Pharm Res (2011) 28:2020–2033
DOI 10.1007/s11095-011-0428-3

RESEARCH PAPER

Formulation, Biological and Pharmacokinetic Studies of Sucrose Ester-Stabilized Nanosuspensions of Oleanolic Acid

Wenji Li • Surajit Das • Ka-yun Ng • Paul W. S. Heng

Received: 25 October 2010 / Accepted: 8 March 2011 / Published online: 9 April 2011

ABSTRACT

Purpose The aim of this study was to develop sucrose ester (SE)-stabilized oleanolic acid (OA) nanosuspensions (NS) for enhanced delivery.

Methods SEOA NS were prepared via O/W emulsion and organic solvent evaporation methods. The particles' size and polydispersity index were measured by nanosizer. Their percent encapsulation efficiency, saturation solubility and *in vitro* dissolution rate were obtained via HPLC. The *in vitro* bioefficacy was analyzed by MTT measurements in A549 human non-small-cell lung cancer cell line. The cellular uptake of OA and *in vivo* pharmacokinetics profile were determined using LC-ESI-MS/MS.

Results Spherical SEOA NS particles (~100 nm in diameter) were produced and found to be physicochemically stable over a month at 4°C. In particular, SEOA 4121 NS (SEL: SEP at 4:1 w/w; SE: OA at 2:1 w/w) produced the greatest increase in saturation solubility (1.89 mg/mL *vs*. 3.43 μg/mL), dissolution rate, cytotoxicity and bioavailability. Preliminary studies indicated that cellular uptake of SEOA NS by A549 cells was temperature-, concentration- and time-dependent.

Conclusion Preparing OA as SE-stabilized NS particles provides a novel method to enhance saturation solubility, *in vitro* dissolution rate, bioefficacy and *in vivo* bioavailability of free OA and/or other potentially useful hydrophobic drugs.

KEY WORDS bioavailability · dissolution rate · hydrophobic drug · oleanolic acid · saturation solubility · sucrose ester · surfactant stabilized nanosuspensions

W. Li • S. Das • K.-y. Ng • P. W. S. Heng (✉)
Department of Pharmacy, National University of Singapore
18 Science Drive 4
Singapore 117543, Singapore
e-mail: phapaulh@nus.edu.sg

INTRODUCTION

Naturally occurring compounds have remained an important source for the search of lead drug compounds. However, many nature-derived drug candidates have problems of low aqueous solubility, low dissolution rate, and, consequently, poor bioavailability, which have limited their clinical applications (1–3). To address these problems, chemically modified derivatives of nature-derived compounds with improved aqueous solubility have been produced (4,5). Although effective, chemical modification takes time, remains expensive, and may not be applicable to all naturally derived compounds (6).

The aqueous solubility, dissolution rate and bioavailability of poorly soluble drugs are often intrinsically related to drugs' particle sizes (7,8). Nanosuspension (NS), which has increased surface area due to reduced particle size, can lead to improved drug dissolution rate and is often accepted as one of the most expeditious and cost-effective methods to improve the dissolution properties of poorly water-soluble drugs. Pharmaceutically, NS refers to a colloidal suspension of drug nanoparticles that are produced by a suitable method (precipitation, milling or high-pressure homogenization) and stabilized by adjuvants such as polymers and surfactants (9–11).

Sucrose ester (SE) is a group of nonionic surfactants synthesized by esterification of sucrose with fatty acids. SEs have been used to prepare micelles (12) and microemulsions (13) and have been found to aid in transdermal drug delivery (14). As surfactants prepared by esterification of nature-derived chemicals, SEs are safe and biodegradable and have been widely used for therapeutic and cosmetic applications (15). Despite these favorable advantages, SEs have not been much used as stabilizers in preparation of nanoparticles or NS until in recent years (16–21).

In the establishment of SEs as effective surfactants to prepare NS for delivery of hydrophobic natural compounds, oleanolic acid (OA) was selected as a model drug because it is a potentially useful triterpenoid with diverse and important pharmacological activities. OA is a poorly water-soluble plant-derived triterpenoid used widely in many Asian countries. It has shown hepatoprotective effects in both acute liver injury and chronic liver fibrosis and cirrhosis (22). In China, it is an over-the-counter drug sold for the treatment of liver diseases. Besides the hepatoprotective effect, OA is reported to have anti-cancer effects including inhibition of tumor initiation and promotion and induction of tumor cell differentiation and apoptosis (23,24). It is also reported to possess other pharmacological effects such as anti-inflammatory (25), hypolipidemia and anti-diabetes (26).

Although NS is a good method to enhance the saturation solubility and oral bioavailability of natural compounds, there is very little research on the OA NS. Chen *et al.* (11) have prepared OA NS stabilized using polysorbate 80 with six-fold increase in saturation solubility, but oral bioavailability was not demonstrated. There are also very few references on the pharmacokinetics profile of OA *in vivo* (27–29). Jeong *et al.* (28) first published the oral bioavailability of OA suspension in rats, and it was as low as 0.7%.

The goal of this study was designed to address these specific knowledge gaps with the following objectives in mind.

a) To investigate the characteristics of OA NS stabilized by blended SEs (sucrose monolaurate (SEL) and sucrose monopalmitate (SEP)) prepared by emulsion/organic solvent evaporation method.
b) Since OA showed anti-cancer properties (23,24) and Gao *et al.* (30) reported OA had weak cytotoxicity effect against A549 cell line, to examine the bioefficacy by comparing the cytotoxcity of both free and nanosized OA using the A549 cell line.
c) To elucidate the cellular uptake mechanism and *in vivo* pharmacokinetics profiles of OA NS and OA coarse suspension using a modified liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) assay (28,29).

