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Nanoparticles of lipid monolayer shell and biodegradable polymer core for controlled release of paclitaxel: Effects of surfactants on particles size, characteristics and *in vitro* performance

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ABSTRACT

This work developed a system of nanoparticles of lipid monolayer shell and biodegradable polymer core for controlled release of anticancer drugs with paclitaxel as a model drug, in which the emphasis was given to the effects of the surfactant type and the optimization of the emulsifier amount used in the single emulsion solvent evaporation/extraction process for the nanoparticle preparation on the particle size, characters and *in vitro* performance. The drug loaded nanoparticles were characterized by laser light scattering (LLS) for size and size distribution, field-emission scanning electron microscopy (FESEM) for surface morphology, X-ray photoelectron spectroscopy (XPS) for surface chemistry, zetasizer for surface charge, and high performance liquid chromatography (HPLC) for drug encapsulation efficiency and *in vitro* drug release kinetics. MCF-7 breast cancer cells were employed to evaluate the cellular uptake and cytotoxicity. It was found that phospholipids of short chains such as 1,2-dilauroylphosphatidylocholine (DLPC) have great advantages over the traditional emulsifier poly(vinyl alcohol) (PVA), which is used most often in the literature, in preparation of nanoparticles of biodegradable polymers such as poly(D,L-lactide-co-glycolide) (PLGA) for desired particle size, character and *in vitro* cellular uptake and cytotoxicity. After incubation with MCF-7 cells at 0.250 mg/ml NP concentration, the coumarin-6 loaded PLGA NPs of DLPC shell showed more effective cellular uptake versus those of PVA shell. The analysis of IC_{50}, i.e. the drug concentration at which 50% of the cells are killed, demonstrated that our DLPC shell PLGA core NP formulation of paclitaxel could be 5.88-, 5.72-, 7.27-fold effective than the commercial formulation Taxol® after 24, 48, 72 h treatment, respectively.

1. Introduction

Nanotechnology has been regarded as one of the most promising approaches to deal with cancer, which is still a leading cause of death all over the world (Ferrari, 2005; Jemal et al., 2008; Farokhzad and Langer, 2009). Nonetheless, there are only a few nanotechnology based drug formulations so far which are approved by FDA for clinical application (Duncan, 2006). It is thus of paramount importance to develop smarter and more powerful drug formulations. Liposomes and polymeric nanoparticle are two of the utmost investigated nanocarriers for anticancer drug delivery. Liposomes, the spherical vesicles of a lipid bilayer or multilayer membrane, have been widely used as a drug delivery vehicle due to high biocompatibility, favorable pharmacokinetic profile and ease of surface modification. In the past few years, several liposomal drug formulations have been approved for clinical use, including Mycet (doxorubicin in liposomes), Doxil (doxorubicin in PEG-liposomes), DaunoXome (daunorubicin in liposomes), DepoCyt (cytarabine in liposomes), etc (Torchilin, 2005). However, liposomes have disadvantages for drug delivery including insufficient drug loading, fast release of hydrophobic drugs and instability (Rai et al., 2008). Polymeric nanoparticles (NPs), featured by their small size, ability to encapsulate drug of poor solubility and permeability, controlled drug release manner, and long circulation half-life, are another dominant platform for drug delivery (Tong and Cheng, 2007; Cho et al., 2008; Zhang et al., 2008b). Up till now, nanoparticle formulations of paclitaxel, one of the best antineoplastic drugs approved by FDA for treatment of a spectrum of cancers, have been intensively investigated (Feng and Chien, 2003; Feng et al., 2004; Kim et al., 2004; Gradishar et al., 2005; Zhang and Feng, 2006; Dong and

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Feng, 2007; Pan and Feng, 2008; Sun et al., 2008). Yet the biocompatibility of NPs formed by most synthetic polymers is not as high as liposomes, especially at the cellular level. It is thus natural to develop novel drug carrier which can combine the advantages and avoid the disadvantages of the liposomes and the nanoparticles of biodegradable polymers.

Recently, several researches have been reported regarding the polymer-lipid hybrid nanoparticles, which are of lipid shell and polymer core produced by various methods, which can be summarized in two categories (De Miguel et al., 2000; Wong et al., 2006a,b, 2007; Thevenot et al., 2007; Li et al., 2008; Zhang et al., 2008a; Chan et al., 2009). The first is to prepare the polymeric core firstly and then merge them with liposomes to form the desired lipid shell–polymer core structure. A typical example is the lipoparticles (De Miguel et al., 2000; Wong et al., 2006a,b, 2007; Thevenot et al., 2007; Li et al., 2008). The formulation process of such structured nanoparticles usually needs two steps, i.e. formation of polymeric NP core and mixing of the core NPs liposomes, resulting in technical complexity and thus lack of control over the final NP physicochemical properties (Zhang et al., 2008a). The second is to produce the core–shell nanoparticles in a single step which combines the nanoprecipiation and self-assembly method (Zhang et al., 2008a; Chan et al., 2009). Such strategies meet the requirement to develop well-defined and predictable lipid–polymer hybrid NPs and facilitate future scale-up.

