTRACT

The aim of this work was to develop a drug delivery system of liposomes, which are coated with D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), a PEGylated vitamin E, with docetaxel as a model drug for enhanced treatment of brain tumour in comparison with the nude liposomes as well as with the so-called stealth liposomes, *i.e.* those coated with polyethylene glycol (PEG), which have been intensive investigated in the literature. Docetaxel or coumarin-6 loaded liposomes were prepared by the solvent injection method and characterized for their particle size, polydispersity, zeta potential and drug encapsulation efficiency. C6 glioma cells were employed as an in vitro model to access cellular uptake and cytotoxicity of the drug or coumarin-6 loaded liposomes. The particle size of the PEG or TPGS coated liposomes was ranged between 126 and 191 nm. High-resolution field-emission transmission electron microscopy (FETEM) confirmed the coating of TPGS on the liposomes. The IC50 value, which is the drug concentration needed to kill 50% cells in a designated time period, was found to be 37.04 ± 1.05, 31.04 ± 0.75, 7.70 ± 0.22, and 5.93 ± 0.57 μg/ml for the commercial Taxotere®, the nude, PEG coated and TPGS coated liposomes, respectively after 24 h culture with C6 glioma cells. The TPGS coated liposomes showed great advantages in vitro than the PEG coated liposomes.

1. Introduction

The poor clinical outcomes with adverse effects of conventional cancer chemotherapy have promoted the development of novel drug delivery platforms (Feng and Chien, 2003). Currently, novel drug delivery platforms developed based on the nanotechnology such as micelles, liposomes, solid lipid nanoparticles and nanoparticles of biodegradable polymers are proposed to improve cancer chemotherapy (Muthu and Feng, 2010). Liposomes are lipid bilayer vesicles, which were first prepared in the 1960s (Huang, 1969). Liposomes can be seen as the simplest artificial biological cells, which have great potential applications in drug delivery, gene therapy, molecular imaging and artificial blood as well as to be used as a model biological cell and cell membrane (Muthu and Feng, 2010). Liposomes may vary in size, most being 200 nm or less, which can be termed as "nanoliposomes". Nanosized liposomes can be made of various natural lipids, and usually composed of phospholipids/cholesterol to encapsulate various active water soluble drug in the hydrophilic core and/or insoluble drug in the hydrophobic membrane for sustained and controlled delivery of imaging and therapeutic agents with enhanced ADME (adsorption, distribution, metabolism and excretion) process to maximize efficacy and minimize side effects. Passive and active targeting can also be realized by enhanced permeability and retention (EPR) and ligand conjugation (Elbayoumi and Torchilin, 2010; Lasic and Papahadjopoulos, 1998; Feng et al., 2004). However, the major drawbacks of conventional liposomes (we call nude liposomes in this article) come with instability, insufficient drug loading, too fast drug release and too short circulation time in the blood (Samad et al., 2007; Dutta, 2007). At the end of 1980s, the landmark in liposome development was the invention of stealth, *i.e.* long circulating liposomes by PEGylation to increase the stability of the liposomes in the blood (Gregoriadis, 1976; Devalapally et al., 2007; Allen et al., 1991). It was suggested that by its excluded volume effect, the long polyethylene glycol

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(PEG) chain coated on the liposome surface prevents the adsorption of plasma protein on to the liposome surface and, as a result, effectively reduces the liposome aggregation in plasma (Yoshioka, 1991; Muthu and Singh, 2009; Yuan et al., 2010).

D-Alpha-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or simply TPGS) is a PEGylated vitamin E, which has greatly improved the pharmaceutical properties of vitamin E and thus has been widely applied in the food and drug industry. TPGS, prepared from the esterification of D-alpha-tocopheryl acid succinate and PEG 1000, is an amphiphilic vitamin E, which is quite stable under normal conditions without hydrolysis. Owing to its hydrophilic–liphophilic balance (HLB) value being between 15 and 19, TPGS has excellent water solubility and it is suitable to serve as an effective surfactant, which can emulsify hydrophobic molecules (Wu and Hopkins, 1999; Varma and Panchagnula, 2005). The co-administration of TPGS has been shown to enhance the solubility, inhibit P-glycoprotein mediated multi-drug resistance, and increase the oral bioavailability of anti-cancer drugs (Dintaman and Silverman, 1999; Boudreaux et al., 1993; Chang et al., 1996; Mu et al., 2005). We have reported TPGS–doxorubicin conjugate as a novel prodrug, which enhanced the therapeutic potential and reduced the systemic side effects of the drug (Cao and Feng, 2008; Anbharasi et al., 2010). Additionally, we studied TPGS as an emulsifier in the preparation of poly (D, L, lactide-co-glycolide) (PLGA) nanoparticles (Mu and Feng, 2003), and as a component of new biodegradable copolymer polylactide–TPGS (PLA–TPGS) for nanoparticle formulation of anti-cancer drugs (Zhang and Feng, 2006). As an effective emulsifier, TPGS has greatly enhanced the performance of nanoparticles, resulting in much higher emulsification efficiency (67 times higher than polyvinyl alcohol) (Mu and Feng, 2002), drug encapsulation efficiency (up to 100%) (Mu and Feng, 2002), cellular uptake, and in vitro cancer cell cytotoxicity, and more desirable in vivo pharmacokinetics (up to 360 h effective treatment for one shot *i.v.* administration) (Feng, 2006; Khin and Feng, 2006). Also, we have recognized the marvelous advantageous of TPGS derivative (TPGS2000) as an effective composition of docetaxel loaded micelles for synergistic effect (Mu et al., 2005; Mi et al., 2011).