The present research is the first to employ SEs as sole stabilizers for preparing NS. It is expected the results of this study may have significant impact on the use of SE-stabilized NS to increase saturation solubility, dissolution rate and bioavailability of hydrophobic natural compounds and may provide them opportunities to be studied for their potential pharmaceutical applications instead of being deemed as unsuitable during the screening stage due to their very poor water solubility.

MATERIALS AND METHODS

Materials

OA was purchased from Nanjing Qinze Pharmaceutics Ltd. Co. (Nanjing, China). SEL (batch M07A001, 90% purity) and SEP (batch M07C003, 90% purity) were obtained from Compass Foods Pte. Ltd. (Singapore). Methyl thiazolyl tetrazolium (MTT) and F12 Ham Kaighn's modification (F12K) medium were purchased from Sigma Aldrich Ltd. Co. (St. Louis, MO, USA). A549 human non-small-cell lung cancer cell line (NSCLC) was purchased from American Type Culture Collection (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories Ltd. Co. (Logan, UT, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Applichem Ltd. Co. (Darmstadt, Germany). MilliQ water (18.2 M Ω cm at 25°C) was obtained from a Millipore Direct-Q ultra-pure water system (Billerica, MA, USA) and used throughout the study.

OA Equilibrium Solubility

Equilibrium solubility of OA was determined by saturation shake-flask method according to the US Pharmacopoeia (USP) XXI (United States Pharmacopeia Convention, Inc., Rockville, MD, USA, 1985). The drug was added to distilled water until excess undissolved drug appeared, which indicated the solution had reached its saturated solubility. Briefly, excess drug was added to conical tubes, each filled with 50 mL distilled water, shaken at 25°C for 24 h, and observed for presence of any undissolved drug. If none was detected, more drug was introduced and the process repeated until undissolved drug crystals appeared and remained. The saturated amount of dissolved drug was determined by filtering through 0.22 μm filter and assaying the filtrate using high performance liquid chromatography (HPLC) assay according to the USP method.

Preparation of NS

NS were prepared by emulsion/organic solvent evaporation method, adapted from the report of Chen *et al.* (11). In brief, SEP and SEL were dissolved in 30 mL MilliQ water in a 50 mL beaker with a magnetic bead at room temperature. The aqueous solution was stirred at 800 rpm on a stir plate (Sybron, East Lyme, CT, USA) for about 30 min until all the surfactants were completely dissolved. OA in 15 mL acetone was added to the solution. The resulting o/w emulsion was stirred at 800 rpm overnight in a fume hood under a small jet of nitrogen to facilitate diffusion and evaporation of organic solvent. This resulted in nanoprecipitation and formation of NS. In the last step of preparation, precipitated materials including OA NS, free

OA and SE were suspended in same volume of water (30 mL) and stirred for 24 h. Next, the suspension with excess undissolved materials was centrifuged (13,000 g×10 min). The supernatant was filtered through a 0.22 μm membrane to give a visually clear NS. The resulting NS was used immediately for determination of OA saturation solubility and percent encapsulation efficiency (EE%). Detailed compositions of various SEOA NS are shown in Table I.

Particle Size Analysis

Dynamic light scattering (DLS) measurements were performed on Zetasizer-3000 (Malvern Instruments, Malvern, UK) at a wavelength of 532 nm. The scattering angle was fixed at 90°, and sample was maintained at 25°C. Particle size determination was carried out using a diluted suspension by adding 4 times its volume with MilliQ water.

Transmission Electron Microscopy (TEM)

Copper grids were coated with 0.25% Formvar film and carbon in sequence. The film faces of the grids were applied with the NS sample and stained with 5% phosphotungstic acid (PTA) subsequently. Excess suspension was carefully blotted off during each step. After drying for over 10 min under bench lamp, the sample was ready for use. TEM photomicrographs were obtained using a transmission electronic microscope (JEM 2010, JEOL Ltd. Co., Tokyo, Japan) operated at 200 kV.

Percent Encapsulation Efficiency (EE%) and Saturation Solubility

HPLC (Model 1100, Agilent, Palo Alto, CA, USA) analysis was carried out using a C18 column (ODS 5 μm, 3.9 mm× 150 mm; Waters, Milford, MA, USA) with 65% acetonitrile and 35% MilliQ water as the mobile phase. Column temperature was set at 24°C. Flow rate was 1 mL/min, and uv detection wavelength was 210 nm. Standard samples were dissolved in methanol. Freshly prepared NS was dissolved in at least 5 times the volumes of methanol to ensure that OA was fully dissolved and fell within the standard calibration curve range. All samples were filtered through 0.22 μm membranes before measurements. The calibration curve over the concentration range of 0.01–0.20 mg/mL was constructed by plotting the peak area of the analyte against the concentration spiked for each media. Seven independently weighed concentrations (0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.20 mg/mL) were used to obtain the calibration curve. The linearity of the assay procedure was determined by calculation of a regression line. Concentrations of OA in diluted SEOA NS samples were obtained from the resulting peak areas and the regression equation of the calibration curve. Saturation solubility of OA was calculated from the amount of OA dissolved in diluted sample multiplied by the dilution factor. For calculation of the EE% of OA, the following equation was used:

$$EE\% = (OA_{NS}/OA_T) \times 100$$

where OA_{NS} indicates amount of OA in NS, and OA_T indicates total amount of OA added in the formulation.