Poly [D,L-lactide-co-glycolide] (PLGA) is one of the FDA approved biodegradable polymers of satisfactory biocompatibility for fabricating nanoparticles for biomedical applications (Feng and Chien, 2003). It has been found that the surface decoration of PLGA NPs by lipids is a promising approach to improve the drug encapsulation efficiency and mediate cellular uptake of the nanoparticles (Feng et al., 2002, 2004). Phospholipids of a hydrophilic headgroup and two hydrophilic chains of various length and bond saturation are natural amphiphilic molecules which form bilayer as the cell membrane (Lodish et al., 2004). They are also a kind of commonly used wetting and emulsifying agents in food industry (Patino et al., 2007). Among the various lipids, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) is widely used as emulsifiers due to its chain saturation, normal molecule length, proper volume of the hydrophilic head, low phase transition temperature below the room temperature, easy processing, and high stability (Nii and Ishii, 2004; Ishii and Nii, 2005). It was suggested that the use of phospholipids as an additive in the emulsification process may be able to improve the performance of the produced PLGA microspheres in blood flow (Garti, 1999), enhance the pulmonary absorption of peptides and proteins (Zhen et al., 1995) and reduce phagocytic uptake of the microspheres (Evora et al., 1998). In the previous research of our group, it has been concluded that the lipids of short and saturated chains such as DLPC, which has the hydrophilic–lipophilic balance index (HLB) of 13 (calculated from the equation: HLB index = Σ (hydrophilic groups) + Σ (lipophilic groups) + 7), could have high emulsification effects for preparation of polymeric particles of the nanoscale size, smooth surface, and desired control release profile of anticancer drugs of high hydrophobicity such as of paclitaxel (Feng and Huang, 2001).

In this research, we continued our earlier work in 2001 to develop a system of biodegradable nanoparticles of DLPC lipid shell and PLGA polymer core for controlled release of paclitaxel employed as a model hydrophobic drug due to its excellent antineoplastic effects against a wide spectrum of cancer. The emphasis was given to optimization of the DLPC amount added in the micro-emulsification process in favor of the particle size and size distribution, surface charge and drug encapsulation efficiency as well as to investigate the advantages of the DLPC-emulsified PLGA NPs over those emulsified by the traditional chemical emulsifier poly(vinyl alcohol) (PVA). The drug loaded PLGA nanoparticles were characterized by laser light scattering (LLS) for size and size distribution, field-emission scanning electron microscopy (FESEM) for surface morphology, X-ray photoelectron spectroscopy (XPS) for surface chemistry, zetasizer for surface charge, and high performance liquid chromatography (HPLC) for drug encapsulation efficiency and *in vitro* drug release kinetics. MCF-7 breast cancer cells were employed to evaluate the cellular uptake of the coumarin-6 loaded PLGA nanoparticles and the cytotoxicity of the drug formulated in the nanoparticles versus Taxol®.

2. Material and methods

2.1. Materials

Paclitaxel (99.8%) was purchased from Dabur Pharma Ltd. (India). Taxol® was provided by National Cancer Center (Singapore). Poly(D,L-lactide-co-glycolide) (PLGA, 75:25, Mw: 90,000–126,000), 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (synonyms: 1,2-dilauroyl-*sn*-glycero-3-phosphocholine or DLPC, $C_{32}H_{64}NO_8P$), poly(vinyl alcohol) (PVA), sucrose, methanol, ethanol, dichloromethane (DCM), acetonitrile (ACN), dimethyl sulfoxide (DMSO), coumarin-6, phosphate buffered saline (PBS, pH 7.4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, penicillin–streptomycin solution, trypsin-EDTA solution and propidium iodide (PI) were all purchased from Sigma–Aldrich (St. Louise, 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. All solvents used in this study were HPLC grade. MCF-7 breast cancer cells were provided by American Type Culture Collection. The water used was pretreated with the Milli-Q® Plus System (Millipore Corporation, Bedford, USA).

2.2. NPs preparation

Preparation of the drug loaded, DLPC shell and PLGA core NPs is based on a modified solvent extraction/evaporation method (Feng et al., 2007). Briefly, weighed amount of PLGA and paclitaxel were dissolved in DCM as the oil phase. The aqueous phase was prepared by dispersing designated amount of DLPC (%, w/v as the unit to present the quantity of DLPC) in ultrapure water by bath sonication as follows: 60 s sonication was followed by 30 s break, which was repeated four cycles under ice bath. Afterwards, the oil phase was mixed with the aqueous phase under stirring and then the mixture was sonicated by probe ultrasonicator in the same way as above described procedure at 20 W output under ice bath. The produced emulsion was placed on magnetic stirrer to evaporate DCM with moderate speeding overnight. The particle suspension was centrifuged at 12,000 rpm for 15 min to collect the NPs. After washing three times, the particles were resuspended in a fixed volume of water with 3% (w/w) sucrose as cryoprotectant and freeze-dried to obtain fine powder. The blank NPs and the coumarin-8 loaded NPs were prepared in a similar procedure. The PVA-emulsified NPs were produced in a similar manner with 2% (w/v) PVA in water as the aqueous phase and 120 s sonication input.

2.3. Particle size and size distribution

Average particle size and size distribution of the NPs were measured by laser light scattering (LLS, 90Plus Particle Sizer, Brookhaven Instruments Co., USA). The dispersion of NPs was diluted by ultrapure water according to the mass concentration and completely sonicated before measurement.

2.4. Surface charge

The surface charge of the NPs in water was determined by Zeta-Plus zeta potential analyzer (Brookhaven Instruments Co., USA) at room temperature. The suspension of NPs was diluted by ultrapure water. The zeta potential was measured under certain pH value and concentration of the dispersion.

2.5. Drug encapsulation efficiency (EE)

The amount of paclitaxel encapsulated in the NPs was measured by high performance liquid chromatography (HPLC, Agilent LC1100). A reversed phase Inertsil® ODS-3 column (250 mm × 4.6 mm, pore size 5 mm, GL Science Inc., Tokyo, Japan) was used. 3 mg freeze-dried NPs were dissolved in 1 ml DCM. After evaporating DCM, 3 ml mobile phase (50:50, v/v acetonitrile/water solution) was added to dissolve the drugs. The solution was then filtered by 0.45 μm polyvinylidene fluoride (PVDF) syringe filter for HPLC analysis. The column effluent was detected at 230 nm with a UV-VIS detector. The drug encapsulation efficiency (EE) is calculated as (actual amount of drug encapsulated in NPs)/(initial amount of drug used in the fabrication of NPs) × 100%.

