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# Cellular uptake of solid lipid nanoparticles and cytotoxicity of encapsulated paclitaxel in A549 cancer cells

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**Abstract**

The aim of this study was to investigate the cellular uptake of solid lipid nanoparticles (SLN) and cytotoxicity of its paclitaxel delivery system. The conjugate of octadecylamine–fluorescein isothiocyanate (ODA–FITC) was synthesized, and used as a marker to prepare fluorescent SLN. The cellular uptakes of fluorescent SLN with different lipid material were evaluated by fluorescence microscopy and the measurement of fluorescence intensity. The order of cellular uptake ability was glycerol tristearate SLN > monostearin SLN > stearic acid SLN > Compritol 888 ATO SLN (ATO888 SLN). The cellular cytotoxicities of paclitaxel were highly enhanced by the encapsulation of lipid matrix. Due to the lower drug entrapment efficiency of glycerol tristearate SLN, monostearin SLN was considered as the best lipid material to improve the cytotoxicity of drug. The polyethylene glycol monostearate (PEG–SA) and the synthesized conjugate of folic acid–stearic acid (FA–SA) were further introduced into monostearin SLN, respectively. The PEG and folate modified SLN could enhance the cellular uptake of SLN and the cellular cytotoxicity of drug by the membrane disturb ability of PEG chains on the SLN surface and the improved endocytosis mediated by folate receptor.


*Keywords:* Solid lipid nanoparticles; Cellular uptake; Cytotoxicity; Fluorescein isothiocyanate; Polyethylene glycol monostearate; Folic acid

## 1. Introduction

Solid lipid nanoparticles (SLN) made from biodegradable solid lipids exist in the submicron size range have attracted increasing attention in recent years. The advantages of SLN are as follows: possibility of controlled drug release and drug targeting, protection of incorporated compound against chemical degradation, no biotoxicity of the carrier, and no problems with respect to large scale production (Marengo et al., 2000; Mehnert and Mäder, 2001; Müller et al., 2000).

Paclitaxel is an effective anti-cancer drug against a wide spectrum of solid tumors, which promotes polymerization of tubulin dimers to form microtubules and stabilizes microtubules by preventing depolymerization (Mo and Lim, 2005). Though paclitaxel has shown high potential as an anti-cancer drug, its practical use is limited due to its poor aqueous solubility. To overcome the problems, nanoparticles (NPs) delivery systems were selectively and efficiently transport paclitaxel to the target organ/cells, and increase the bioavailability (Serpe et al., 2004; Dong and Feng, 2005).

SLN formulations encapsulating paclitaxel have been studied by several groups (Müller et al., 2000; Serpe et al., 2004). However, most SLN studies are either focusing on formulation preparation and characterization or in vivo biodistribution of SLN. Currently, relatively few studies were devoted to the efficacy aspect of SLN formulations at cellular level. Only Stevens et al. and Wong et al. have provided a series of in-depth studies (Stevens et al., 2004; Wong et al., 2006a,b). However, their works were related with paclitaxel-prodrug or doxorubicin. In our knowledge, few detail works were conducted to investigate the effect of SLN composition on the cellular uptake and cytotoxicity of paclitaxel loaded SLN.

Fluorescence techniques is a widely used method for investigating cellular uptake and in vivo distribution of NPs. It can be visualized by either confocal or fluorescence microscopy or quantified by analyzing the extracted fluorescent dye (Zauner et al., 2001). As showed in our group's previous research (Yuan et al., 2007), the ODA–FITC labeled lipid nanoparticles indicated above 97% ODA–FITC incorporation efficiency, and the leakage of ODA–FITC from lipid nanoparticles was lower

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than 7% in vivo transport. Due to this reason, the ODA–FITC labeled SLN was used to investigate the in vivo transport of SLN, but not used for the cellular uptake evaluation of SLN.

In this study, the chemical conjugate of ODA–FITC was synthesized to incorporate into SLN for evaluating cellular uptake of SLN composed of different lipid materials. Paclitaxel was then chosen as the model drug, and A549 cancer cells were chosen as the model cells, to investigate the cellular cytotoxicities of drug loaded SLN. The physicochemical properties of nanoparticles, such as particle size, zeta potential, drug entrapment efficiency, drug loading, and in vitro drug release profiles were also researched. Furthermore, the polyethylene glycol monostearate (PEG–SA) and folic acid–stearic acid (FA–SA) were incorporated into SLN to reach the higher cellular uptake and cytotoxicity by the enhanced endocytosis.

## 2. Materials and methods

### 2.1. Materials

Monostearin, stearic acid (SA) and glycerol tristearate (GT) was supplied by Shanghai Chemical Reagent Co. Ltd., China. Compritol 888 ATO (ATO888) was purchased from Gattefossé, France. Polyethylene glycol monostearate (PEG–SA, the polymerization degree of ethylene glycol is 40) was purchased from Tokyo Kasei Kogyo Co. Ltd., Japan. The surfactant, poloxamer 188, was provided by Shenyang Pharmaceutical University Jiqi Co. Ltd., China. Octadecylamine (ODA) was purchased from Fluka, USA. Fluorescein isothiocyanate (FITC) and folic acid (FA) was purchased from Acros Organic, USA. Paclitaxel was purchased from Zhanwang Biochemical Co. Ltd., China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Trypsin and RPMI Medium 1640 were purchased from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co. Ltd., Zhejiang, China. All other solvents were analytical or chromatographic grade.

### 2.2. Synthesis of ODA–FITC and FA–SA

The conjugate of ODA–FITC was synthesized by the reaction between amino group of ODA and isothiocyanate group of FITC as the previous report (Yuan et al., 2007). To obtain the conjugate, 60 ml *N*,*N*-dimethylformamide (DMF) dissolved 7 mg ODA completely by sonicate treatment (Sonic Purger CQ250, Academy of Shanghai Shipping Electric Instrument). Meanwhile, 5 ml DMF dissolved 20 mg FITC. The two kinds of DMF solution were mixed and then in 50 °C water bath, shaked (HZ-8812S, Hualida Co. Ltd.) for 48 h. The gained reaction mixture was cooled to room temperature, and then the ODA–FITC to be precipitated by adding 20 ml of distilled water. Collecting the precipitate by filtration with 0.45 μm millipore filter, using 20 ml distilled water to wash twice. The obtained conjugate (ODA–FITC) was lyophilized and stored in dark.