In the literature, replacement of PEG by TPGS for liposome surface coating has shown even longer circulation time (Wang et al., 2005; Zhai et al., 2008). Recent studies also showed the feasibility of forming TPGS containing liposomes, which showed improvement in the permeation of dextran through Caco-2 cells (Transwell® model) without any cytotoxicty effect (Parmentier et al., 2010, 2011). Nevertheless, their investigation was focused on oral drug delivery for better permeability and stability across the gastro intestinal (GI) tract. Therefore, further investigation of preparation, characterization, in vitro and in vivo therapeutic effects on the advantages of the TPGS coated liposomes need to be investigated further for cancer therapeutics.

Docetaxel (N-debenzoyl-N-tert-butoxycarbonyl-10-deacetylpaclitaxel) is used in this research as a model anticancer drug, which is a semi-synthetic derivative of the taxoid family of anti-neoplastic agents (Bissery et al., 1991, 1995). We aim in this research to enhance its therapeutic effects and reduce its side effects by formulation of the drug in the TPGS-coated liposomes in close comparison with the clinical Taxotere® (docetaxel formulated in polysobate 80), which causes side effects despite of higher patient response than Taxol® (paclitaxel formulated in Cremophor EL). The docetaxel loaded liposomes (nude, PEG-coated and TPGS-coated) were prepared by the solvent injection method, which were characterized for their size and size distribution, surface morphology, surface charge, drug encapsulation efficiency and drug release profile. Fluorescent coumarin-6 loaded TPGS coated liposomes were prepared for quantitative and qualitative investigation of cellular uptake by C6 glioma cells. In vitro cytotoxicity of C6 glioma cells was assessed and IC50 values were obtained to evaluate the therapeutic effects of the three types of docetaxel formulations in comparison.

2. Materials and methods

2.1. Materials

Docetaxel of purity 99.56% was purchased from Jinhe Bio-Technology Co. Ltd. (Shanghai, China). 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was acquired from Avanti Polar Lipids (Alabama, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE–mPEG-2000) was a generous gift from Lipoid GmbH (Ludwigshafen, Germany). D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) $C_{33}O_5H_{54}$ $(CH_2CH_2O)_{23}$ was from Eastman chemical company (Kingsport, TN, USA). Cholesterol, acetone, methanol, ethanol, phosphate buffer saline (PBS), coumarin-6, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), penicillin–streptomycin solution, trypsin–EDTA solution and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tween 80 was from ICN Biomedicals, Inc. (OH, USA). Triton X-100 was provided by USB Corporation (OH, USA), fetal bovine serum (FBS) was purchased from Gibco Life Technologies (AG, Switzerland). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen Corporation. C6 glioma cells were provided by American Type Culture Collection. Clinical formulation Taxotere® was supplied by Aventis Pharmaceuticals, USA. All solvents such as acetonitrile, methanol and ethanol were of high performance liquid chromatography (HPLC) grade. All chemicals were used without further purification. Millipore water was prepared by a Milli-Q Plus System (Millipore Corporation, Breford, USA).

2.2. Preparation of docetaxel or coumarin-6 loaded liposomes

Conventional (non-coated), DSPE–mPEG-2000 coated (PEG coated) and TPGS coated liposomes were prepared according to the solvent injection method (Wang et al., 2005). In brief, docetaxel/coumarin-6, DPPC, cholesterol and either DSPE–mPEG-2000 or TPGS (added only in the preparation of coated liposomes) were dissolved in 0.3 ml of ethanol at 60 °C, according to the formulae (Table 1). The mixtures were injected into 2.7 ml of 1 mM phosphate buffered saline, pH 7.4 isothermally at 60 °C. The suspensions formed were kept at 60 °C under stirring for 60 min to form multilamellar vesicles (MLV). The small unilamellar vesicles were prepared from the MLV suspensions by ultrasonication (Vibra Cell™, 130w, 20 kHz) for 5 min followed by filtration through 0.22 μm filter while keeping the lipids above the phase transition temperature (T_m). Finally, liposomes were centrifuged at 11,000 rpm for 15 min to remove the excess non-incorporated drug and stored at 4 °C. Liposomes formulae were established to get higher drug encapsulation, controlled drug release and stability (Fig. 1A–C).

2.3. Liposome characterization

2.3.1. Particle size, polydispersity, zeta potential and surface morphology

Size, polydispersity and zeta potential of the liposomes were measured by photon correlation spectroscopy (PCS) using Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK). The liposome samples were analyzed after 50 times dilution with deionized water to a count rate of 100–300 kcps.

Table 1
Formulas of liposomes.

Batches	Lipid compositions	Molar ratio	Docetaxel (mg)	Coumarin-6 (mg)
DTX	DPPC:cholesterol	8:7.7	1.0	–
DTX–mPEG	DPPC:cholesterol:DSPE–mPEG 2000	8:7.7:1	1.0	–
DTX–TPGS	DPPC:cholesterol:TPGS	8:7.7:1	1.0	–
CM6	DPPC:cholesterol	8:7.7	–	0.1
CM6–mPEG	DPPC:cholesterol:DSPE–mPEG 2000	8:7.7:1	–	0.1
CM6–TPGS	DPPC:cholesterol:TPGS	8:7.7:1	–	0.1

DTX: docetaxel loaded conventional liposomes prepared with DPPC and cholesterol.
DTX–mPEG: docetaxel loaded liposomes prepared with DSPE–mPEG-2000.
DTX–TPGS: docetaxel loaded liposomes prepared with TPGS.
CM6: coumarin-6 loaded conventional liposomes prepared with DPPC and cholesterol.
CM6–mPEG: coumarin-6 loaded liposomes prepared with DSPE–mPEG-2000.
CM6–TPGS: coumarin-6 loaded liposomes prepared with TPGS.