2.6. Particle morphology

The shape and surface morphology of the NPs were investigated by field-emission scanning electron microscope (FESEM, JSM-6700F, JEOL, Japan) at an accelerating voltage of 5 kV. For the sample preparation, one drop of the NP dispersion was dribbled on copper tape supported by the stub and the water was evaporated under reduced pressure. The resulted dry particle layers were coated by platinum which was carried out by the Auto Fine Coater (JEOL, Tokyo, Japan) for 30 s at 30 mA current.

2.7. Surface chemistry

The existence of DLPC coating on the surface of paclitaxel-loaded NPs was confirmed by X-ray photoelectron spectroscopy (XPS, AXIS His-165 Ultra, Kratos Analytical, Shimadzu Corporation, Japan). The elements on the NP surface were identified according to the specific binding energy (eV), which was recorded from 0 to 1200 eV with pass energy of 80 eV under the fixed transmission mode. The data were processed by specific XPS software.

2.8. Controlled drug release

The drug loaded NPs were dispersed in PBS (0.1 M, pH 7.4) containing 0.1% (w/v) Tween-80, which can improve the solubility of paclitaxel in PBS. The dispersion was then put in an orbital shaker shaking at 120 rpm with water bath at 37 °C. At designated time intervals, the suspension was centrifuged at 11,000 rpm for 30 min. The pellet was resuspended in fresh medium to continue the drug release. The drug released in the supernatant was extracted by DCM and transferred in the same mobile phase. After the evaporation of DCM, paclitaxel quantity was determined by the same HPLC procedure as mentioned above. The error bars were obtained from triplicate samples.

2.9. Cell culture

MCF-7 breast cancer cells from American Type Culture Collection (ATCC) were employed. DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin was utilized as cell culture medium. Cells were cultivated in medium at 37 °C in humidified environment with 5% CO_2. Before experiment, the cells were precultured until confluence was reached to 75%.

2.10. In vitro cellular uptake

For quantitative study, MCF-7 cells were seeded into 96-well black plates (Costar, IL, USA) at 5×10^3 cells/well (0.1 ml) and after the cells reached 80% confluence, the medium was changed to the suspension of coumarin-6 loaded NPs at a NP concentration of 0.250 mg/ml for 0.5, 1.0, 2.0 and 4.0 h, respectively. After incubation, the NP suspension in the testing wells was removed and the wells were washed with 0.1 ml PBS three times to remove the NPs outside the cells. After that, 50 μl of 0.5% Triton X-100 in 0.2 N NaOH solution was added to lyse the cells. The fluorescence intensity present in each well was then measured by microplate reader (Genios, Tecan) with excitation wavelength at 430 nm and emission wavelength at 485 nm.

For qualitative study, MCF-7 cells were cultivated in the 8-well coverglass chamber (LAB-TEK®, Nagle Nunc, IL) till 70% confluence. The courmarin-6 loaded NPs dispersed in the cell culture medium at concentration of 0.250 mg/ml were added into the wells. Cells were washed three times after incubation for 0.5 and 2 h and then fixed by 70% ethanol for 20 min. The cells were further washed twice by PBS and the nuclei were then counterstained by PI for 45 min. The cell monolayer was finally washed thrice by PBS and observed by confocal laser scanning microscope (CLSM, Olympus Fluoview FV1000).

2.11. In vitro cell cytotoxicity

MCF-7 cells were incubated in 96-well transparent plates (Costar, IL, USA) at 5×10^3 cells/well (0.1 ml) and after 12 h, the old medium was removed and the cells were incubated for 24, 48 and 72 h in the media containing Taxol® or paclitaxel-loaded NPs suspension at an equivalent paclitaxel concentration of 25, 10, 2.5, 0.25 μg/ml. The NPs were sterilized with UV irradiation for 1 day prior to use. At given time intervals, the cultured cells were assayed for cell viability with MTT. The wells were washed twice with PBS and 10 μl of MTT supplemented with 90 μl culture medium was added. After 3 h incubation, the medium was removed and the precipitate was dissolved in DMSO. The absorbance of the wells was measured by the microplate reader (Genios, Tecan) with wavelength at 570 nm and reference wavelength at 620 nm. Cell viability was calculated by the following equation: cell viability = $Int_s/Int_{control} \times 100\%$, where Int_s is the absorbance of the wells containing the cells incubated with the NP suspension and $Int_{control}$ is the absorbance of the wells containing the cells incubated with the culture medium only (positive control).

2.12. Statistical analysis

Data were expressed as the means with 95% confidence intervals. Statistical tests were performed with the Student's *t* test. For all tests, *P* values less than 0.05 were considered to be statistically significant. All statistical tests were two-tailed.

3. Results

3.1. Particle size and size distribution

Table 1 illustrates the size and size distribution of the paclitaxel-loaded NPs of DLPC shell and PLGA core with five different DLPC amount levels from 0.01% to 0.10% (w/v) used in the preparation process, which were obtained from LLS. The general sizes of the NPs of the 0.02–0.10% (w/v) DLPC are in the range of 200–300 nm in diameter with polydispersity of 0.13–0.24. The NPs in such size range may be desired for high cellular uptake (Khin and Feng, 2005). The trend of the size change with lipid quantity was shown in Fig. 1(B), i.e. increase of lipid quantity would result in smaller

Table 1
Characteristics of the paclitaxel-loaded DLPC shell and PLGA core NPs of various DLPC amount used in the nano-emulsification process: particle size, size distribution, initial drug loading and encapsulation efficiency.