Referred to the literature (Benns et al., 2002), the conjugate of FA–SA was prepared. Firstly, 5 ml DMF dissolved 20 mg stearic acid (SA), and then at room temperature, 20 mg EDC was added into the stearic acid DMF solution and reacted 1 h. Secondly, 30 mg folic acid and 0.5 ml pyridine was added to the stearic acid DMF solution, and further reaction overnight. To precipitate the FA–SA, 20 ml distilled water was added into reaction mixture. To remove unreacted folic acid, EDC and water soluble side product, the dispersion was dialysised against distilled water for 3 days by using cellulose membrane (8000 molecular weight cut-off). Finally, after collecting by filtration with 0.45 μm millipore filter, the precipitate was lyophilized.

$^1$H NMR spectrum was used to analysis of synthesized ODA–FITC and FA–SA. The samples were measured at 298 K with about 5 wt.% $CDCl_3$ or $(CD_3)_2SO$ solution using a NMR Spectrometer (AC-80, Bruker Biospin, Germany).

### 2.3. Preparation of SLN

The blank SLN and drug loaded SLN were prepared by solvent diffusion method in an aqueous system referred to our previous study (Hu et al., 2005). Briefly, 6 ml ethanol completely dissolved 60 mg lipid materials with or without 3 mg paclitaxel in 70 °C water bath. Then the obtained organic solution was quickly dispersed into 60 ml distilled water under mechanical agitate (DC-40, Hangzhou Electrical Engineering Instruments, China) with 400 rpm in 70 °C water bath for 5 min. The resultant pre-emulsion (melted lipid droplet) was cooled to room temperature to obtain the SLN dispersion.

To prepare the fluorescent SLN, PEG modified SLN and FA modified SLN, 9 mg ODA–FITC, 6 mg PEG–SA and 6 mg FA–SA was used instead of the same amount of lipid materials, respectively.

The pH value of the above obtained SLN dispersion was adjusted to 1.20 by addition of 0.1 M hydrochloric acid to form the NPs aggregation. The aggregate of NPs was received by centrifugation (20,000 rpm for 15 min, 3K30, Sigma, Germany). The collected SLN precipitate was then re-suspended in 3 ml distilled water containing 0.1% poloxamer 188 by probe-type ultrasonic treatment with 20 times (200 W, active every 2 s for a 3 s duration) (JY92-α, Scientz Biotechnology Co. Ltd., China), and used for further in vitro cellular uptake and cytotoxicity tests.

### 2.4. Characterization of SLN

The blank or drug loaded SLN in dispersion were diluted 20 times with distilled water, of which the volume average diameter and zeta potential were determined with Zetasizer (3000HS, Malvern Instruments, UK).

### 2.5. Drug entrapment efficiency determination

After the SLN was received by the centrifugation, the drug content in the supernatant was determined in triplicate by HPLC (Agilent 1100 series, USA). The column was Diamohsil$^{TM}$ C18 (250 mm × 4.6 mm) with 5 μm particles. A mixture of acetonitrile and water (50:50 v/v) was used as the mobile phase, and was delivered at a flow rate of 1.0 ml/min. The column effluent

was detected at 227 nm with a variable wavelength detector (G1314A, JP11615541, UV detector, USA). The column temperature was maintained at 35 °C. Injected volume of the sample was 20 μl (Hu et al., 2006). The calibration curve for the quantification of paclitaxel was linear over the range of standard concentration of paclitaxel at 0.5–120 μg ml$^{-1}$ with a correlation coefficient of $R^2 = 0.9994$. The limit of detection was 0.01 μg ml$^{-1}$.

The separated drug loaded SLN were re-dispersed using 100 ml of PBS (pH 7.4) with 2 M sodium salicylate and surged by vortexing (XW-80A, Instruments Factory of Shanghai Medical University, China) for 3 min to dissolve the adsorbed drugs on the surface of SLN. Then the dispersions were centrifuged at 20,000 rpm for 15 min (3K30, Sigma, Germany). The drug content in the obtained supernatant was measured by HPLC as described above. The drug entrapment efficiency (EE) and drug loading (DL) of SLN were calculated from Eqs. (1) and (2)

$$\mathrm{EE} = \frac{W_\mathrm{a} - W_{\mathrm{s}_1} - W_{\mathrm{s}_2}}{W_\mathrm{a}} \times 100\% \quad (1)$$

$$\mathrm{DL} = \frac{W_\mathrm{a} - W_{\mathrm{s}_1} - W_{\mathrm{s}_2}}{W_\mathrm{a} - W_{\mathrm{s}_1} - W_{\mathrm{s}_2} + W_\mathrm{L}} \times 100\% \quad (2)$$

where $W_\mathrm{a}$ is the weight of drug added in system, $W_{\mathrm{s}_1}$ the analyzed weight of drug in supernatant after the first centrifugation, $W_{\mathrm{s}_2}$ the analyzed weight of drug in supernatant after the second centrifugation, and $W_\mathrm{L}$ is the weight of lipid added in system.

### 2.6. In vitro release study

The precipitate of drug loaded nanoparticles were dispersed in 100 ml of PBS (pH 7.4) containing 2 M sodium salicylate and surged by vortexing (XW-80A, Instruments Factory of Shanghai Medical University) for 3 min, and then shaken horizontally (SHELLAB1227-2E, SHELLAB, USA) at 37 °C and 60 strokes/min. One millilitre of the dispersion was withdrawn from the system at definite time interval and filtrated with 0.22 μm filter. The filtrate was determined by HPLC method as described above.

### 2.7. Cellular uptake of fluorescent blank SLN

In a 24-well plate, A549 cells were seeded at a density of 10,000 cells per well in 1 ml of growth medium and incubated for 24 h to attach. Cells were then incubated with ODA–FITC loaded SLN suspension (the concentration were 50, 100 and 200 μg ml$^{-1}$, respectively) in growth medium for different time. Cells were washed twice with PBS (pH 7.4) and directly observed under a fluorescence microscope (OLYMPUS America, Melville, NY).

To assay cellular uptake quantitatively, after washing the cells with PBS, 40 μl trypsin PBS solution (2.5 mg/ml) was added. After incubated for 5 min, the cells were harvested by adding 1 ml PBS, and then treated by probe-type ultrasonication for 5 times (4 °C, 200 W, active every 5 s for a 60 s duration) to obtain the cell lysate. Finally, the cell lysate was centrifuged at 10,000 rpm for 10 min, and the supernatant was supplied to fluorescence assay by using fluorometer (F-4000, HITACHI Co., Japan) (excitation: 490 nm; emission: 528 nm). The cellular uptake percentage of fluorescent SLN was calculated from Eq. (3)

$$\text{cellular uptake percentage of fluorescent SLN}\,(\%) = \frac{I}{I_0} \times 100\% \quad (3)$$

where $I$ is the fluorescence intensity in different time and $I_0$ is the initial fluorescence intensity of the fluorescent nanoparticles.