2.3.2. Characterization by atomic force microscopy

The surface properties of TPGS coated liposomes were visualized by an atomic force microscope (AFM) (Digital 3000 Nanoscope, Santa Barbara, California, USA) under normal atmospheric conditions. Explorer atomic force microscope was set in tapping mode, using high resonant frequency (F_0 = 346 kHz) pyramidal cantilevers with silicon probes having force constants of 41 N/m. Scan speed was set at 1 Hz. The sample was diluted 50 times with distilled water and then dropped onto glass slides, followed by vacuum drying during 24 h at 25 °C. The measurements were obtained using AFM image analysis software (Nanoscope 5.30).

2.3.3. Morphological study

Surface morphology was also studied by a field-emission transmission electron microscopy (FETEM) system (JEM 2010F, JOEL, Japan). Samples of conventional non-coated, PEG coated and TPGS coated liposomes were prepared by placing one drop on a copper grid and dried under vacuum pressure. The dried liposomes being stained with 1% phosphotungstic acid for 30 s were finally examined.

2.3.4. Determination of drug/dye encapsulation efficiency

The docetaxel encapsulated in the liposomes was measured by HPLC (Agilent LC1100, Agilent, Tokyo, Japan). A reverse-phase HPLC column (Agilent Eclipse XDB-C18, 4.6 mm × 250 mm, 5 μm) was used. Briefly, 20 μl of drug loaded liposomes were mixed with 80 μl of methanol for liposomes disruption and dissolved in mobile phase consisting of acetonitrile and deionized water (50:50, v/v) to 1 ml. The solution was filtered through 0.45 μm syringe filter before transferred into HPLC vial. The flow rate of mobile phase was set at 1.0 ml/min. The column effluent was detected with a UV/VIS detector at 230 nm. The calibration curve was linear in the range of 50–50,000 ng/ml with a correlation coefficient of R^2 = 0.999. The drug encapsulation efficiency was defined as the ratio between the amount of docetaxel encapsulated in the liposomes and that added in the liposomes preparation process.

For fluorescent liposomes, the coumarin-6 encapsulation efficiency was determined by the same dilution process as described for the drug-loaded liposomes. The fluorescence was measured by HPLC with a flow rate of 1.3 ml/min mobile phase consisting of acetonitrile/deionized water (60:40 v/v). The excitation and emission wavelength were set at 462 nm and 502 nm, respectively, using a fluorescence detector module.

2.3.5. In vitro drug release

The dialysis bag diffusion technique was used to study the in vitro drug release from the docetaxel loaded liposomes (Muthu et al., 2009). The drug loaded liposomes of a volume equivalent to 100 μg of docetaxel were placed in the dialysis bag (cellulose membrane, molecular weight cut off 1000 Da), hermetically sealed and immersed into 20 ml of phosphate buffered saline (pH 7.4) containing 0.1% (w/v) Tween 80. The entire system was kept at 37 ± 0.5 °C with continuous shaking at 100 rpm/min. Samples were withdrawn from the receptor compartment at predetermined time intervals and replaced by fresh medium. Docetaxel has low solubility in phosphate buffered saline (pH 7.4). Therefore, sink conditions

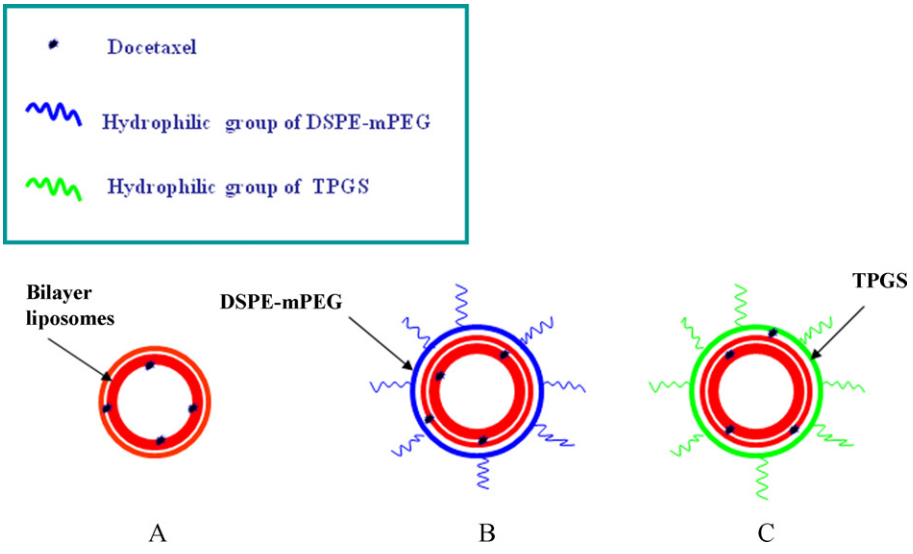

Fig. 1. Schematic diagram of (A) the conventional (non-coated) liposomes, (B) the PEG coated liposomes, and (C) the TPGS coated liposomes.

were maintained for release studies by adding 0.1% (w/v) Tween 80 in the release medium. The samples were filtered through 0.45 μm syringe filter before transferred into HPLC vial. The drug content in the samples was determined by HPLC (Agilent LC1100, Agilent, Tokyo, Japan) as described in the drug encapsulation efficiency determination. The drug release profiles were calculated.

2.4. Cellular uptake of liposomes

For quantitative study, confluent C6 glioma cell line (American Type Culture Collection) were harvested with 0.125% Gibco trypsin–EDTA solution (Invitrogen) and seeded into 96 well assay plates (Corning Incorporated) at 5×10^3 viable cells/well. After the cells reached confluence, the cells were incubated with 100 μl of 10 μg/ml coumarin 6-loaded liposomes (all three types) in the DMEM supplemented with 10% Hyclone fetal bovine serum (FBS, Thermo Scientific) and 1% Gibco penicillin–streptomycin (Invitrogen) at 37 °C for 30 min, 1 h and 2 h. At designated time period, the suspension was removed and the wells were washed three times with 50 μl cold PBS. After that, 50 μl of 0.5% Triton X-100 in 0.2 N NaOH was introduced into each well for cell lysis. The fluorescence intensity of each sample well was measured by Tecan microplate reader (GENios) with excitation wavelength at 430 nm and emission wavelength at 485 nm. Cellular uptake efficiency was expressed as the percentage of cells-associated fluorescence after washing vs the fluorescence present in the feed suspension.