Table I Detailed Compositions of NS formulations

Group	SEL Weight (mg)	SEP Weight (mg)	OA Weight (mg)
SELOA	250.00	0.00	25.00
SEPOA	0.00	250.00	25.00
SEOA 91101	225.00	25.00	25.00
SEOA 9121	225.00	25.00	125.00
SEOA 9151	225.00	25.00	50.00
SEOA 4121	200.00	50.00	125.00
SEOA 4151	200.00	50.00	50.00
SEOA 2151	167.00	83.00	50.00

SELOA (SEL: OA at 10:1 w/w), SEPOA NS (SEP: OA at 10:1 w/w), SEOA91101 NS (SEL: SEP at 9:1 w/w; SE: OA at 10:1 w/w), SEOA9121 NS (SEL: SEP at 9:1 w/w; SE: OA at 2:1 w/w), SEOA9151 NS (SEL: SEP at 9:1 w/w; SE: OA at 5:1 w/w), SEOA4121 NS (SEL: SEP at 4:1 w/w; SE: OA at 2:1 w/w), SEOA4151 NS (SEL: SEP at 4:1 w/w; SE: OA at 5:1 w/w), SEOA2151 NS (SEL: SEP at 2:1 w/w; SE: OA at 5:1 w/w)

Lyophilization of SEOA NS and Free OA Solution

SEOA NS and free OA solution were frozen at −80°C overnight and then freeze-dried (Labconco Corp., Kansas City, MO, USA) for 24 h at −70°C and 0.02 mbar.

Stability Study

Stability of Storage in Suspension Form

The effect of storage time on the stability of SEOA NS at 4°C was investigated. After filtration, the concentration changes of OA in NS recovered after storage of 1 and 3 months were determined by HPLC.

OA Stability in Plasma

SEOA4121 NS (SEL: SEP at 4:1 w/w; SE: OA at 2:1 w/w) was diluted with 60 times the volumes of normal rat plasma, vortexed for 1 min and incubated in a shaking (100 rpm) water bath at 37°C. Samples were removed at 0, 1, 2, 4 and 24 h after incubation and centrifuged (13,000 g× 10 min). Supernatant (50 μL) of each sample was removed and placed into a new tube. Ethyl acetate (1 mL) containing 1000 ng glycyrrhetinic acid (GA) as the internal standard (IS)

was then added, vortexed for 1 min and centrifuged (13,000 g×10 min). The supernatant was carefully removed, dried under nitrogen flow for 1 h at 40°C and reconstituted in 1 mL methanol for LC-ESI-MS/MS measurement. The LC-ESI-MS/MS analysis method will be discussed in "Chromatography and Tandem Mass Spectrometry Analysis." All experiments were in triplicates, and results averaged.

Stability in Simulated Gastric and Intestinal Fluids

Non-enzyme-simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8 and 7.4) were prepared following USP 29 (United States Pharmacopeia Convention, Inc., Rockville, MD, USA, 2006) with modification. SGF was prepared by dissolving 2 g of sodium chloride in 0.2 N hydrochloric acid and diluting with sufficient distilled water to make a 1000 mL solution and adjusting to pH 1.2±0.1. SIF was prepared by dissolving 6.8 g of monobasic potassium phosphate in 250 mL distilled water, mixing and adding 77 mL of 0.20 N sodium hydroxide. The resulting solution was adjusted with either 0.2 N sodium hydroxide or 0.2 N hydrochloric acid to pH 6.8±0.1 and 7.4±0.1 and made up with distilled water to 1000 mL.

SEOA4121 NS was separately diluted with 10 x volumes of SGF (pH 1.2) or SIF (pH 6.8 and 7.4), vortexed for 1 min and incubated in a shaking (100 rpm) water bath at 37°C. Samples were removed after 0, 0.5, 1, 2, 4 and 24 h incubation and centrifuged (13,000 g×10 min); supernatant (50 µL) of each sample was transferred to a new tube for lyophilization and subsequent dilution for HPLC analysis. All experiments were in triplicates, and results averaged.

In Vitro Dissolution Test

Dissolution experiments were carried out using a dissolution apparatus (Model 2100c; Distek, North Brunswick, NJ, USA) according to the USP 29 Apparatus 2 (United States Pharmacopeia Convention, Inc., Rockville, MD, USA, 2006). The dissolution medium (500 mL) was pH 7.4 phosphate buffer solution containing 1% sodium dodecyl sulfate (SDS), thermostated at 37±0.5°C with 100 rpm rotating speed. SEOA 4121 NS, SEOA 4121 NS lyophilized powder, OA coarse suspension (suspended in N,N-DMAC:PEG400:water in the ratio of 2:4:1 v/v/v), and SEOA4121 NS in dialysis bags (MWCO 2,000; Spectrum Medical Industries Inc., Singapore) were all added into the dissolution media; each bag contained an estimated amount equivalent to 8 mg OA. Samples (3 mL) were withdrawn at predetermined time intervals and filtered through 0.22 µm filters. After each withdrawal, an equal volume of the dissolution medium was added to maintain the volume constant. The content of dissolved OA was determined using HPLC. All dissolution experiments were performed in triplicates, and all sample analyses were carried out in triplicates.

Cytotoxicity of OA and SEOA NS

A549 human NSCLC cells were cultured in F12 Ham Kaighn's modification (F12K) medium, supplemented with 10% FBS, 10 mM HEPES, 100 U/mL penicillin G and 100 µg/mL streptomycin. The cells were maintained at 37°C in a 5% CO_2 humidified incubator. To determine cytotoxicity of OA and SEOA NS, A549 cells were seeded in 96-well plates at a density of 6×10^3 cells per well and incubated for 24 h at 37°C in a 5% CO_2 humidified incubator. Culture media were then removed and replaced with 100 µL fresh media (blank) or fresh media containing 0.5% DMSO (control) or different concentrations of OA (in media with 0.5% DMSO) or SEOA NS or the same ratio blank SE NS without OA. After 24 and 72 h incubation, 10 µL MTT solution (5 mg/mL in PBS) was added to each well. After incubation at 37°C for 4 h, the mixtures in the wells were removed, and 110 µL DMSO was added to each well and shaken at 100 rpm for 30 min. Absorbance was measured using a multiplate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm. Proliferation rate (%) was calculated as ((sample reading-blank reading)/(control reading-blank reading))×100.