### 2.8. Cytotoxicity

In a 96-well plate, A549 cells were seeded at a density of 10,000 cells per well in 0.2 ml of RPMI1640 with 10% FBS and antibiotics, and then cultured at 37 °C for 24 h. After culture, the growth medium was removed and growth medium containing paclitaxel solution (a 50:50 mixture of Cremophor EL and ethanol) with different concentration, blank SLN with different concentration and paclitaxel loaded SLN with different drug concentration was added, respectively. The cells were further incubated for 48 h or 72 h. Then 100 μl of fresh growth medium containing 50 mg MTT was added to each well and cells were incubated for 4 h. After removing the unreduced MTT and medium, each well was washed with 100 μl of PBS and 180 μl of DMSO were then added to each well to dissolve the MTT formazan crystals. Finally, the plates were shaken for 20 min and the absorbance of formazan product was measured at 570 nm in a microplate reader (BioRad, Model 680, USA). Survival percentage was calculated as compared to mock-treated cells (100% survival). All the experiments were performed in triplicate.

### 2.9. Cellular uptake of paclitaxel

A549 cells were seeded in a 24-well plate at a seeding density of 10,000 cells per well in 1 ml of growth medium and allowed to attach for 24 h. Cells were then incubated with paclitaxel loaded monostearin SLN, paclitaxel loaded monostearin SLN with 10 wt.% PEG–SA (PEGylated SLN), paclitaxel loaded monostearin SLN with 10 wt.% FA–SA (folated SLN) (drug concentration: 2 μg ml$^{-1}$) in growth medium for 1, 2, 4, 12, 24, and 48 h. After the cells were washed with PBS, 100 μl trypsin PBS solution (2.5 μg ml$^{-1}$) was added. The cells were further incubated for 5 min. The cells were then harvested by adding 400 μl methanol. The cell lysate was centrifuged at 10,000 rpm for 10 min. The drug content in the supernatant after centrifugation was measured by HPLC method. The protein content in the cell lysate was measured using the Micro BCA protein assay kit. The uptake percentages of drug were calculated from Eq. (4)

$$\text{drug uptake percentage}\,(\%) = \frac{C/M}{C_0/M_0} \times 100\% \quad (4)$$

where $C$ is the intracellular concentration of paclitaxel in different time, $M$ the unit weight (milligram) of cellular protein in different time, $C_0$ the initial concentration of paclitaxel, and $M_0$ is the initial unit weight (milligram) of cellular protein.

### 2.10. Statistical analysis

Data were expressed as means of three separate experiments, and were compared by analysis of variance (ANOVA). A *P*-value < 0.05 was considered statistically significant in all cases.

## 3. Results and discussion

### 3.1. Preparation of SLN

The fluorescent blank SLN and paclitaxel loaded SLN were prepared by solvent diffusion method in an aqueous system. The properties such as zeta potential, volume average diameters and its polydispersity indexes of resulted fluorescent blank and drug loaded SLN composed of different lipid materials are listed in Table 1. Due to the SLN used in the in vitro cellular uptake and cytotoxicity tests were the SLN redispersions in 0.1% poloxamer 188 solutions. The results were the data of SLN redispersions.

From Table 1, it is obvious that the volume average diameter of drug loaded SLN were higher than that of fluorescent SLN with the same lipid matrix. The bigger size of drug loaded SLN caused from two reasons. The one is the incorporation of drug into SLN increased the amounts of solid phase. The other is the incorporated paclitaxel and ODA–FITC had different properties. The paclitaxel is hydrophobic, and the FITC fragment of ODA–FITC is hydrophilic. The incorporation of ODA–FITC into SLN could enhance the hydrophilicity of SLN surface, and formed steric hindrance to stable SLN. As a result, the sizes of fluorescent SLN were smaller than that of drug loaded SLN. Among glycerol tristearate, monostearin, stearic acid and ATO888 SLN, the glycerol tristearate SLN had smallest particle size, and no clear difference was found in other SLN. Comparing FA–SA and PEG–SA modified monostearin SLN with monostearin SLN, the FA–SA and PEG–SA modified monostearin SLN had smaller particle. There may also be caused by the enhanced hydrophilicity of SLN surface due to the incorporation of FA–SA and PEG–SA.

Zeta potential is a key factor to evaluate the stability of colloidal dispersion. In general, particles could be dispersed stably when absolute value of zeta potential was above 30 mV due to the electric repulsion between particles (Müller et al., 2001). As shown in Table 1, all of the absolute value of zeta potential for fluorescent SLN and drug loaded SLN were higher than 30 mV, and no obvious difference was found between fluorescent SLN and drug loaded SLN. This demonstrated that the SLN redispersions obtained by present method were physically stable systems.

Table 2
Drug entrapment efficiency (EE) and drug loading (DL) of paclitaxel loaded SLN ($n = 3$)

| Material | EE (%) | DL (%) |
|---|---|---|
| Monostearin | 61.83 ± 1.70 | 2.92 ± 0.15 |
| Stearic acid | 76.29 ± 0.81 | 3.85 ± 0.04 |
| Glycerol tristearate | 36.20 ± 2.52 | 2.01 ± 0.14 |
| ATO888 | 77.41 ± 1.05 | 3.96 ± 0.05 |
| Monostearin with 10 wt.% PEG–SA | 60.83 ± 2.57 | 2.90 ± 0.16 |
| Monostearin with 10 wt.% FA–SA | 59.34 ± 1.34 | 2.69 ± 0.06 |

### 3.2. Drug entrapment efficiency and loading capacity

The data of drug entrapment efficiency (EE) and drug loading (DL) for drug loaded SLN prepared by solvent diffusion method are given in Table 2. It is seen from Table 2 that excepting glycerol tristearate SLN, the EE of other kinds of SLN was mostly about 60–70%, while the DL was about 3–4%. Some researchers have reported that the particle size related with the drug loading, the higher drug loading had bigger particle size (Maschke et al., 2007). In present research, glycerol tristearate SLN with smallest particle size indicated the lowest EE and DL. In addition, the PEG–SA and FA–SA modified monostearin SLN were no obvious change in EE and DL comparing with monostearin SLN.

### 3.3. In vitro release study

The drug release profiles from the SLN prepared by the solvent diffusion method at 70 °C were shown in Fig. 1. Stearic acid SLN showed complete release in 12 h, however, about 40% burst drug release was found in the initial 1 h. Glycerol tristearate SLN released nearly 75% of drug in 48 h with a burst drug release nearly 30% of drug within the initial 1 h. ATO888 SLN only released 32.39% of drug even after 48 h. Monostearin SLN showed no burst drug release within the initial 2 h, and released nearly 50% of drug in 48 h. When adding 10 wt.% PEG–SA, monostearin SLN showed a relative burst drug release at the initial 2 h, while adding 10 wt.% FA–SA, the release character had little change.