DLPC concentration (%, w/v)	Particle size (nm)	Polydispersity	Initial drug loading[a] (%)	Encapsulation efficiency[b] (%)
0.1	239 ± 4.1	0.239 ± 0.037	5	41.8 ± 0.07
0.05	243 ± 4.2	0.138 ± 0.043	5	43.8 ± 0.06
0.04	244 ± 8.6	0.139 ± 0.035	5	56.1 ± 0.07
0.02	265 ± 4.2	0.174 ± 0.018	5	36.9 ± 4.17
0.01	435 ± 28.5	0.324 ± 0.013	5	15.05 ± 0.01

Data represent mean ± SE, $n = 3$.
[a] Drug loading = (amount of drug used in the fabrication)/(amount of PLGA used + amount of drug used in the fabrication).
[b] Encapsulation efficiency = (amount of drug encapsulated in the yielded NPs)/(amount of drug used in the fabrication).

NPs. This is understandable since the role of the emulsifier is to stay in the oil (the drug and the polymer in the organic solvent) – water (the aqueous phase with the lipid) interface to lower the surface tension of the lipid monolayer, which is actually the surface energy intensity of the interface, and thus facilitate the nanoparticle formulation, namely less surface energy would be needed to form a NP of a give surface area. The emulsifier amount used in the micro/nano-emulsification process can be used to quantitatively control the nanoparticle size, which is a dominant factor to determine the key characters of the drug loaded nanoparticles. It has been a common sense in the past years in the controlled release area that the smaller the nanoparticles, the better performance would be expected. Nevertheless, our earlier research showed that too small NPs, for example those of diameter less than 100 nm, would not be certainly in favor of desired NP drug formulation. This is understandable. Firstly, too small NPs would result in low drug encapsulation efficiency since they would have larger surface area to volume ratio and thus causing more drug loss into the aqueous phase in the NP preparation process (Mu and Feng, 2002; Ruan and Feng, 2003). Secondly, internalization of the NPs by the biological cells is a process in which the surface energy of the NPs is sacrificed to provide the bending energy when the curvature of a piece of the lipid bilayer membrane continues to increase and finally to form a shell on the nanoparticle surface in the endocytosis process. It seems that NPs of diameter less than 100 nm would not have enough surface energy to provide the needed bending energy to form the curvature of the designated size (Khin and Feng, 2005). This has also been confirmed by computer simulation of the NP internalization process (Decuzzi and Ferrari, 2007).

3.2. Drug encapsulation efficiency (EE)

The drug encapsulation efficiency (EE) of the NPs is an important of the NP drug formulation since anticancer drugs are usually quite expensive and the EE is also crucial for clinical applications since more NPs would have to be used for a given dose if the EE is low. Table 1 also lists the drug encapsulation efficiency of the NP formulations with various amount of DLPC used in the nano-emulsification process. It can be seen from Table 1 that the DLPC amount is a decisive factor for EE but the relation could be com-

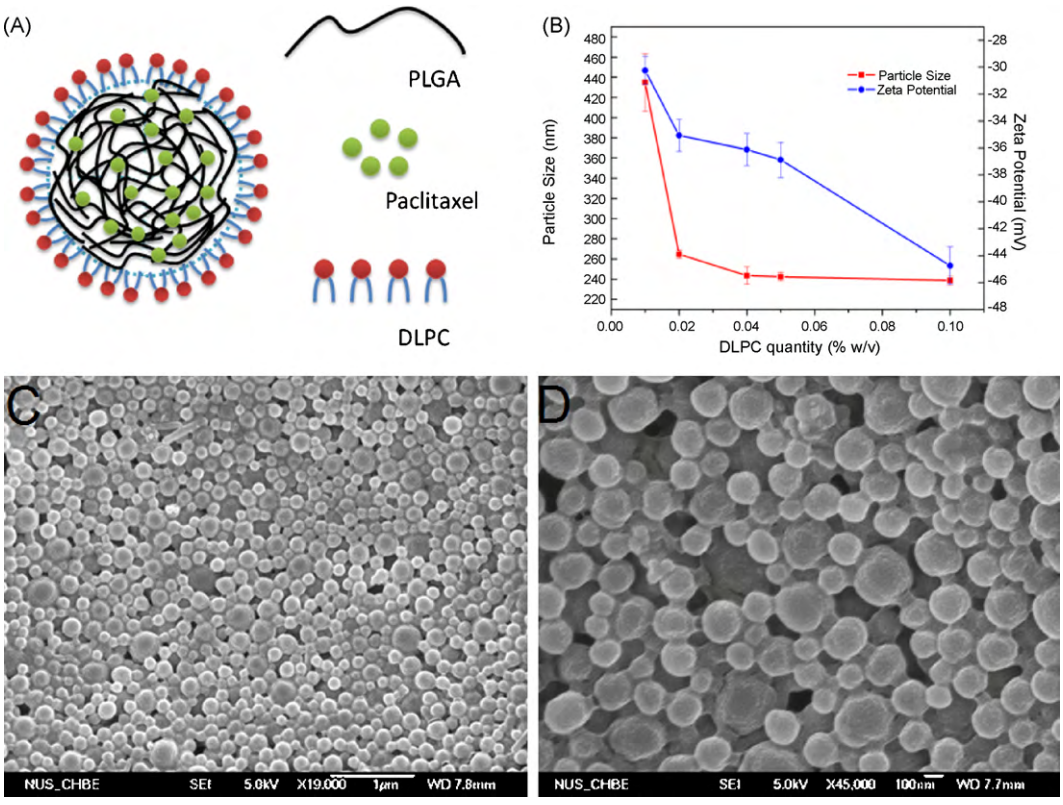

Fig. 1. Structure and characteristics of the paclitaxel-loaded DLPC shell PLGA core NPs. (A) Schematic illustration of the NP structure. (B) Effect of the DLPC amount used in the nano-emulsification process on the NP size and zeta potential. (C) FESEM image of the paclitaxel-loaded 0.04% (w/v) DLPC shell and PLGA core NPs. (D) The zoom-in FESEM image of (C).