Cellular Uptake Study

A549 cells (100,000 cells/mL) were seeded into each well of the 6-well plates (Falcon; Becton Dickinson, Sparks, MD, USA) and allowed to attach for 24 h. Next, the cell culture media were replaced with fresh media containing different concentrations of SEOA NS. After incubation for 1 and 3 h, respectively, the cells were washed thrice with cold PBS (4°C, pH 7.4, 10 mM). The cells were then lysed by incubating with 0.2 mL cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). After brief sonication on ice, the cell lysates were processed to determine the OA levels by LC-ESI-MS/MS using a modified published method (28,29), which will be elaborated in "Chromatography and Tandem Mass Spectrometry Analysis."

To determine the levels of cellular uptake of NS, 5 µL of glycyrrhetinic acid (GA) solution (1 µg/mL) as the internal standard (IS) was added to 50 µL of cell lysate sample and vortexed for 1 min. The sample was extracted with ethyl acetate (900 µL) by vortex-mixing (1 min) and centrifuged (13,000 g×10 min) at room temperature. The organic layer was carefully transferred and dried under nitrogen flow at 40°C. The residue was dissolved in methanol and transferred to clean vials for sample injection. Calibration standards were prepared with 50 µL blank cell lysate samples by the same method. Cell protein was assayed by

BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the kit protocol. Results were expressed as amount (μg) of OA per mg total cell protein.

To study the effect of temperature on SEOA NS uptake, as control groups, A549 cells were pre-incubated in regular growth medium at 4°C for 30 min and co-incubated with SEOA NS (15 μg/mL) at 4°C for 3 h. Normal groups were pre-incubated in regular growth medium at 4°C for 30 min, then co-incubated with SEOA NS (15 μg/mL) at the higher temperature of 37°C for 3 h. The dose and time effects on cellular uptake of NS were also examined. Dose-dependent NS uptake effect was studied by incubating cells in different concentrations of NS (15 and 30 μg/mL) for 1 h. For the time-dependent NS uptake effect study, cells were incubated with NS (15 μg/mL) for 1 and 3 h.

Pharmacokinetics Study

Intravenous and Oral Administration of OA to Rats

The study design and animal handling protocol of this pharmacokinetic study were modified from our previous study (31) and approved by the Institutional Animal Care and Use Committee of the National University of Singapore. Adult male Sprague–Dawley rats (250–300 g) were purchased from the Laboratory Animal Center of the National University of Singapore. The rats were housed under temperature (22 ± 1°C) and relative humidity (60–70%)-controlled environment in Animal Holding Unit of the university operated at a 12-h light/dark cycle. The rats were given free access to food and water before surgery. On the day before the pharmacokinetic study, a polyethylene tube (i.d. 0.58 mm, o.d. 0.965 mm, Becton Dickinson, Sparks, MD, USA) was placed into the right jugular vein through surgical implant under anesthesia. The intravenous (IV) drug administration and blood sample collection were performed through this cannula. The rats were randomly divided into four groups (n = 5 per group). Group 1 received IV administration of OA, while three other groups received oral dosing through gavage. It is known that oral absorption may be influenced by different dietary regimens and the inherent bile salt solubilization capacity in the intestine. Hence, the rats for oral administration (groups 2–4) were kept in fasting condition overnight prior to the oral gavage and during blood collection, but free access to water was allowed. However, such restriction was not applied to the rats that received IV administration. Rats in groups 2 and 3 were administered single dose of SEOA 4121 NS by oral gavage at the dose of 10 and 20 mg/kg, respectively. As controls and comparisons, rats in groups 1 and 4 would receive either SEOA4121 NS by IV administration (2 mg/kg) or oral administration of coarse OA suspensions in N, N-DMAC: PEG400: water (2:4:1 v/v/v) at the dose of 20 mg/kg. Serial blood samples (200 μL) were collected from each animal at 1, 5, 15, 30, and 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after IV administration and at 5, 15, and 30 min, and 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after oral administration. The cannula was flushed, and blood was replaced by an equivalent volume of heparin-saline (20 IU/mL heparin in normal saline) after each draw of blood sample. Plasma samples were collected after centrifugation (3,000 g × 5 min) of the blood samples and stored at −80°C until LC-ESI-MS/MS analysis.

Sample Preparation and Calibration

The sample preparation method (liquid-liquid extraction) was adopted from our previous study with minor modification (31). The plasma sample (100 μL) was spiked with methanol solution (5 μL) of GA (20 μg/mL) as IS and mixed briefly in a clean 2 mL centrifuge tube. Then ethyl acetate (300 μL) was added to the tube and mixed for 1 min to facilitate the extraction procedure. After this liquid-liquid extraction, the tube was centrifuged (13,000 g × 10 min), and the ethyl acetate layer was carefully transferred to another clean tube. The extraction procedure was repeated for two extra times, and the ethyl acetate layer was collected in the same tube. The sample was then dried under nitrogen flow at 40°C. The residue was reconstituted with methanol (75 μL) and centrifuged (13,000 g × 5 min). The supernatant was injected (10 μL) into the HPLC column. Calibration standards were prepared with 100 μL blank plasma samples in the same way. The calibration curve was obtained from the samples prepared by spiking OA and internal standard into pooled rat plasma. It was linear (r^2 = 0.9907) within the range of 20–2,000 ng/mL of OA.

The within-day and between-day accuracy and precision were evaluated at three concentration levels (40, 100 and 800 ng/mL) based on five measurements carried out in a single day and over five days of validation period, respectively, according to previous report with modifications (29). The accuracy was expressed as bias (the percentage of difference between the measured and spiked concentration over that of the spiked value), whereas the precision was presented as the relative standard deviation (R.S.D.%). The absolute recovery of the extraction was determined by comparing the peak area obtained from the plasma sample with peak areas obtained by the direct injection of pure OA standard solutions in the HPLC column at three different concentration levels. The quantification of the chromatogram was performed by using peak area ratios of OA to internal standard.