Table 1
Properties of resulted fluorescent blank SLN and paclitaxel loaded SLN ($n = 3$)

| Material | Fluorescent SLN | | | Paclitaxel loaded SLN | | |
|---|---|---|---|---|---|---|
| | $d_v$ (nm) | PI (–) | $\zeta$ (mV) | $d_v$ (nm) | PI (–) | $\zeta$ (mV) |
| Monostearin | 272.7 ± 43.6 | 0.101 ± 0.015 | 36.4 ± 2.1 | 437.3 ± 68.2 | 0.436 ± 0.068 | 41.2 ± 5.2 |
| Stearic acid | 233.8 ± 52.4 | 0.243 ± .0.028 | 30.6 ± 6.8 | 391.9 ± 59.7 | 0.239 ± 0.031 | 39.5 ± 5.4 |
| Glycerol tristearate | 159.8 ± 37.2 | 0.235 ± 0.039 | 32.6 ± 3.2 | 185.0 ± 40.8 | 0.187 ± 0.019 | 31.7 ± 3.8 |
| ATO888 | 248.3 ± 68.0 | 0.171 ± 0.032 | 33.6 ± 3.4 | 401.8 ± 63.4 | 0.352 ± 0.035 | 38.7 ± 4.7 |
| Monostearin with 10 wt.% PEG–SA | 255.9 ± 36.9 | 0.480 ± 0.061 | 31.1 ± 2.0 | 374.3 ± 54.9 | 0.358 ± 0.037 | 32.9 ± 3.6 |
| Monostearin with 10 wt.% FA–SA | 263.4 ± 43.6 | 0.538 ± 0.075 | 34.3 ± 3.3 | 369.3 ± 49.3 | 0.465 ± 0.055 | 32.2 ± 2.9 |

$d_v$, PI and $\zeta$ indicate the volume average diameter, polydispersity index and zeta potential, respectively.

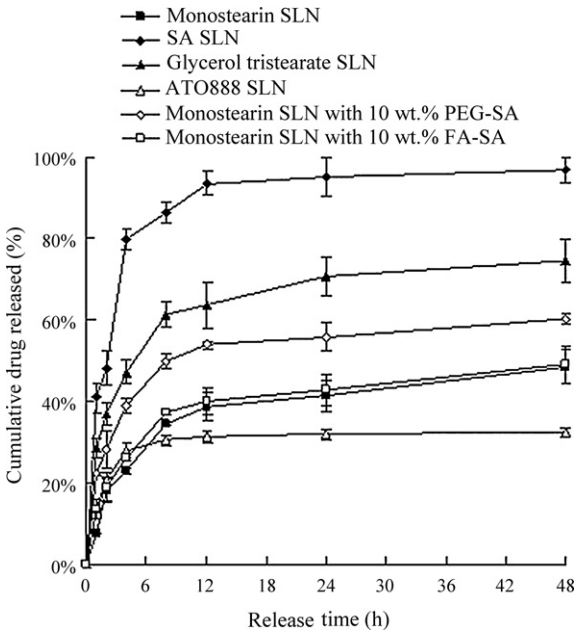


Fig. 1. In vitro paclitaxel release profiles of paclitaxel loaded SLN composed of different lipid materials. Mean ± S.D., $n = 3$.

### 3.4. Cellular uptake of SLN with different lipid materials

The cellular uptakes of SLN composed of different lipid materials were qualitatively and quantitatively evaluated by using ODA–FITC incorporated SLN and employing fluorescence microscopy and the measurement of fluorescence intensity. Using A549 as model cells, at first, the cells were incubated with 200 μg $ml^{-1}$ fluorescent SLN composed of different lipid materials for different time. The cellular uptakes of fluorescent SLN were viewed by fluorescence microscopy. Fig. 2 shows the fluorescence image when the cells was incubated with fluorescent SLN composed of different lipid materials for 1, 2, 4, 12, and 24 h, respectively. As a control, there was no fluorescence inside A549 cells were observed after the cells were incubated with FITC solution for 24 h.

As shown in Fig. 2, there was no significant fluorescence inside A549 cells after the cells were incubated with fluorescent monostearin SLN, stearic acid SLN, glycerol tristearate SLN and ATO888 SLN in 2 h. After 2 h, the obvious fluorescence images were observed, and the fluorescence intensity were enhanced with the incubation time. It means the cellular uptakes of SLN were time-dependent. Nanoparticles are generally internalized into cells via fluid phase endocytosis (Schoepf et al., 1998), receptor-mediated endocytosis (Moore et al., 1998) or phagocytosis (Weissleder et al., 1997). For the past decades, significant effort has been devoted to surface modification of various drug carriers with PEG to improve their internalization efficiency. It has been demonstrated that particles with PEG-modified surfaces cross-cell membranes was enhanced. The possible mechanism for this uptake is that PEG can dissolve in both polar and nonpolar solvents and have high solubility in cell membranes (Storm et al., 1995). However, the cellular uptake of PEG modified nanoparticles is a non-specific cellular uptake. One strategy to realize efficient and specific cellular uptake of nanoparticles is to modify the nanoparticle surface with a ligand that is efficiently taken up by target cells via receptor-mediated endocytosis (Lowry et al., 1998). It was reported that the intracellular accumulation may be further optimized through receptor-mediated endocytosis of the paclitaxel-loaded NPs (Mo and Lim, 2005). Cancerous cells divide rapidly and need folic acid for DNA synthesis, thus the amount and activity of folic acid receptor in the membrane of cancer cells was higher than normal cells. Folic acid has been linked to drugs, proteins, liposomes, and cationic polymers for enhanced uptake by cancerous cells (Zhou et al., 2002). In this study, the conjugate of FA–SA was incorporated into monostearin SLN to increase the cellular uptake mediated by folate receptor. From Fig. 2, it is clear that the obvious fluorescence images were found when the cells were incubated with PEG–SA and FA–SA modified monostearin SLN in 1 h. The cellular uptakes of SLN were highly enhanced by the incorporation of PEG–SA and FA–SA into SLN. Moreover, it is also obvious that the fluorescent SLN accumulated in the cytoplasm in all cases. This result was consistent with the literature (Benis et al., 1994). Since the action target of paclitaxel is microtubules in the cytoplasm, SLN are suitable carriers to deliver paclitaxel into cells for the targeting therapy.