For the qualitative study, confluent C6 glioma cells were harvested with 0.125% Gibco trypsin–EDTA solution (Invitrogen) and seeded in LABTEK® cover glass chambers (Nagle Nunc®) having DMEM at a concentration of 5×10^3 viable cells/chamber. The cells were incubated overnight and were subsequently incubated with coumarin 6-loaded liposomes in the DMEM (concentration of 10 μg/ml) at 37 °C. After 2 h, the cells were washed 3 times with cold PBS and fixed by 75% ethanol for 20 min. Then, the cells were washed twice with cold PBS. The nuclei were stained by incubating with propidium iodide (PI, Sigma) for another 10 min. The cell monolayer was washed three times with PBS and observed by confocal laser scanning microscopy (CLSM) (Nikon C1, Nikon Corporation, Japan) with imaging software, NIS-Element AR 3.0.

2.5. Cytotoxicity of docetaxel formulated in liposomes

Cytotoxicity of docetaxel formulated in the drug-loaded liposomes was investigated by the MTT assay. 100 μl of C6 glioma cells were seeded into 96 well plates (Costar, IL, USA) at the density of 5×10^3 viable cells/well with DMEM and incubated at least overnight to allow cell attachment. The spent medium was discarded and the cells were incubated with all three types of docetaxel loaded liposomes and their cytotoxicty was assessed in comparison with Taxotere® at 0.025, 0.25, 2.5 and 25 μg/ml equivalent drug concentration for 24 h. After 24 h, the medium was removed and the wells were washed twice with cold PBS. Following that, 100 μl of MTT solution (0.5 mg/ml) prepared in the DMEM was added to each well of the plate. The plates were further incubated for 3–4 h in the incubator. Finally, MTT in medium was removed and 50 μl of DMSO was added into each well of transformed MTT crystals and the absorbance of the transformed MTT solution in the wells was measured at 450 nm wavelength using a microplate reader. Cell viability was calculated by the followed equation:

$$\text{Cell viability}(\%) = \frac{\text{ABS of sample}}{\text{ABS of control}} \times 100$$

where ABS of sample is the absorbance of the transformed MTT in cells incubated with the liposomes while the ABS of control is the absorbance of transformed MTT in cells incubated with the culture medium only (positive control). IC_{50}, the drug concentration at which 50% of the cell population in a designated period was destroyed in comparison with that of the control sample, was calculated by regression (curve fitting) of the cell viability data.

2.6. Statistical analysis

Results are given as mean ± standard deviation (S.D). Mean values of encapsulation efficiency and in vitro data were compared using the Student's *t*-test. Differences are considered significant at a level of $P < 0.05$.

3. Results and discussion

3.1. Particle size, polydispersity and zeta potential analysis

The mean particle size and polydispersity of docetaxel loaded liposomes were shown in Table 2. PCS measurements were undertaken in multimodal analysis to get a true reflection of particle size distribution (Muthu and Singh, 2008). The particle size distribution curves for all the samples were unimodal. The sizes of docetaxel loaded liposomes prepared by the solvent injection method and its population standard deviation were 161.5 ± 1.1, 126.4 ± 0.8 and 183.8 ± 3.1 nm for batches of the drug loaded, non-coated liposomes (DTX), the PEG-coated liposomes (DTX–mPEG) and the TPGS coated liposomes (DTX–TPGS), respectively. The size of the corresponding coumarin-6 loaded liposomes prepared by the solvent injection method and its population standard deviation were 133.9 ± 1.2, 105.3 ± 1.1 and 191.1 ± 1.5 nm for batches of the coumarin-6 loaded, non-coated liposomes (CM6), the PEG-coated liposomes (CM6–mPEG) and the TPGS coated liposomes (CM6–TPGS), respectively (Table 2). The polydispersity of all the liposomes showed quite narrow size distribution, which is nearer to 0.2. The particle size results indicate that the PEG coating leads to decrease in the liposome size. Instead, increase in the sizes of liposomes was observed with the TPGS coating (Table 2).

The zeta potential of all the liposomes were found to be negatively charged (Carafa et al., 2010). Here, stability of the TPGS coated liposomes (Fig. 1C) was found to be higher than that of the conventional liposomes (Fig. 1A) owing to the surfactant property of TPGS, which was coated on the liposomes. Also, the PEG coating showed more negative charge on the PEG coated liposome surface (Fig. 1B and Table 2). This may be attributed to the negatively charged functional groups of DSPE–mPEG layer formed on the liposome surface. It confirms the presence of DSPE–mPEG on the liposome surface.

3.2. Encapsulation efficiency

The drug encapsulation efficiency of the docetaxel loaded liposomes were 42.74 ± 0.57%, 55.56 ± 0.60% and 64.10 ± 0.57% for the DTX, DTX–mPEG and DTX–TPGS liposomes, respectively. Similarly, dye encapsulation efficiency of the coumarin-6 loaded liposomes were 42.12 ± 0.50%, 52.97 ± 0.69% and 64.34 ± 0.22% for the CM6, CM6–mPEG and CM6–TPGS liposomes, respectively (Table 2). The inclusion of a small amount of cholesterol increased the amount of docetaxel/dye that could be loaded. The reasons may be that it reduced the fluidity and increased the stability of lipid bilayer membranes formed by DPPC (Zhai et al., 2008). The TPGS coated liposomes showed significant increase ($P < 0.05$) in the drug/dye encapsulation efficiency compared with the conventional and the PEG coated liposomes. It is clear that the TPGS coating provides better protection of the drug loss in the liposomal formulation process than the PEG coating. The difference in the encapsulation efficiency between the TPGS coated and DSPE–mPEG-2000 or PEG coated liposomal formulations were related to the different molecular

Table 2
Particle size, polydispersity, zeta potential and encapsulation efficiency of liposomes.