Chromatography and Tandem Mass Spectrometry Analysis

The concentrations of OA in plasma were determined by a previously reported LC-ESI-MS/MS method with modifications (28,29). Briefly, the HPLC system was an Agilent

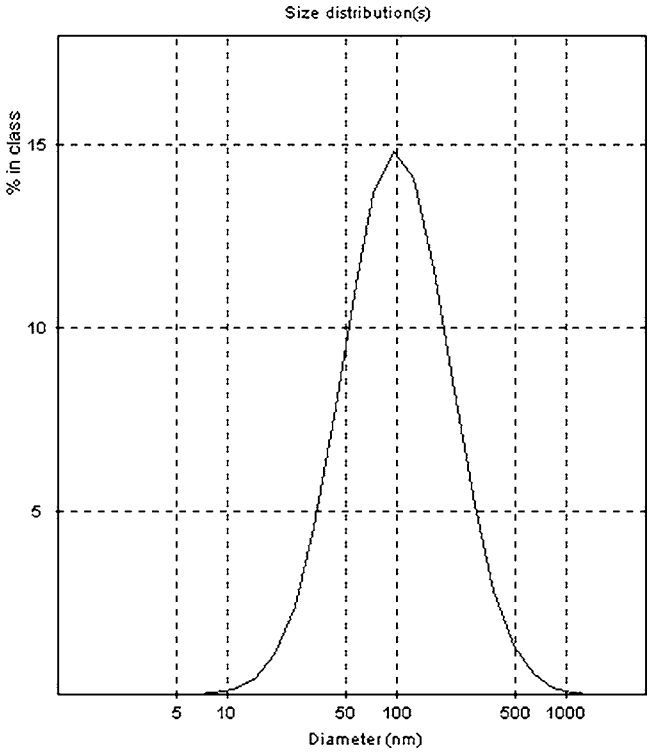

Fig. 1 Representative particle size distribution data obtained from Zetasizer Instrument by the intensity of signal.

1100 series machine equipped with a G1312A binary pump and a G1379A degasser (Agilent, Palo Alto, CA, USA). The HPLC column was a C18 column (300 mm × 2. mm i.d.) packed with 3 μm ODS stationary phase (Hypersil Aquasil, Thermo Scientific, Waltham, MA, USA) which was protected with a guard column (Inertsil ODS-3; GL Sciences, Tokyo, Japan). The HPLC mobile phase consisted of acetonitrile/10 mM ammonium acetate buffer pH 6.5 (15: 85, v/v). The flow rate was set at 0.30 mL/min, and analysis was performed in isocratic mode. The mass spectrometer was Qtrap 2000 model with an electrospray ionization (ESI) interface (Applied Biosystems, Toronto, Canada). Negative ion ESI with the collision energy −30 V, curtain gas 10 psi and ion source temperature 200°C were used. Quantification was performed with multiple selected reaction monitoring (MRM) mode. The transition of OA is 455.5/455.5 (m/z) and GA (IS) is 469.5/425.5 (m/z) with a scan time of 100 ms per transition.

Results Analysis

WinNonlin standard Version 5.01 (Scientific Consulting Inc., Apex, NC, USA) was used to analyze the pharmacokinetic parameters, and non-compartmental model was adopted for the analysis. The area under the plasma concentration (AUC) *versus* time curve ($AUC_{0\rightarrow t}$) in rats that received oral administration (Groups 2–4) was calculated by the linear trapezoidal rule, whereas the $AUC_{0\rightarrow t}$ in rats that received IV dosing (Group 1) was calculated through the same rule except the logarithmic scale was taken. Clearance (Cl) values were calculated using the equation: $Cl = \frac{Dose}{AUC_{0\rightarrow t}}$. Absolute bioavailability, F (%) of OA after oral administration (Groups 2–4) was calculated using the following equation:

$$F(\%) = \frac{\frac{AUC_{0\rightarrow t}(\text{Group 2,3 or 4})}{\text{Dose(Group 2,3 or 4)}}}{\frac{AUC_{0\rightarrow t}(\text{Group 1})}{2\text{mg/kg}}} \times 100$$

Statistical Analysis

Results were presented as mean ± standard deviation (std). Statistical significance of the results was analyzed using two-tail independent sample *t*-test or one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

Table II Comparison of SEOA NS Properties

Group	Size (nm)	PDI	EE%	Saturation Solubility (mg/mL)
SELOA	101.60 ± 4.00 [e, f]	0.60 ± 0.09 [d, e]	79.40 ± 0.06 [d, e, f, g, h]	0.66 ± 0.01 [d, g, h]
SEPOA	92.20 ± 2.10 [e, f, h]	0.61 ± 0.02 [d, e]	79.82 ± 3.20 [d, e, f, g, h]	0.67 ± 0.03 [d, g, h]
SEOA91101	103.60 ± 5.30 [e, f]	0.57 ± 0.08 [d, e].	79.29 ± 2.69 [d, e, f, g, h]	0.66 ± 0.02 [d, g, h]
SEOA9121	171.40 ± 3.40 [a, b, c, d, f, g, h]	0.41 ± 0.04 [a, b, c, g, h]	18.07 ± 0.58 [a, b, c, d, f, g, h]	0.75 ± 0.02 [d, g, h]
SEOA9151	133.70 ± 1.20 [a, b, c, d, e, g, h]	0.49 ± 0.02 [h]	40.73 ± 3.02 [a, b, c, e, g, h]	0.68 ± 0.05 [d, g, h]
SEOA4121	96.60 ± 2.30 [e, f, h]	0.41 ± 0.03 [a, b, c, g, h]	45.38 ± 1.81 [a, b, c, e]	1.89 ± 0.08 [a, b, c, e, f, g, h]
SEOA4151	93.10 ± 1.50 [e, f, h]	0.59 ± 0.08 [d, e]	54.31 ± 0.74 [a, b, c, e, f]	0.91 ± 0.01 [a, b, c, d, e, f]
SEOA2151	110.60 ± 6.80 [b, d, e, f, g]	0.66 ± 0.07 [d, e, f]	49.15 ± 2.37 [a, b, c, e, f]	0.82 ± 0.04 [a, b, c, d, e, f]