For further quantitatively evaluate the cellular uptake of SLN with different lipid materials, the measurement of fluorescence intensity was carried out after the cells were incubated with fluorescent SLN composed of different lipid materials for different time. Fig. 3 shows the cellular uptake percentage of fluorescent SLN composed of different lipid materials against incubation time. It can be seen that the order of cellular uptake ability for SLN was glycerol tristearate SLN > monostearin SLN > stearic acid SLN > ATO888 SLN, and the cellular uptake percentage of fluorescent SLN increased with the incubation time. It was reported that the affinity between fatty acid and cell membrane was related with the melting point of fatty acid, and the length and saturation degree of the carbon chain (Tranchant et al., 1997). The melting point of monostearin, stearic acid, glycerol tristearate and ATO888 is 55–60, 67–69, 71–73 and 68 °C. As cells prefer uptaking substance with lower melting point, the uptake of monostearin SLN was higher than stearic acid SLN and ATO888 SLN. According to above reason, the glycerol tristearate SLN should show the lowest cellular uptake. However, the cellular uptake of glycerol tristearate SLN was fastest. The fastest cellular uptake of glycerol tristearate SLN may be due to the smallest particle size (Table 1). Because the difference length of the carbon chain between stearic acid and ATO888, the cellular uptake of stearic acid SLN was faster than that of ATO888 SLN. Although glycerol tristearate SLN showed the best cellular uptake ability, owing to the superior EE and DL of monostearin, it was chosen over glycerol tristearate as lipid material to prepare the PEGylated and folated SLN. The quantitative results also indicated that the introduction of PEG–SA and FA–SA into SLN could highly enhance the cellular uptake of SLN. It was reported that the surface of monostearin SLN showed a great deal of roughness after modifying with PEG2000, and could accelerate the endocytosis of SLN by A549 cells (Ding et al., 2004).

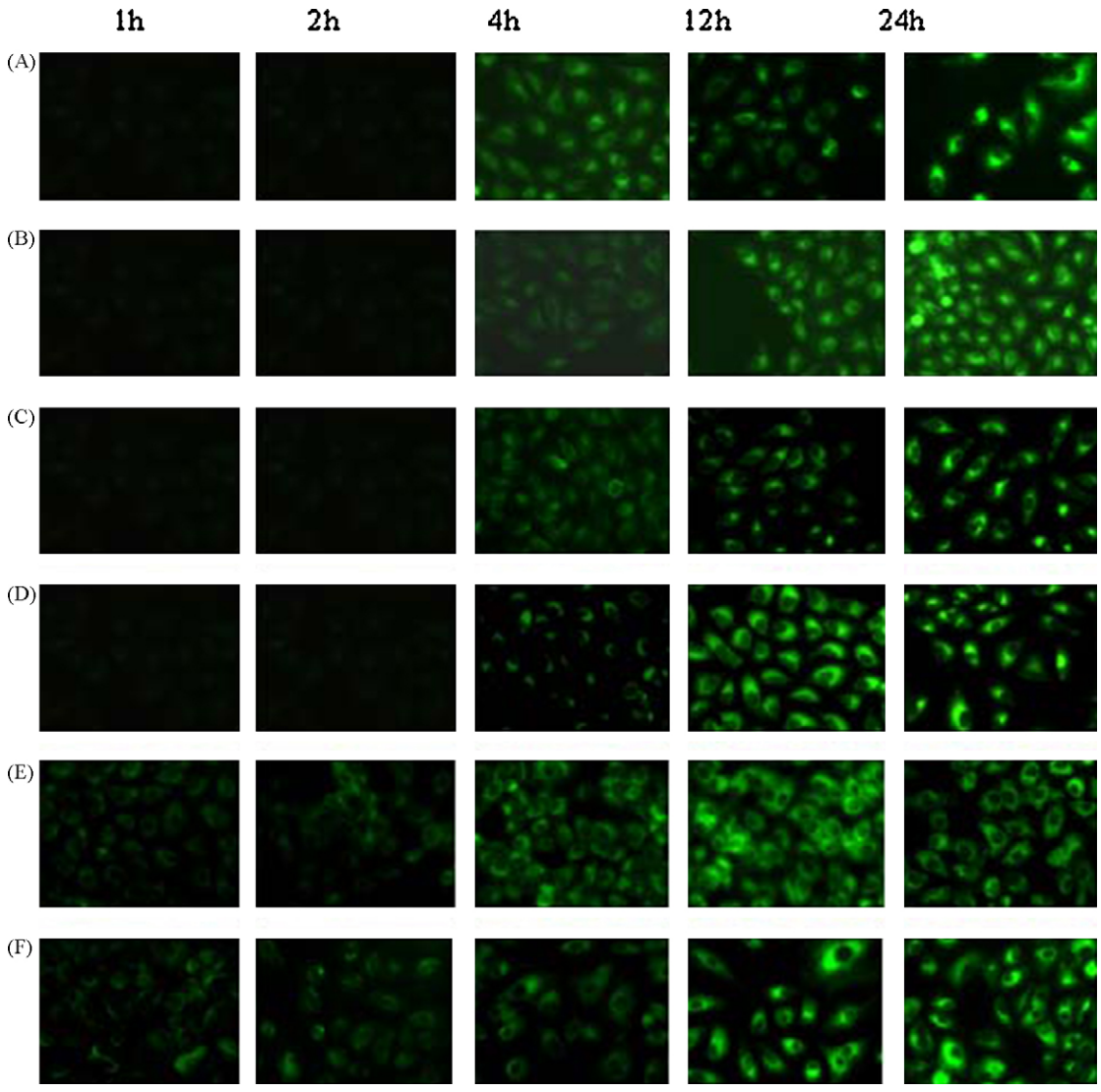


Fig. 2. Fluorescence image after the cells were incubated with the fluorescent SLN composed of different lipid materials: (A) monostearin SLN; (B) stearic acid SLN; (C) glycerol tristearate SLN; (D) ATO888 SLN; (E) monostearin SLN with 10 wt.% PEG–SA; (F) paclitaxel loaded monostearin SLN with 10 wt.% FA–SA (the lipid concentrations of SLN were 200 μg ml$^{-1}$).

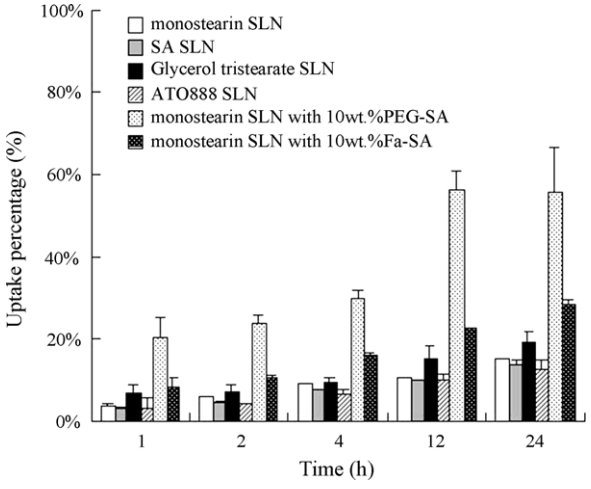


Fig. 3. The cellular uptake percentage of fluorescent SLN composed of different lipid materials in A549 cells. The lipid concentrations of SLN were 200 μg ml$^{-1}$, mean ± S.D., $n = 3$.