Batches	Particle size (nm) (mean ± S.D[a])	Polydispersity (mean ± S.D[a])	Zeta potential (mV) (mean ± S.D[a])	Encapsulation efficiency (%) (mean ± S.D[a,b])
DTX	161.5 ± 1.1	0.092 ± 0.009	−2.56 ± 0.19	42.74 ± 0.57
DTX–mPEG	126.4 ± 0.8	0.164 ± 0.027	−32.97 ± 0.65	55.56 ± 0.60
DTX–TPGS	183.8 ± 3.1	0.142 ± 0.013	−2.41 ± 0.30	64.10 ± 0.57
CM6	133.9 ± 1.2	0.249 ± 0.013	−3.41 ± 0.20	42.12 ± 0.50
CM6–mPEG	105.3 ± 1.1	0.146 ± 0.010	−33.20 ± 0.44	52.97 ± 0.69
CM6–TPGS	191.1 ± 1.5	0.176 ± 0.009	−3.43 ± 0.22	64.34 ± 0.22

S.D: standard deviation.
DTX: docetaxel loaded conventional liposomes prepared with DPPC and cholesterol.
DTX–mPEG: docetaxel loaded liposomes prepared with DSPE–mPEG-2000.
DTX–TPGS: docetaxel loaded liposomes prepared with TPGS.
CM6: coumarin-6 loaded conventional liposomes prepared with DPPC and cholesterol.
CM6–mPEG: coumarin-6 loaded liposomes prepared with DSPE–mPEG-2000.
CM6–TPGS: coumarin-6 loaded liposomes prepared with TPGS.
[a] $n = 3$.
[b] Encapsulation efficiency (%) = (amount of drug or dye loaded in liposomes/amount of drug or dye added during fabrication) × 100.

weight of PEG chains exposed on the liposome surface. The molecular weight of the hydrophilic part was 1000 for TPGS, but 2000 for DSPE–mPEG-2000. The higher solubilization effect of DSPE–mPEG-2000 (due to its hydrophilic part) in comparison to TPGS leads to less encapsulation efficiency for PEG coated liposomes (Zhai et al., 2008).

3.3. AFM study

The AFM image of individual DTX–TPGS liposome shown in Fig. 2 shows its smooth surface without any noticeable pinholes or cracks. AFM also revealed that the TPGS coated liposome was spherical in shape of size below 200 nm, which agrees quite well with the result measured by PCS.

3.4. Morphological study

Fig. 3 shows field-emission transmission electron microscope of (A) an individual non-coated liposome in 50 nm scale, (B) an individual PEG coated liposome also in 50 nm scale, and (C) an individual TPGS coated liposome in 200 nm scale, which revealed that the docetaxel loaded non-coated liposomes (DTX), the PEG coated liposomes (DTX–mPEG) and the TPGS coated liposomes (DTX–TPGS) were spherical in shape (Fig. 3A–C). The liposome size as observed by FETEM correlated, which also agrees well with that measured by PCS. Additionally, FETEM images also confirmed the presence of TPGS coating on the liposomes surface (Figs. 1C and 3C).

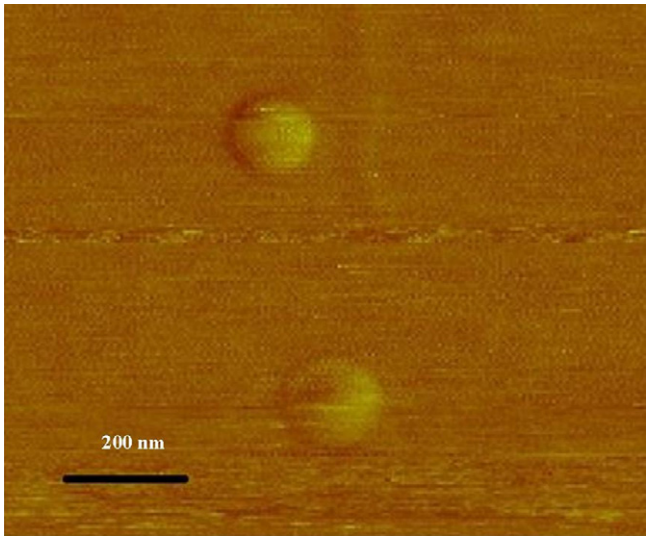

Fig. 2. Atomic force microscopy (AFM) image of the TPGS coated liposomes showing individual liposomes in 200 nm scale.

3.5. In vitro drug release

Fig. 4 shows the accumulated percentage release of docetaxel from the drug loaded non-coated liposomes (DTX), the PEG coated liposomes (DTX–mPEG) and the TPGS coated liposomes (DTX–TPGS) in phosphate buffered saline (pH 7.4) containing 0.1% (w/v) Tween 80. Only the DTX–mPEG showed initial burst release followed by controlled release (Yuan et al., 2010). The DTX and DTX–TPGS liposomes showed controlled release for more than 1 day without any burst release.