Table 2
Comparison of the characteristics of DLPC shell PLGA core NPs and PVA coated PLGA NPs under same initial drug loading (5%): particle size, size distribution, zeta potential and encapsulation efficiency.

Shell compound	Concentration (%, w/v)	Particle size (nm)	Polydispersity	Zeta potential (mV)	Encapsulation efficiency (%)
DLPC	0.04	244 ± 8.6	0.139 ± 0.035	−36.1 ± 1.18	56.1 ± 0.07
PVA	2	294 ± 4.8	0.143 ± 0.023	−26.5 ± 1.54	43.1 ± 4.98

Data represent mean ± SE, $n = 3$.

plicated since more surfactant will result in smaller NPs which the could lower the EE. However, more surfactant on the NP surface would increase the surface pressure (thus lower the surface tension) which would become an obstacle for the drug leak from the nanoparticles to the aqueous phase. We pursued an optimization by using 0.04% (w/v) of DLPC used in the nano-emulsification process, which resulted in 56.14 ± 0.07% EE.

Table 2 shows a comparison of the characteristics of DLPC-emulsified and PVA-emulsified PLGA NPs at the same initial drug loading (5%), from which it can be seen that as emulsifier, DLPC has advantages over the traditional PVA (1) DLPC has much higher emulsification efficiency than PVA. To form the same amount NPs, the DLPC needed is only 1/50 of the PVA; (2) DLPC emulsified would result in much higher drug encapsulation efficiency. Moreover, DLPC is a natural product and the PVA is a chemical. The former can thus cause fewer side effects than the latter.

3.3. Surface charge

Surface charge is an important indication for the stability of a colloidal system in medium as well as for their interaction with the biological cells *in vivo*. The zeta potential values of the DLPC-emulsified PLGA NPs were schematically presented in Fig. 1(B), which demonstrated that the more DLPC used in the preparation process, the larger absolute value of the negative charge would be resulted. The negative charge of the NPs could be due to both of the polymer and the emulsifier. Although DLPC is neutral, the DLPC coated NPs could still exhibit non-zero mobilities in an external electric field that may result in higher negative charge since some anions could bind to the neutral lipids, making the surface more negatively charged (Makino et al., 1991). Typically the zeta potential of the 0.04% (w/v) DLPC coated PLGA NPs was measured as −36.12 ± 1.18 mV. The values prove the high stability of the NPs suspending in aqueous medium.

3.4. Particle morphology

Field-emission scanning electron microscope (FESEM) was employed to image the morphology of the particles (Fig. 1(C) and (D)). It is revealed from the images that the 0.04% (w/v) DLPC coated NPs are generally spherical in shape with narrow size distribution. The rough surface of the NPs shown in the zoom-in image might be due to the lipid layers coated on the PLGA cores. The particle size observed from the FESEM image is in good agreement with that determined above by LLS. There are a few larger spheres attached with each other, which might be attributed to the low solubility of DLPC in water, thereby the spare amount of which still surrounds the solid polymeric particles or simultaneously forms large vesicle-like aggregators.

3.5. Surface chemistry

The surface chemistry of the drug loaded NPs was analyzed by XPS. The elements on the NPs surface were identified according to their specific binding energy, which was recorded from 0 to 1200 eV with pass energy of 80 eV under the fixed transmission mode. The data were processed by specific XPS processing software provided by the instrument manufacturer. For proving the successful surface coating of lipids on PLGA cores, phosphorous was specifically scanned in that phosphorous only exists in DLPC molecules. From Fig. 2, the distinct peak of signals from 2p orbital of phosphorous (P 2p) qualitatively verifies that lipid molecules embrace PLGA cores since only lipid molecules consist of phosphorous. Therefore, it can be confirmed that the DLPC layer has been successfully coated on the PLGA core. Also, from the quantification report generated by the software Kratos PC Processing, the mass concentration percent of phosphorous and nitrogen was 3.16% and 2.14%, respectively. According to the molecular formula of DLPC, $C_{32}H_{64}NO_8P$, the mass percent of phosphorous and nitrogen is 4.98% and 2.25%, respectively. Since the mass percent of phosphorous of the NPs is lower than that of the DLPC molecules and the percent of nitrogen of the NPs is quite close to that of the DLPC molecules, it is postulated that some paclitaxel molecules might exist on the NPs surface.

3.6. In vitro drug release profile

The *in vitro* drug release profile of the paclitaxel-loaded DLPC coated PLGA NPs in 168 h was shown in Fig. 3, from which it can be seen that there is an initial burst of 32.48% in the first 12 h. Such a fast drug release may be due to the drug molecules on and near the surface of the NPs. The initial burst could be helpful to suppress the growth of cancer cells in short time. In the following 72 h, the cumulative release percent reached 75.83%, and the release presents a sustained manner, which provides the possibility to continually fight against cancer cells, resulting in the decreased cancer cell viability as shown in the section of *in vitro* cytotoxicity below. The cumulative release percent almost achieved 100% after 7 days, showing a full release ability of the NP formulation. The generally sustained and controlled release profile of paclitaxel facilitates the application of the NPs for the delivery of anticancer drugs. Rather, from Fig. 3,

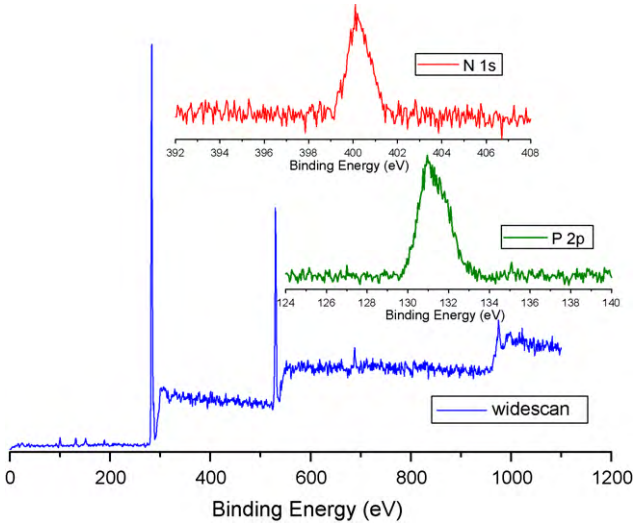

Fig. 2. XPS spectrum of the paclitaxel-loaded DLPC shell PLGA core NPs with 0.04% (w/v) DLPC used in the nano-emulsification process: the widescan spectrum, P 2p spectrum, and N 1s spectrum.