Figs. 4 and 5 show the cellular fluorescence image and cellular uptake percentage of fluorescent SLN after the cells was incubated with monostearin SLN having different concentration for 24 h. From Figs. 4 and 5, it is obvious the fluorescence intensity inside cells and the cellular uptake percentage of fluorescent SLN was increased with the incubation concentration of SLN at the same incubation time. These results mean the cellular uptake percentage of fluorescent SLN was concentration dependent.

### 3.5. Cytotoxicity

The cytotoxicities of blank SLN are shown in Table 3. The 50% growth inhibition ($IC_{50}$) values of cell for four biocompatible solid lipid materials were 300–500 μg ml$^{-1}$, which were less cytotoxicity. It means these lipid materials were suitable to prepare SLN for the use of drug carriers. After adding 15 wt.% ODA–FITC, 10 wt.% FA–SA or 10 wt.% PEG–SA, the $IC_{50}$ of SLN were still upper than 300 μg ml$^{-1}$.

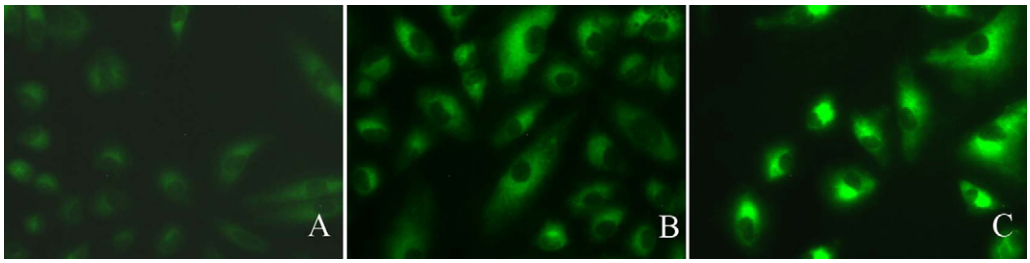


Fig. 4. Fluorescence image after the cells were incubated with the fluorescent monostearin SLN for 24 h: (A) the lipid concentrations of SLN was 50 μg ml$^{-1}$; (B) the lipid concentrations of SLN was 100 μg ml$^{-1}$; (C) the lipid concentrations of SLN was 200 μg ml$^{-1}$.

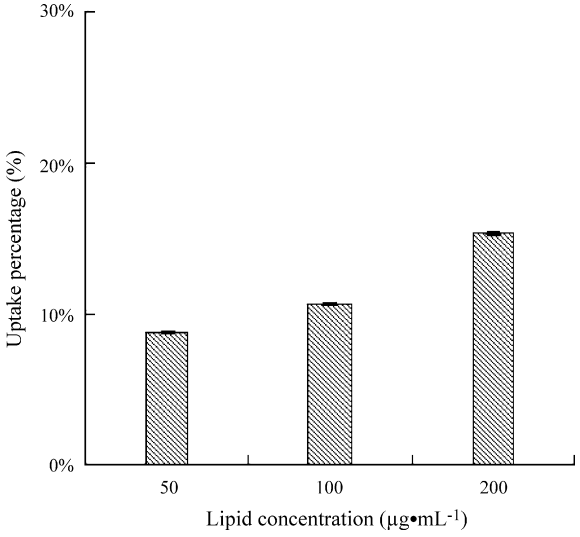


Fig. 5. The cellular uptake percentage of fluorescent monostearin SLN in A549 cells for 24 h at different lipid concentration. Mean ± S.D., $n = 3$.

The PEG–SA, synthesized ODA–FITC and FA–SA were safe materials for the modifications of drug carriers.

Table 4 reports 50% growth inhibition ($IC_{50}$) values for paclitaxel solution and paclitaxel loaded SLN composed of different lipid materials on the A549 cells for 48 and 72 h. Figs. 6 and 7 show the cell survival curves of A549 cells after exposure to paclitaxel solution and paclitaxel loaded SLN composed of different lipid materials for 48 and 72 h, respectively. From Table 4, Figs. 6 and 7, it was found that higher cytotoxicities was observed at 72 h than at 48 h, however, the change tendency of the cytotoxicities was coincidence. All of the $IC_{50}$ value of drug loaded SLN composed of different materials were lower than that of the formulation for drug solution. This means the drug internalization into cell could be enhanced by the encapsulation of SLN matrix and the endocytosis of SLN (Serpe et al., 2004). Drug loaded monostearin SLN showed the lowest cytotoxicity comparing to drug loaded SLN composed of the other lipid materials ($P < 0.05$). Although the cellular uptake of glycerol tristearate SLN was faster than that of monostearin SLN, the cytotoxicity was lower than that of monostearin SLN ($P < 0.05$). It possibly due to the burst drug release behavior of

Table 3
Cytotoxicities of blank SLN composed of different lipid materials

| Materials | $IC_{50}$ (μg ml$^{-1}$) |
|---|---|
| Monostearin | 396.82 ± 33.19 |
| Stearic acid | 331.12 ± 34.12 |
| Glycerol tristearate | 401.49 ± 27.68 |
| ATO888 | 471.48 ± 30.91 |
| Monostearin with 15 wt.% ODA–FITC | 362.88 ± 37.24 |
| Monostearin with 10 wt.% PEG–SA | 347.89 ± 42.40 |
| Monostearin with 10 wt.% FA–SA | 308.72 ± 41.89 |

Table 4
The cytotoxicities after 48 and 72 h exposure of A549 cells to paclitaxel solution and paclitaxel loaded SLN ($n = 3$)

| Formulation | $IC_{50}$ (μg ml$^{-1}$) | |
|---|---|---|
| | 48 h | 72 h |
| Paclitaxel solution | 2.36 ± 0.13 | 1.82 ± 0.04 |
| Paclitaxel loaded monostearin SLN | 0.75 ± 0.03 a, b, c | 0.54 ± 0.03 d, e, f |
| Paclitaxel loaded stearic acid SLN | 1.06 ± 0.07 a, g | 0.82 ± 0.02 d, h |
| Paclitaxel loaded glycerol tristearate SLN | 0.92 ± 0.04 b, g | 0.72 ± 0.03 e, h |
| Paclitaxel loaded ATO888 SLN | 1.45 ± 0.12 c | 1.14 ± 0.05 f |
| Paclitaxel loaded monostearin SLN with 10 wt.% PEG–SA | 1.86 ± 0.13 | 1.49 ± 0.03 f |
| Paclitaxel loaded monostearin SLN with 10 wt.% FA–SA | 0.21 ± 0.02 | 0.17 ± 0.01 g, h |

a, b, c, d, e, f indicate significant difference ($P < 0.05$). g, h: indicate no significant difference ($P > 0.05$).