After 24 h of dialysis in phosphate buffered saline (pH 7.4), the percentage of docetaxel released from the liposomes was 50%, 77% and 64% for the DTX, DTX–mPEG and DTX–TPGS, respectively (Fig. 4). The effect of surface coatings on the drug release was studied. The PEG surface coating increased the drug release kinetics. However, TPGS coating in the liposomes showed slower drug release kinetics. The $t_{50\%}$, at which 50% of the drug encapsulated in the liposomes has released in the phosphate buffered saline (pH 7.4) was about 24, 2.8 and 17.2 h for the DTX, DTX–mPEG and DTX–TPGS liposomes, respectively (Fig. 4). There was significant ($P < 0.05$) increase in the in vitro drug release from the surface coated liposomes (*i.e.* DTX–mPEG and DTX–TPGS) compared with the DTX liposomes (Fig. 4). The faster rate of drug releases obtained from the surface coated liposomes may be explained by the influence of hydrophilic and solubilization property of PEG or TPGS on docetaxel (Mu et al., 2005; Sheu et al., 2003).

3.6. Cellular uptake

The quantitative cellular uptake of C6 glioma cells after incubation with the coumarin-6 loaded conventional liposomes (DTX), PEG coated liposomes (DTX–mPEG) and TPGS coated liposomes (DTX–TPGS) were shown in Fig. 5. It can be noticed from Fig. 5 that there was no trend of general increase of liposomes uptake by the cells with the incubation time. The absence of time-dependent behavior could be explained by presence of active and maximum endocytosis process within the system on or before 30 min (Gupta et al., 2007). Among the three-liposome formulations studied, it was clearly shown that the cellular uptake of the TPGS coated liposomes (DTX–TPGS) was significantly higher compared with

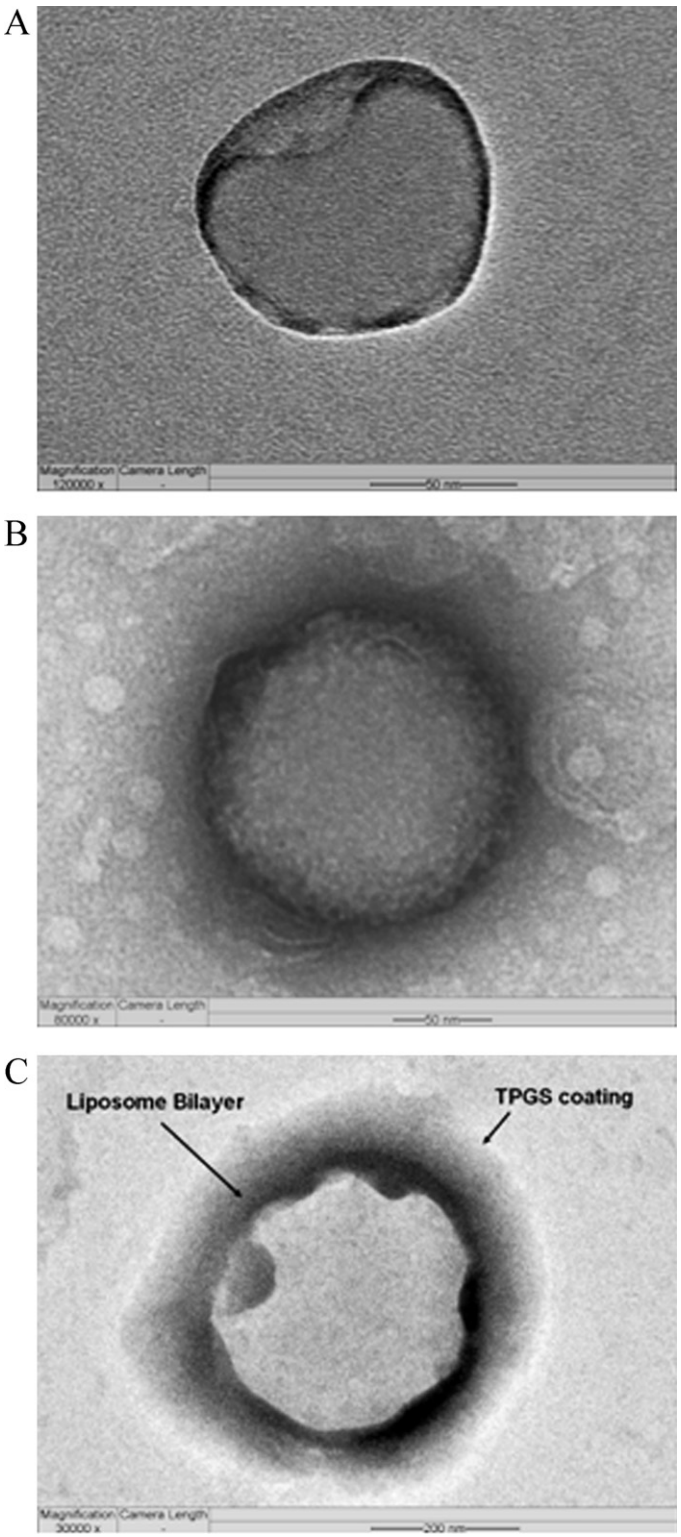

Fig. 3. Field-emission transmission electron microscope (FETEM) image of (A) an individual non-coated liposome in 50 nm scale, (B) an individual PEG coated liposome also in 50 nm scale, and (C) an individual TPGS coated liposome in 200 nm scale.

the conventional liposomes (DTX) and the PEG coated liposomes (DTX–mPEG) ($P < 0.05$). It may be most likely due to the effect of TPGS, which enhanced the absorption of the liposomes.

The confocal images of C6 glioma cells after incubation with the coumarin-6 loaded conventional liposomes (DTX), the PEG coated liposomes (DTX–mPEG) and the TPGS coated liposomes (DTX–TPGS) were shown in Fig. 6. To better compare the intensity of fluorescence among the cells treated with the three types of liposomes, the images were taken under the same imaging parameters such as sensitivity, gain, offset, and laser power constant throughout the cell imaging process. It can be observed that the green fluorescence in the C6 glioma cells, which corresponds to the TPGS coated liposomes (DTX–TPGS) (the right column), was stronger than that of the conventional liposomes (DTX) (the left column) and the PEG coated liposomes (DTX–mPEG) (the middle column), respectively. More regions in the cytoplasm were stained in green, implying enhanced uptake of the TPGS coated liposomes (DTX–TPGS) (row 3) (Fig. 6).