Data represent three independent experiments, repeated in triplicates. Values are presented as means ± std. [a] significantly different compared with SELOA ($p < 0.05$). [b] significantly different compared with SEPOA ($p < 0.05$). [c] significantly different compared with SEOA91101 ($p < 0.05$). [d] significantly different compared with SEOA4121 ($p < 0.05$). [e] significantly different compared with SEOA9121 ($p < 0.05$). [f] significantly different compared with SEOA9151 ($p < 0.05$). [g] significantly different compared with SEOA4151 ($p < 0.05$). [h] significantly different compared with SEOA2151 ($p < 0.05$)

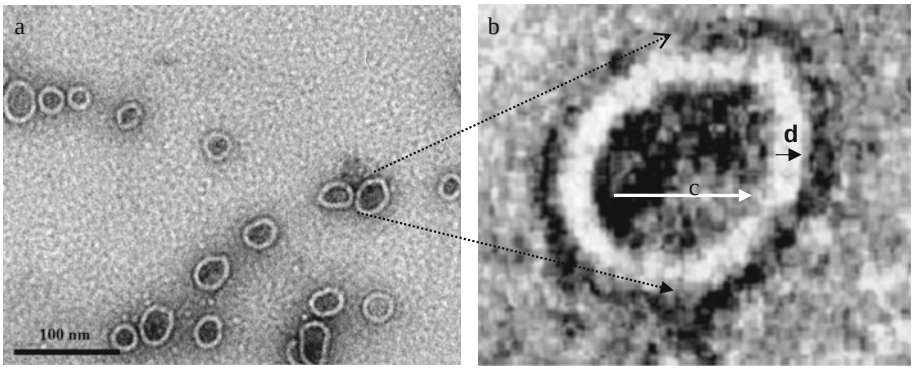

Fig. 2 Representative TEM of SEOA-NS produced by emulsion/organic solvent evaporation method. (c is encapsulated OA; d is thickness of SE coating.)

RESULTS AND DISCUSSIONS

Characteristics of SEOA NS

Particle Size and Polydispersity Index of Different SEOA NS

The size and polydispersity index (PDI) of SEOA NS were characterized by dynamic light scattering (DLS) measurement. Figure 1 shows a typical size distribution curve of OA NS prepared with SEL and SEP. The graph shows the distribution of particle sizes, with the mode of particles around 100 nm. With the exception of SEOA9121 NS (SEL: SEP at 9:1 w/w; SE: OA at 2:1 w/w) ($p<0.01$), which had a mean size of 171.40 nm, the mean sizes of all other particles were all at around 100 nm range. The PDIs were high, showing considerable variation in the size of the NS particles and suggesting possible existence of some aggregated moieties. SEOA 4121 NS and SEOA 9121 NS have the smallest PDI reading of 0.41 (see also Table II).

As indicated in Table II, it is noticed that the weight ratio of SEL to SEP may influence the size of NS. Among all the formulations, when the ratio of SEL to SEP equals 9:1, the particle sizes are among the largest. For instance, between SEOA 4121 and SEOA 9121, which only differ from the SEL to SEP weight ratio, the latter is much larger. The condition is same with SEOA 2151(SEL: SEP at 2:1 w/w; SE: OA at 5:1 w/w), SEOA 4151(SEL: SEP at 4:1 w/w; SE: OA at 5:1 w/w) and SEOA 9151(SEL: SEP at 9:1 w/w; SE: OA at 5:1 w/w). This may suggest that at the above surfactant ratio (SEL: SEP at 9:1 w/w), the particles of SEOA NS were coated by much thicker surfactant layers, more OA encapsulated and/or agglomerated moieties existed.

Morphology Determination by TEM

Morphology of SEOA NS was studied using transmission electronic microscopy (TEM). Figure 2 shows examples of the TEM photos of SEOA NS. The resulting OA particles were generally spherical in shape with a mean diameter of around 30–40 nm. TEM images show the particles presented as clustered agglomerates, encapsulated by distinct SE membrane, which appears like an "outer shell." This outer shell may function as steric barrier that had ensured the stability of OA NS.

Table III Stability of SEOA NS

Group	Original (%)	1 month relative concentration (%)	3 month relative concentration (%)
SEOA4151	100.00	82.52 ± 1.63 [e]	67.75 ± 0.56 [c, e, g]
SEPOA	100.00	86.32 ± 1.12 [e]	65.35 ± 0.70 [c, e, g]
SEOA2151	100.00	88.50 ± 1.81 [e]	82.24 ± 0.79 [a, b, g, h]
SELOA	100.00	88.27 ± 4.79 [e]	76.79 ± 0.33 [g]
SEOA91101	100.00	98.61 ± 4.44 [a, b, c, d, f, g, h]	88.21 ± 1.30 [a, b, f, g, h]
SEOA9151	100.00	84.69 ± 4.63 [e]	71.12 ± 1.52 [e, g]
SEOA9121	100.00	80.54 ± 2.97 [e]	44.88 ± 0.06 [a, b, c, d, e, f, h]
SEOA4121	100.00	87.06 ± 4.86 [e]	68.17 ± 0.70 [c, e, g]

Data represent three independent experiments, repeated in triplicates. Values are presented as means ± std. [a] significantly different compared with SEOA4151 ($p<0.05$). [b] significantly different compared with SEPOA ($p<0.05$). [c] significantly different compared with SEOA2151 ($p<0.05$). [d] significantly different compared with SELOA ($p<0.05$). [e] significantly different compared with SEOA91101 ($p<0.05$). [f] significantly different compared with SEOA9151 ($p<0.05$). [g] significantly different compared with SEOA9121 ($p<0.05$). [h] significantly different compared with SEOA4121($p<0.05$)

Table IV Stability of SEOA 4121 NS After Incubation with Rat Plasma

Time (h)	Relative Concentration (%)	Std (%)
0	100.00	0.00
1	96.94	9.63
2	88.99	1.95
4	89.91	10.38
24	83.94	5.05

Data represent three independent experiments, repeated in triplicates.