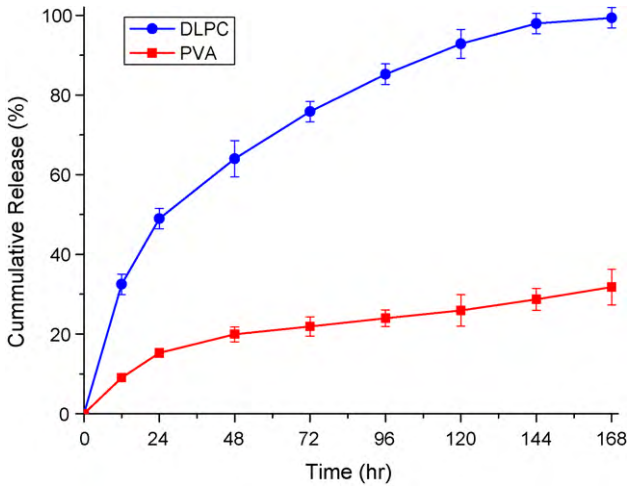

Fig. 3. *In vitro* paclitaxel release profile from the PVA-emulsified PLGA NPs (red curve) and the paclitaxel-loaded DLPC shell PLGA core NPs with 0.04% (w/v) DLPC used in the nano-emulsification process (blue curve). Data represent mean ± SE, $n = 3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

it can also be seen that the release rate of paclitaxel from PVA-emulsified PLGA NPs was much slower. The possible reason can be attributed to the much higher molecular weight of PVA (30,000–75,000) than DLPC resulting in denser coating on the PLGA core and thus slower diffusion of the drugs into water in the same time.

3.7. *In vitro* cellular uptake

The cellular uptake of the coumarin-6 loaded NPs was firstly examined qualitatively to visualize the internalization of the DLPC-emulsified PLGA NPs. The Fig. 4 shows the confocal laser scanning microscope (CLSM) images of the MCF-7 human adenocarcinoma cells after 0.5 h (for the pictures in the upper row) and 2 h (for the pictures in the lower row) incubation with the coumarin-6 loaded DLPC-emulsified PLGA NPs respectively. The two pictures in the left column (A and D) show the green fluorescence from the coumarin-6 loaded NPs which have been internalized in the MCF-7 cells. The fluorescence was stained in FTIC channel. The two pictures in the middle column show the red fluorescence from the cell nuclei stained in the propidium iodide (PI) channel. The two pictures in the right column are the combination of the corresponding left and middle pictures which are obtained from the merge channels of the FITC and PI. It can be seen from Fig. 4 that the red fluorescence representing the nucleus stained by PI is circumvented by green fluorescence representing the coumarin-6 loaded NPs internalized in the cytoplasm. In addition, after incubating 2 h, the fluorescent NPs taken up by the cells (as shown in the pictures of the upper row) are more than those incubated for 0.5 h (as shown in the pictures in the lower row), which was confirmed by the brighter green fluorescence in cytoplasm of the cells with 2 h incubation under the same exciting laser intensity.

A quantitative investigation has also been conducted by measuring the fluorescence intensity of the CLSM images to demonstrate the possible advantages of the DLPC-emulsified PLGA NPs versus the PVA-emulsified PLGA NPs. The same concentration of well dispersed coumarin-6 loaded DLPC-emulsified or PVA-emulsified PLGA NPs (250 μg/ml) was used for all four cases of the MCF-7 cells after 0.5, 1, 2, 4 h incubation at 37 °C, respectively. The results are summarized in Fig. 5. We can see from this figure that the fluorescence intensity (a.u.) from the DLPC-emulsified PLGA NPs taken up by the cells was 9726 ± 424, 12478 ± 437, 21081 ± 1148, and 27340 ± 1729 in comparison with 7712 ± 365, 9958 ± 354, 12529 ± 569, and 16404 ± 1643 for the PVA-emulsified PLGA NPs, respectively (Fig. 5, student's t test, $P < 0.05$). This means that the former is 26.1%, 25.3%, 68.2%, and 66.7% more effective than the latter after 0.5, 1, 2, and 4 h incubation with the MCF-7 cells, respectively.