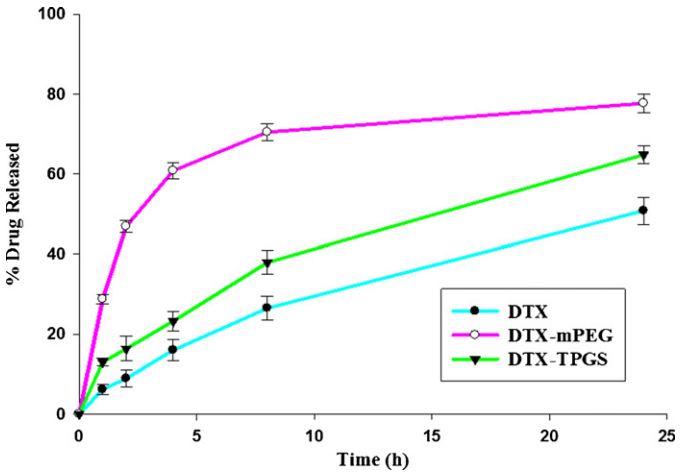

Fig. 4. In vitro drug release from the docetaxel loaded liposomes in phosphate buffered saline (pH 7.4). Bar represent ±SD ($n = 3$).

3.7. Cytotoxicity of liposomes

In vitro cytotoxicity of the docetaxel formulated in the non-coated liposomes (DTX), the PEG-coated liposomes (DTX–mPEG) and the TPGS coated liposomes (DTX–TPGS) were investigated in comparison with Taxotere® at the same equivalent drug concentration on C6 glioma cells after 24 h incubation at 37 °C and the results are shown in Fig. 7. It is worthy to note that the docetaxel loaded liposomes achieved the higher cytotoxicity when compared with commercial Taxotere® in all equivalent drug concentration levels applied. This could be due to the effect of controlled docetaxel release from the liposomes (Fig. 7 and Table 3). Also, the docetaxel loaded TPGS liposomes (DTX–TPGS) resulted in higher cytotoxicity compared with the non-coated and the PEG coated liposomes (Table 3). Also, increase in the drug concentration from 0.025 μg/ml

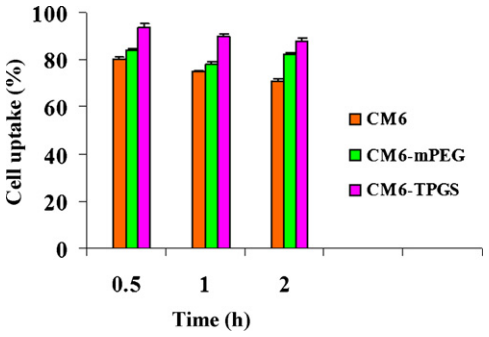

Fig. 5. Cellular uptake efficiency of the coumarin-6 loaded liposomes by C6-glioma cells after 0.5, 1 and 2 h incubation.