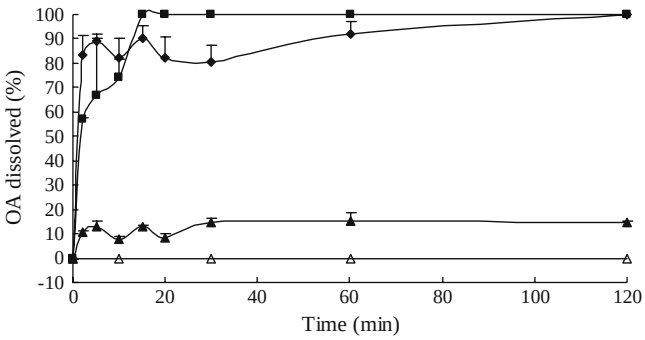

Fig. 3 Dissolution profiles of OA coarse suspension (▲) (suspended in N,N-DMAC:PEG400:Water at 2:4:1 v/v/v), SEOA 4121 NS (■), SEOA 4121 lyophilized powder(◆), and SEOA4121 NS in dialysis bag (△) in pH 7.4 phosphate buffer solution containing 1% sodium dodecyl sulfate (SDS) Data is presented as mean (%) ± std from three independent experiments repeated triplicate.

Free OA Equilibrium Aqueous Solubility at 25°C, SEOA NS Percent Encapsulation Efficiency, Saturation Solubility and Stability

After characterizing the size and morphology of the SEOA NS, we carried out studies whereby we (1) compared the EE% of NS prepared with either pure SE or mixture of different SE combined at predetermined weight ratios and (2) studied which formulation of these NS would give rise to the highest OA saturation solubility.

Since free OA equilibrium aqueous solubility was too low to be detected directly, the OA in equilibrium aqueous solution were freeze-dried and concentrated in methanol solution for HPLC detection. By our HPLC settings, OA was detected as a single peak with 6.8 min retention time, and equilibrium solubility of OA in water at 25°C was determined to be approximately 3.43 ± 0.11 (μg/mL), which is close to the value reported by Chen *et al.* (4.37 μg/mL) (11). SE did not have any effect on the separation of OA, and the elution peak of SE was merged with that of organic solvent in the early stage.

From Tables II and III, of all the SEOA NS, SEOA4121 NS had the highest OA saturation solubility at 1.89 mg/mL, which was almost 550-fold higher than free OA. Meanwhile, SEOA91101 NS has the highest percent encapsulation efficiency (79.29%) and highest stability.

According to Ostwald-Freundlich's equation, $\log(C_s/C_\alpha) = 2\sigma V/(2.303RT\rho r)$ (32), where C_s is the saturation solubility, C_α is the solubility of the solid consisting of large particles, σ is the interfacial tension of substance, V is the molar volume of the particle material, R is the gas constant, T is the absolute temperature, ρ is the density of the solid and r is the radius. By decreasing the particle size (r), NS can increase the saturation solubility of OA (C_s). Compared with a previous report by Chen *et al.* (11), the saturation solubility increase of 191- to 550-fold by SE-stabilized NS is much more effective than the 6-fold increase by polysorbate 80. This is the first report of making SEL- and SEP-stabilized NS.

From the chemical structure, SEL has shorter fatty acid chain than SEP, and according to the literature (33,34) and manufacturer, SEL is more hydrophilic than SEP with smaller logP value (1.5 to 3.2) and larger HLB value (13–15). To stabilize NS with the combination of SEL and SEP could exert advantageous properties of both more affinity to the hydrophobic drug and ability to more readily dissolve in aqueous solution. It would also be expected that this complementary nature of these two SEs should lead to the formation of a more stable NS. Indeed, when the surfactant-to-drug ratio (10:1) was kept constant for SELOA NS (SEL: OA at 10:1 w/w), SEPOA NS (SEP: OA at 10:1 w/w) and SEOA91101 NS (SEL: SEP at 9:1 w/w; SE: OA at 10:1 w/w), the three OA NS formulations all exhibited similar values in their mean particle sizes, encapsulation efficiencies and OA solubility. However, SEOA91101 NS was more stable in storage than SELOA and SEPOA (Table III).

The weight ratio of surfactant (SEL and SEP) to OA may have influenced the NS properties. Interestingly, when

Table V Stability of SEOA 4121 NS after inCubation in SGF and SIF

Media	Sample Time (h) 0.5	1	2	4	24
	Relative Concentration (%)				
pH 7.4	97.97 ± 1.13	97.60 ± 1.31	93.05 ± 0.38	93.05 ± 0.28	92.21 ± 1.13
pH 6.8	100.00 ± 0.36	99.62 ± 0.51	97.46 ± 1.13	94.97 ± 0.51	94.97 ± 1.13
pH 1.2	100.00 ± 6.68	98.42 ± 1.31	98.16 ± 0.47	95.71 ± 0.24	95.60 ± 0.85

Data represent three independent experiments, repeated in triplicates. Values are presented as means ± std.

the SEL: SEP weight ratios were kept constant and the amount of OA to SE was increased (from 10:1 to 2:1) for the three OA NS (SEOA 91101, SEOA9151, and SEOA9121), it resulted in an increase in the particle size (from 103.60 nm to 171.40 nm) and OA saturation solubility, but a decrease in the EE% (from 79.29% to 18.07%), and stability (Tables II and III). The findings indicated that adding OA brought about an increase in the