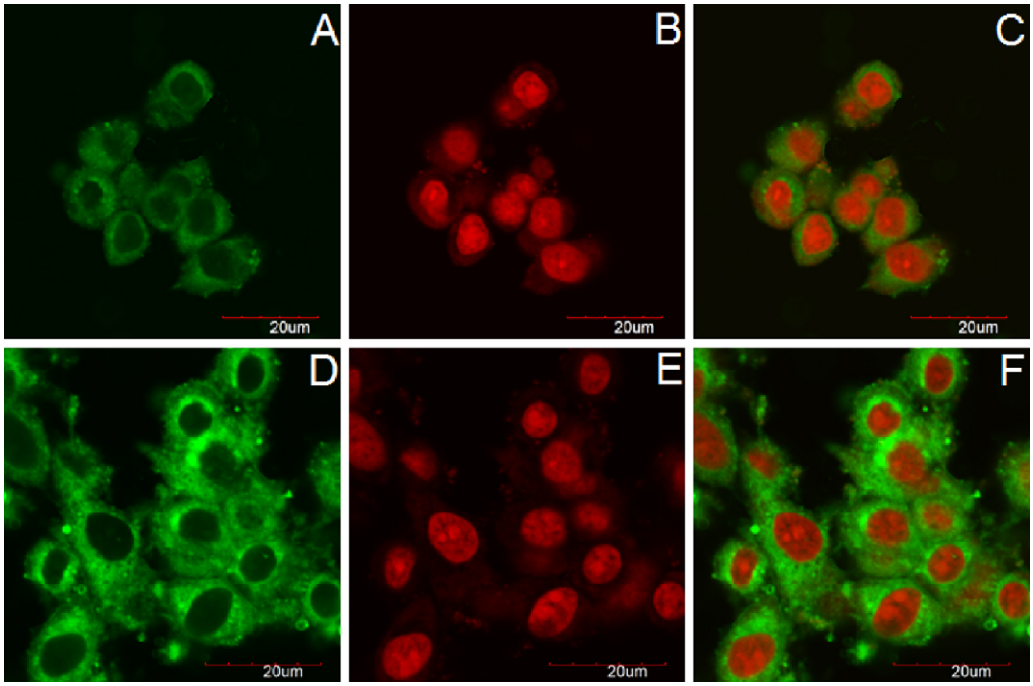

Fig. 4. The confocal laser scanning microscopy (CLSM) images of MCF-7 cancer cells after 0.5 (the upper row) and 2 (lower row) hour incubation with the coumarin-6 loaded DLPC shell and PLGA core NPs at 250 μg/ml NP concentration at 37 °C. Left column (A and D) shows the green fluorescence in the cells stained in FTIC channel. Middle column shows the red fluorescence from the cell nuclei stained in the propidium iodide (PI) channel. Right column are the combination of the corresponding left and middle pictures in the same row which are obtained from the merge channels of the FITC and PI. All the scale bars represent 20 μm.

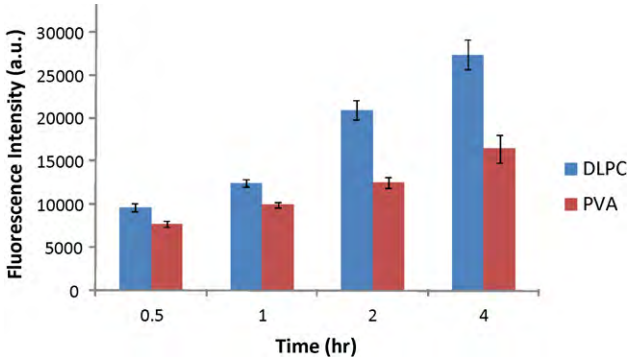

Fig. 5. Cellular uptake efficiency of the coumarin-6 loaded DLPC- or PVA-emulsified PLGA NPs by MCF-7 cells after 0.5 h, 1 h, 2 h, and 4 h incubation at 250 μg/ml NP concentration, respectively. Data represent mean ± SE, $n = 6$.

3.8. In vitro cell cytotoxicity

Fig. 6 is a 3D plot of the *in vitro* cell viability of MCF-7 cancer cells after 24, 48, 72 h incubation with Taxol® or the paclitaxel-loaded DLPC-emulsified PLGA NPs at the various designated equivalent paclitaxel dose of 25, 10, 2.5, 0.25 μg/ml, respectively. T25, T10, T2.5, T0.25 and NP25, NP10, NP2.5, NP0.25 denote the cases of Taxol® and the NP formulation at 25 μg/ml, 10 μg/ml, 2.5 μg/ml, and 0.25 μg/ml dose repectively. From this figure, the effects of the drug dose and the incubation time can be clearly observed. It can be seen from Fig. 6 that the cell viability was 32.9 ± 1.87%, 51.6 ± 3.24%, 59.1 ± 4.30%, and 66.0 ± 7.18% for Taxol® after 24 h incubation at 37 °C respectively. Instead, the viability decreased to 4.82 ± 0.30%, 23.1 ± 0.92%, 23.8 ± 1.19%, 24.1 ± 1.53%, and 1.05 ± 0.09%, 10.22 ± 0.43%, 11.84 ± 1.02%, 12.77 ± 1.64% after 48 and 72 h treatment at the four designated drug concentrations, respectively. It is straightforward to understand that the lower cell viability corresponds to the higher concentration of drugs and longer treating time. As for the NP formulation, the same concentrations 25, 10, 2.5, and 0.25 μg/ml of drug encapsulated in the NPs were applied to measure cell viability. The cell viability under after 24 h treatment at those four concentrations was 31.2 ± 2.45%, 34.5 ± 2.33%, 41.9 ± 3.05%, and 58.5 ± 2.64%, respectively. After 48 and 72 h treatment, the viability decreased to 12.44 ± 0.71%, 13.87 ± 0.96%, 15.18 ± 0.69%, 36.23 ± 1.49, and 7.28 ± 0.29%, 7.44 ± 0.55%, 8.88 ± 0.68%, and 13.51 ± 2.65%, respectively (data represent mean ± SE, $n = 6$. The standard error was not shown on the figure. In all cases, $P < 0.05$ under the two-tailed student's t test).