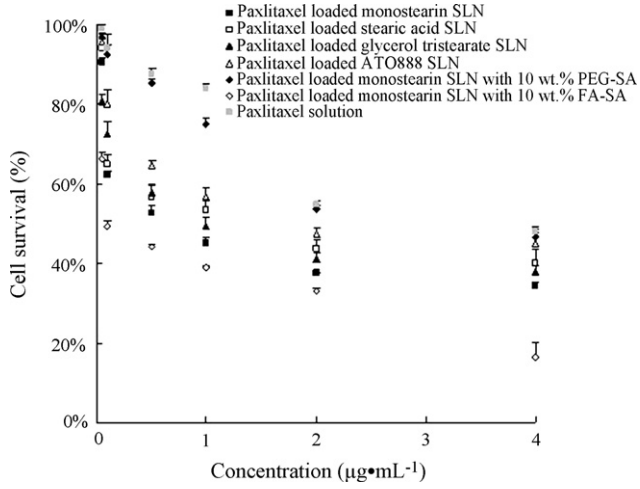


Fig. 6. Cell survival curves of A549 cells against drug concentration after the cells were incubation with paclitaxel solution and paclitaxel loaded SLN composed of different lipid materials at 48 h.

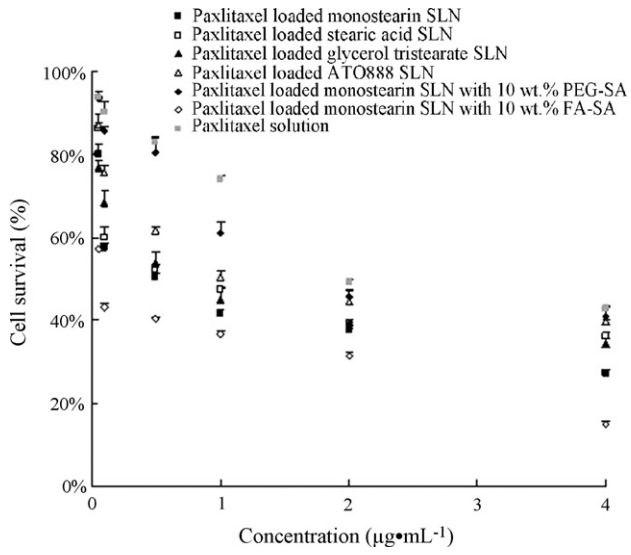


Fig. 7. Cell survival curves of A549 cells against drug concentration after the cells were incubation with paclitaxel solution and paclitaxel loaded SLN composed of different lipid materials at 72 h.

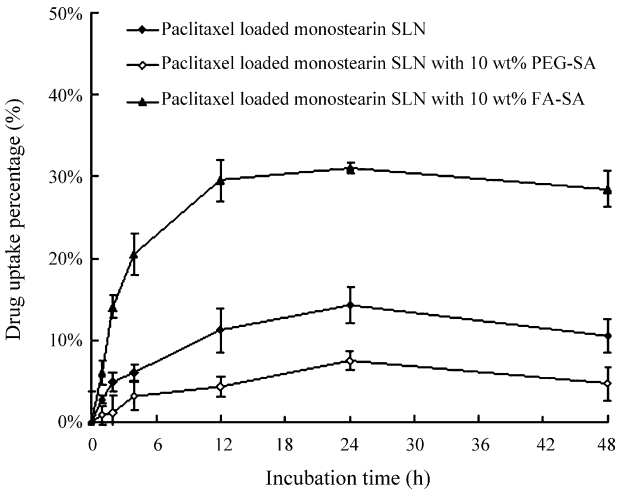


Fig. 8. The drug uptake percentage against incubation time after the cells were incubation with paclitaxel loaded monostearin SLN, paclitaxel loaded monostearin SLN with 10 wt.% PEG–SA, paclitaxel loaded monostearin SLN with 10 wt.% FA–SA. Mean ± S.D., $n = 3$.

drug loaded glycerol tristearate SLN at the initial stage, which led to the lower drug concentration internalized into cells. After the monostearin SLN modified with PEG–SA, the cytotoxicity of drug delivery did not increase comparing with that of drug loaded monostearin SLN. The possible reason is the PEG–SA modified monostearin SLN had a relative burst drug release at the initial stage (Hu et al., 2007), considerable amount of drug was released from PEG modified SLN before the cellular uptake. On the other hand, after the drug loaded monostearin SLN modified with FA–SA, the cytotoxicity was enhanced significantly compared to that of paclitaxel solution and drug loaded monostearin SLN. The enhanced cytotoxicity of FA–SA modified drug loaded monostearin SLN could be contributed to the faster cellular uptake, which caused by the enhanced endocytosis mediated by the folate receptor.

### *3.6. Cellular uptake of paclitaxel*

To clear the relationship between the cytotoxicity and the drug concentration in cell, the cellular uptakes of paclitaxel measurement were conducted. After the physical mixture of drug loaded SLN and A549 cells was treated with methanol at 70 °C, the extraction efficiency of drug could reach up to 94.3%.

Fig. 8 shows the cellular uptakes of paclitaxel delivered by monostearin SLN, folated monostearin SLN and PEGylated monostearin SLN, respectively. Incubating cells with drug loaded monostearin SLN within 24 h, the intracellular drug concentration increased with incubation time prolonged. However, the highest drug uptake percentage was only 14.3% (24 h), and then decreased. The reduction of drug uptake percentage in the later incubation stage may be caused by the drug metabolism in cells. On the other hand, the drug uptake percentages were higher than that of drug loaded monostearin SLN, when the cells were incubated with drug loaded folated monostearin SLN. The highest drug uptake percentage could reach up to 31.1% (24 h). The enhanced intracellular drug concentration could contribute to the drug transport mediated by the folate receptor. The drug uptake percentages were even lower than that of drug loaded monostearin SLN due to the burst drug release in the initial stage, when the cells were incubated with drug loaded PEGylated monostearin SLN. This is the reason why PEG enhances SLN uptake more than folate but the latter formulation enhances cytotoxicity much more.