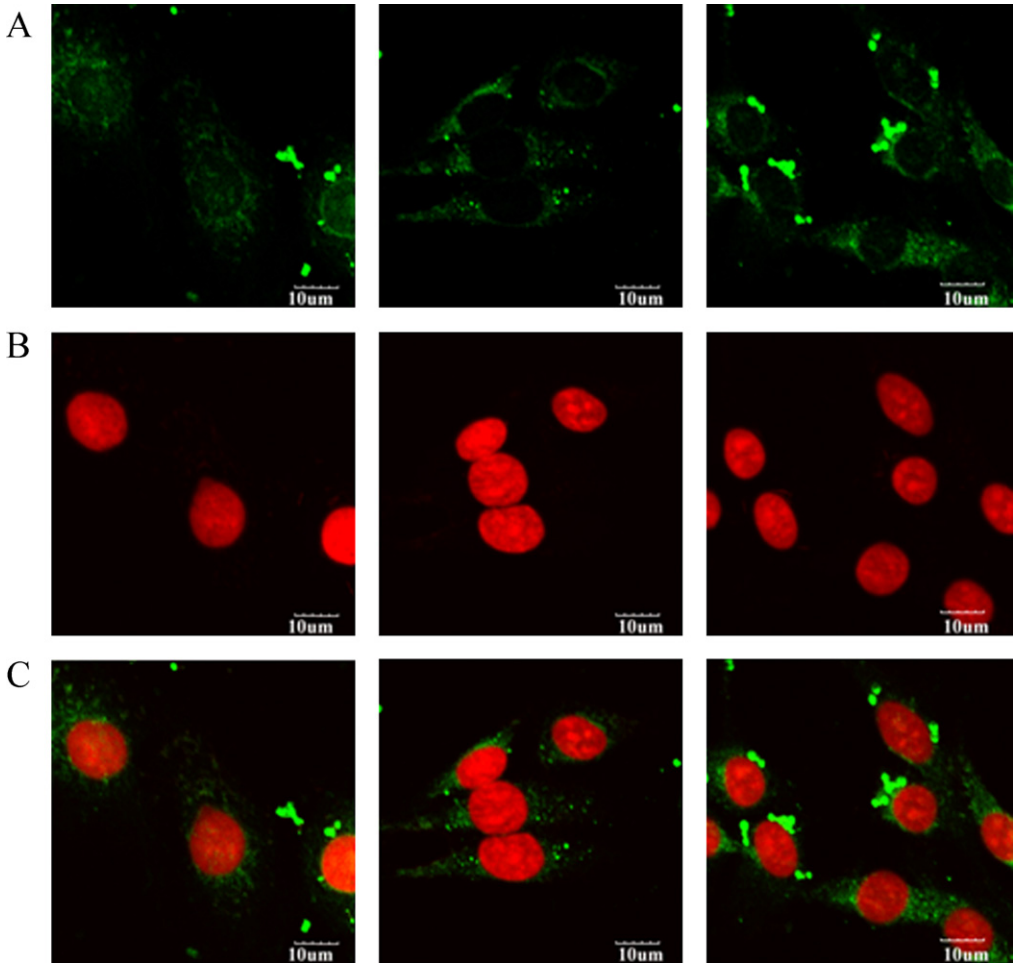

Fig. 6. Confocal laser scanning microscopy (CLSM) of C6 glioma cells after 2 h incubation with the fluorescent coumarin-6 loaded conventional liposomes (left column), the PEG coated liposomes (middle column) and the TPGS coated liposomes (right column). Row (A): fluorescein isothiocyanate (FITC) channels showing the green fluorescence from the liposomes distributed in cytoplasm, (B): PI channels showing the red fluorescence from propidium iodide stained nuclei, and (C): merged channels of FITC and PI. Scale bar = 10 μm.

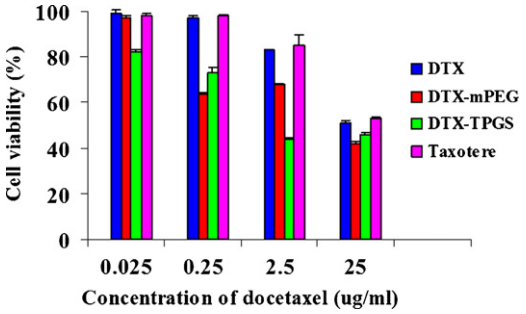

Fig. 7. Cytotoxicity of docetaxel formulated in the liposomes on C6 glioma cells for 24 h culture at 37 °C. Cell viability is studied in comparison with commercial formulation Taxotere®.

to 25 μg/ml resulted in higher cytotoxicity in presence of endocytic mechanism (Wu et al., 2006).

In order to demonstrate the advantages of the docetaxel loaded liposomes, a quantitative index of inhibitory concentration, IC_{50}, which is the drug concentration required to induce the death of 50% cells incubated in a designated period, was determined. For instance, after 24 h incubation, IC_{50} for each of the three types of liposomes, namely the DTX, DTX–mPEG and DTX–TPGS liposomes vs Taxotere® were determined from the cytotoxicity data to be 31.04 ± 1.05, 7.70 ± 0.22, 5.93 ± 0.57 vs 37.04 ± 1.05 μg/ml, respectively, which imply that the liposomal formulations of docetaxel are 16.2%, 79.2% and 84.1% more efficient than Taxotere® after 24 h treatment, respectively (Table 3 and Fig. 7). This indicates that in order to kill the same number of cancer cells, the TPGS

Table 3
IC_{50} values of docetaxel formulated as liposomes or Taxotere® for C6-glioma cells ($n = 6$).

Incubation time (h)	IC_{50} (μg/ml)			
	C6 glioma cells			
	Taxotere®	DTX	DTX–mPEG	DTX–TPGS
24	37.04 ± 1.05	31.04 ± 0.75	7.70 ± 0.22	5.93 ± 0.57

DTX: docetaxel loaded conventional liposomes prepared with DPPC and cholesterol.
DTX–mPEG: docetaxel loaded liposomes prepared with DSPE–mPEG-2000.
DTX–TPGS: docetaxel loaded liposomes prepared with TPGS.

coated liposomes (DTX–TPGS) require much lower drug concentration in comparison with the non-coated and the PEG coated liposomes (Table 3). This enhanced cytotoxicity can be possibly attributed to reversal of the multi-drug resistance (MDR) effect of the cancer cells by TPGS (Woodcock et al., 1992; Collnot et al., 2007). Most importantly, the TPGS coated liposomes could bring high concentration of drug to brain cancer cells and reduce the systemic side effects as efficient and cost-effective drug delivery system for clinical application.

4. Conclusion

We developed the TPGS coated liposomes for better performance in drug delivery than the conventional (non-coated) and the PEG coated liposomes for brain cancer chemotherapy. Our study confirms that the solvent injection method is suitable for preparation of the docetaxel loaded liposomes. The TPGS coated liposomes showed maximum docetaxel encapsulation efficiency up to 64%. The PCS, AFM and FETEM analysis showed the TPGS coated liposomes are well structured in nano size. In vitro drug release profiles revealed the better controlled release property of the TPGS coated liposomes. Compared with the non-coated and the PEG coated liposomes, the TPGS coated liposomes showed higher cellular uptake and cytotoxicity. The docetaxel formulated in the TPGS coated liposomes achieved 84.0%, 80.9% and 23.0% decrease in the IC_{50} value compared with that of Taxotere®, the non-coated liposomes and the PEG coated liposomes, respectively after 24 h incubation with C6 glioma brain cancer cells. Moreover, the TPGS coated liposomes further improved the liposomal stability and thus protected the docetaxel from reticuloendothelial system (RES) than the PEG coated liposomes – the so-called stealth liposomes (Feng, 2008; Muthu and Feng, 2009). Given the higher in vitro cytotoxicity, we may speculate that this new formulation of the drug in our TPGS coated liposomes would have greater in vivo efficacy than the PEGylated liposomes. Moreover, The TPGS coated liposomes also have great potential to be developed for targeted drug delivery by ligand conjugation and as a multi-functional nanocarrier with imaging agents such as iron oxides and quantum dots co-encapsulated (Muthu and Wilson, 2010; Maruyama, 2011). It should be pointed out, however, that since TPGS may be in favor of endocytosis of the drug-loaded liposomes, which may occurs for both of cancer cells and healthy cells, the side effects may increased for those of non-targeting effects, *i.e.* the TPGS-coated liposomes without ligand conjugation.

Conflict of interest

The authors report no conflict of interest.

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