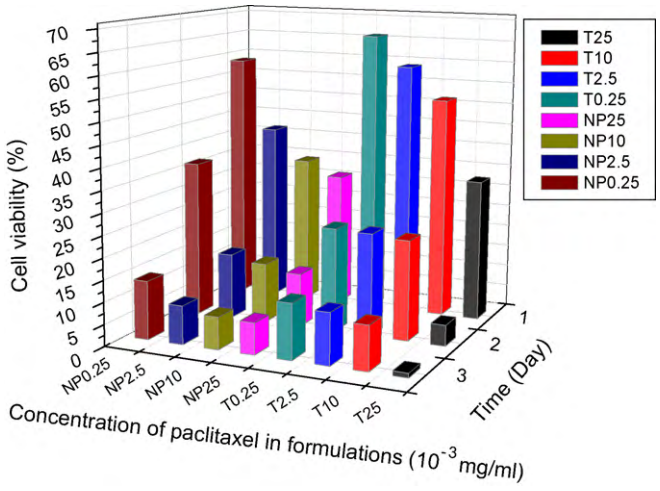

Fig. 6. 3D plot of *in vitro* cell viability of MCF-7 cancer cells after 24 h, 48 h, and 72 h incubation with Taxol® or the paclitaxel-loaded DLPC-emulsified PLGA NPs at the equivalent paclitaxel dose of 25 μg/ml, 10 μg/ml, 2.5 μg/ml, and 0.25 μg/ml, respectively. T25, T10, T2.5, T0.25 and NP25, NP10, NP2.5, NP0.25 denote the cases of Taxol® and the NP formulation at 25 μg/ml, 10 μg/ml, 2.5 μg/ml, and 0.25 μg/ml dose repectively.

A quantitative evaluation of the *in vitro* therapeutic effect of a dosage form is IC_{50}, which is defined as the drug concentration needed to kill 50% of the incubated cells in a designated time period. It can be calculated from the above *in vitro* cell viability data that the IC_{50} for 24, 48, 72 h treatment was 5.06, 0.0163, 0.00897 μg/ml for Taxol® and 0.86, 0.00285, 1.23×10^{-6} μg/ml, for the NP formulation, respectively. This means that our DLPC shell PLGA core NP formulation of paclitaxel could be 5.88-, 5.72-, 7.27-fold effective than the commercial formulation Taxol® after 24, 48, 72 h treatment, respectively.

4. Discussion

The schematic structure of the paclitaxel-loaded DLPC shell and PLGA core NPs developed in this work is represented in Fig. 1(A). The NPs were produced by the oil-in-water (O/W) single emulsion solvent extraction/evaporation method with DLPC as emulsifier. After emulsification of the oil phase in the aqueous phase by applying ultrasonication, the amphiphilic lipids were adsorbed onto the surface of the oil droplets containing PLGA and paclitaxel by hydrophobic interaction. Followed by the evaporation of DCM under continuous magnetic stirring, the drug loaded NPs were then collected. DLPC was introduced as the emulsifier to facilitate stable formation of the solid PLGA cores. The hydrophilic–lipophilic balance index (HLB) of DLPC is calculated to be around 13. It is hence an amphiphilic molecule that is appropriate to stabilize the oil-in-water emulsions to form solid nanoparticles with high drug encapsulation efficiency.

Among various physicochemical and pharmaceutical properties of the drug loaded nanoparticles of biodegradable polymers, the particle size and surface property may be two of the most important characters that would determine their *in vitro* and *in vivo* performance, which may include the drug encapsulation efficiency, the cellular uptake efficiency, the cytotoxicity, the pharmacokinetics and biodistribution, and thus therapeutic effects of the formulated drug. It is thus crucial to find a way to control the particle size and surface coating in the nano-emulsification process. It is obvious that the amount of DLPC used as emulsifier has significant impact on the particle sizes. Too little amount of DLPC would not be enough to cover the entire surface of the nanoparticles to stabilize the oil droplets in the O/W emulsion, thus leading to nanoparticles of large size. Too much amount of DLPC, however, would facilitate formation of smaller nanoparticles, which would result in lower drug encapsulation efficiency and lower cellular uptake efficiency (Zauner et al., 2001; Mu and Feng, 2002; Ruan and Feng, 2003; Khin and Feng, 2005; Chithrani et al., 2006; Decuzzi and Ferrari, 2007). Furthermore, the spare DLPC molecules would cause particle adhesion in the aqueous phase. In this research we have optimized the amount of DLPC used as emulsifier, which seems to be 0.04%. We demonstrated that DLPC has advantages versus the traditional emulsifier PVA for the PLGA NP formulation of anticancer drugs with paclitaxel as a model drug and the drug formulated in such DLPC-emulsified PLGA NPs showed ideal particle size and drug encapsulation efficiency and excellent performance in *in vitro* cellular uptake and cytotoxicity. It should be pointed out, however, that these advantages should be further confirmed by *in vivo* evaluation such as the pharmacokinetics, the half-life in the blood system, the biodistribution of the formulated drug and the xenograft tumor model. We have reported the *in vivo* evaluation of the paclitaxel-loaded, TPGS-emulsified PLGA NPs as well as the paclitaxel-loaded

NPs of the PLA-TPGS copolymers (Khin and Feng, 2006; Feng et al., 2007; Zhang et al., 2008c). The excellent *in vivo* performance of the DLPC shell and PLGA core NPs could thus be expected.

5. Conclusions

We have successfully developed a system of nanoparticles of DLPC shell and PLGA core for sustained and controlled release of anticancer drugs with paclitaxel as a model drug. We continued our earlier work of using phospholipids as emulsifier in the nanoparticle formulation with the focus that the amount of DLPC used in the nano-emulsification process would play a key role to determine particle size and surface coating, which are the two most decisive for the physicochemical properties and *in vitro*/*in vivo* performance of the drug loaded NPs, which has been optimized to be 0.04% (w/v). We concluded that DLPC has great advantages versus traditional PVA with higher emulsification efficiency, higher drug encapsulation efficiency. We demonstrated that after incubation with MCF-7 cells at 0.250 mg/ml NP concentration, the coumarin-6 loaded PLGA NPs of DLPC shell showed higher cellular uptake efficiency versus those of PVA shell. The analysis of IC_{50}, i.e. the drug concentration at which 50% of the cells are killed, demonstrated that our DLPC shell PLGA core NP formulation of paclitaxel could be 5.88-, 5.72-, 7.27-fold effective than the commercial formulation Taxol® after 24, 48, 72 h treatment, respectively.

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