## 4. Conclusion

Herein, the chemical conjugate of ODA–FITC was synthesized to prepare fluorescent SLN for the cellular uptake evaluation of SLN composed of different lipid materials. The cellular uptake of SLN indicated time and concentration dependent, and related with melting point of lipid material, length of carbon chain for lipid and the particle size. Due to the internalized SLN were accumulated in the cellular cytoplasm, the paclitaxel was used as model drug to evaluate the cytotoxicity of SLN drug delivery systems. The cytotoxicity could be enhanced by the encapsulation of SLN matrix, and the cytotoxicity related with the ability of cellular uptake and the drug loading of SLN. Although the incorporation of PEG–SA into SLN could enhance the cellular uptake, it did not increase the cytotoxicity. The introduction of FA–SA into SLN could enhance the cellular uptake and cytotoxicity, which revealed a potential application for the targeting therapy of tumor.

## References

Benis, S., Chapey, C., Couvreur, P., Robert, J., 1994. Enhanced cytotoxicity of doxorubicin encapsulated in polyisohexylcyanoacrylate nanospheres against multi-drug resistant cells in culture. Eur. J. Cancer 30A, 89–93.

Benns, J.M., Mahato, R.I., Kim, S.W., 2002. Optimization of factors influencing the transfection efficiency of folate–PEG–folate-graft-polyethylenimine. J. Control. Release 79, 255–269.

Ding, J.Ch., Hu, F.Q., Yuan, H., 2004. Uptake of monostearin solid lipid nanoparticles by A549 cells. Acta Pharmaceutica Sinica 39, 876–880.

Dong, Y.C., Feng, S.S., 2005. Poly(D,L-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs. Biomaterials 26, 6068–6076.

Hu, F.Q., Jiang, S.P., Du, Y.Z., Yuan, H., Ye, Y.Q., Zeng, S., 2005. Preparation and characterization of stearic acid nanostructured lipid carriers by solvent diffusion method in an aqueous system. Colloids Surf. B: Biointerfaces 45, 167–173.

Hu, F.Q., Ren, G.F., Yuan, H., Du, Y.Z., Zeng, S., 2006. Shell cross-linked stearic acid grafted chitosan oligosaccharide self-aggregated micelles for controlled release of paclitaxel. Colloids Surf. B: Biointerfaces 50, 97–103.

Hu, Y., Xie, J.W., Tong, Y.W., Wang, C.H., 2007. Effect of PEG conformation and particle size on the cellular uptake efficiency of nanoparticles with the HepG2 cells. J. Control. Release 118, 7–17.

Lowry, M.B., Duchemin, A.M., Robinson, J.M., Anderson, C.L., 1998. Functional separation of pseudopod extension and particle internalization during Fc gamma receptor-mediated phagocytosis. J. Exp. Med. 187, 161–176.

Marengo, E., Cavalli, R., Caputo, O., Rodriguez, L., Gasco, M., 2000. Scale-up of the preparation process of solid lipid nanospheres. Int. J. Pharm. 205, 3–13.

Maschke, A., Becker, C., Eyrich, D., Kiermaier, J., Blunka, T., Göpferich, A., 2007. Development of a spray congealing process for the preparation of insulin-loaded lipid microparticles and characterization thereof. Eur. J. Pharm. Biopharm. 65, 175–187.

Mehnert, W., Mäder, K., 2001. Solid lipid nanoparticles: production, characterization and applications. Adv. Drug Deliv. Rev. 47, 165–196.

Mo, Y., Lim, L.Y., 2005. Preparation and in vitro anticancer activity of wheat germ agglutinin (WGA)-conjugated PLGA nanoparticles loaded with paclitaxel and isopropyl myristate. J. Control. Release 107, 30–42.

Moore, A., Basilion, J.P., Chiocca, E.A., Weissleder, R., 1998. Measuring transferrin receptor gene expression by NMR imaging. Biochim. Biophys. Acta 1402, 239–249.

Müller, R., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.

Müller, R.H., Jacobs, C., Kayser, O., 2001. Nanosuspensions as particulate drug formulations in therapy: rationale for development and what we can expect for the future. Adv. Drug Deliv. Rev. 47, 3–19.

Schoepf, U., Marecos, E., Melder, R., Jain, R., Weissleder, R., 1998. Intracellular magnetic labeling of lymphocytes for in vivo trafficking studies. BioTechniques 24, 642.

Serpe, L., Catalano, M.G., Cavalli, R., Ugazio, E., Bosco, O., Canaparo, R., Muntoni, E., Frairia, R., Gasco, M.R., Eandi, M., Zara, G.P., 2004. Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line. Eur. J. Pharm. Biopharm. 58, 673–680.

Stevens, P.J., Sekido, M., Lee, R.J., 2004. A folate receptor-targeted lipid nanoparticle formulation for a lipophilic paclitaxel prodrug. Pharm. Res. 21, 2153–2157.

Storm, G., Belliot, S.O., Daemen, T., Lasic, D.D., 1995. Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. Adv. Drug Deliv. Rev. 17, 31–48.

Tranchant, T., Besson, P., Hoinard, C., Delarue, J., Antoine, J.M., Couet, C., Goré, J., 1997. Mechanisms and kinetics of α-linolenic acid uptake in Caco-2 clone TC7. Biochim. Biophys. Acta 1345, 151–161.

Weissleder, R., Cheng, H.C., Bogdanova, A., Bogdanov, A., 1997. Magnetically labeled cells can be detected by MR imaging. J. Magn. Reson. Imaging 7, 258–263.

Wong, H.L., Bendayan, R., Rauth, A.M., Xue, H.Y., Babakhanian, K., Wu, X.Y., 2006a. A mechanistic study of enhanced doxorubicin uptake and retention in multidrug resistant breast cancer cells using a polymer-lipid hybrid nanoparticle system. J. Pharmacol. Exp. Ther. 317, 1372–1381.

Wong, H.L., Rauth, A.M., Bendayan, R., Manias, J.L., Ramaswamy, M., Liu, Z.S., Erhan, S.Z., Wu, X.Y., 2006b. A new polymer-lipid hybrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug-resistant human breast cancer cells. Pharm. Res. 23, 1574–1585.

Yuan, H., Chen, J., Du, Y.Z., Hu, F.Q., Zeng, S., 2007. Studies on oral absorption of stearic acid SLN by a novel fluorometric method. Colloids Surf. B: Biointerfaces 58, 157–164.

Zauner, W., Farrow, N.A., Haines, A.M., 2001. In vitro uptake of polystyrene microspheres: effect of particle size cell line and cell density. J. Control. Release 71, 39–51.

Zhou, W., Yuan, X., Wilson, A., 2002. Efficient intracellular delivery of oligonucleotides formulated in folate receptor-targeted lipid vesicles. Bioconjug. Chem. 13, 1220.