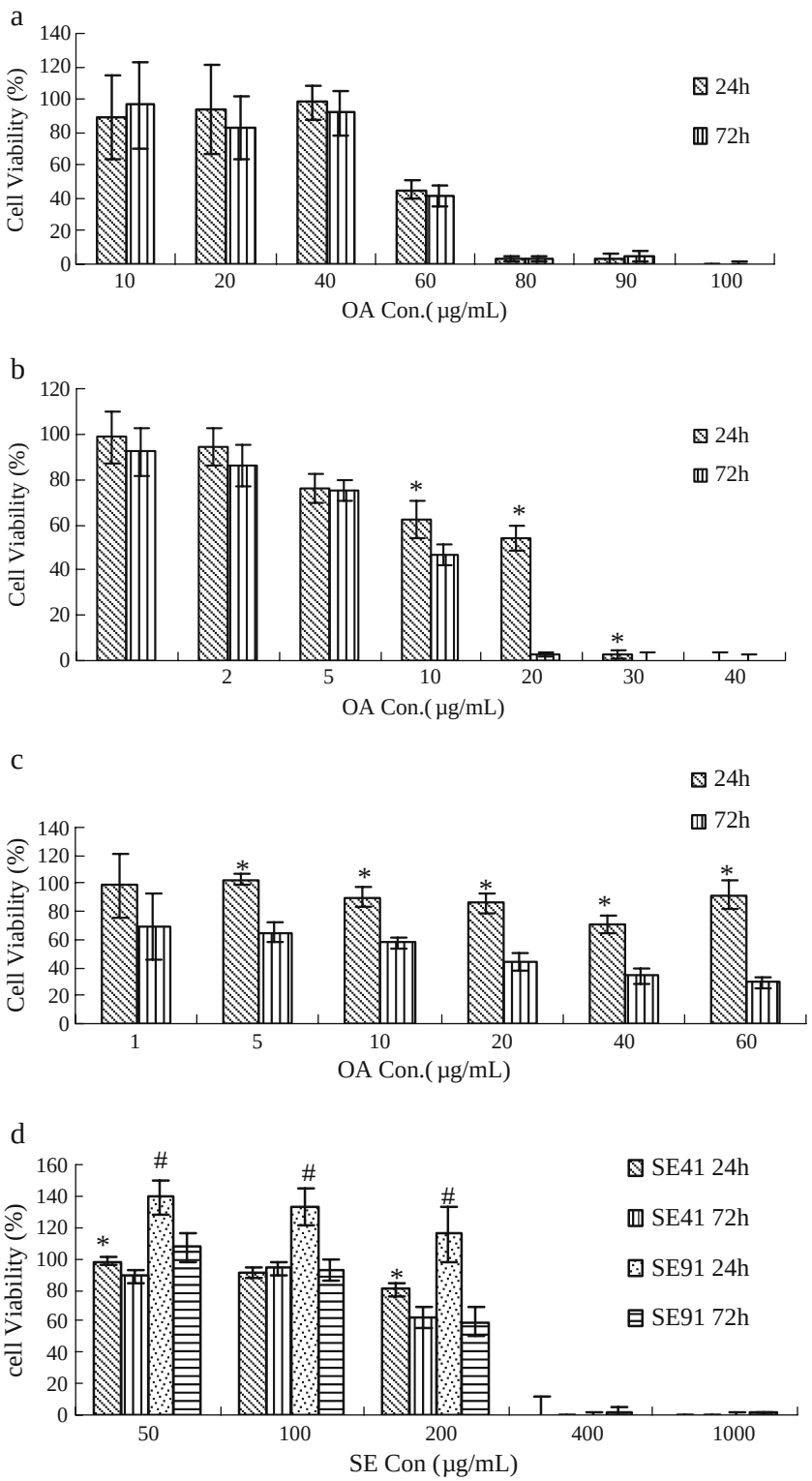

Fig. 4 Dose- and time-dependent growth inhibition of A549 cells by (**a**) free OA dissolved in media containing 0.05% DMSO, (**b**) SEOA91101 NS (*, $p < 0.05$ between 24 h and 72 h) (**c**) SEOA4121 NS(*, $p < 0.05$ between 24 h and 72 h) (**d**) blank SE 41 NS (SEL: SEP at 4:1 w/w) and blank SE 91 NS (SEL: SEP at 9:1 w/w) without OA. *, $p < 0.05$ between SE41 24 h and SE41 72 h; #, $p < 0.05$ between SE91 24 h and SE91 72 h. Data are presented as mean (μg/mL) ± std from three independent experiments repeated in quadruplicate.

saturation solubility of SE-stabilized OA NS, but it did not keep on increasing and the increase was at the expense of sacrificing encapsulation efficacy and stability. Since the amount of SE is constant among all the formulations, if the increase of OA beyond the stabilized ability of SE, it may probably cause the decrease in the EE% and stability.

Table III demonstrates the stability comparison among the SEOA NS. It was noticed that most of the NS were relatively stable (>80%) for 1 month of storage at 4°C but not quite stable for 3 months of storage. Among them, SEOA91101 NS was the most stable. Since SEOA 4121 NS was also stable in the first month storage and with the highest OA content, it was selected for further *in vitro* study and *in vivo* pharmacokinetics applications. From Table IV and Table V, either in mimic plasma situation or in SGF and SIF circumstances, SEOA4121 NS was shown to be relatively stable over the first 24 h *in vitro*.

In Vitro Dissolution

In order to determine whether the aim of improving the dissolution rate of OA was achieved, *in vitro* dissolution profiles of different OA formulations were determined (Fig. 3). The dissolution rate of OA coarse suspension (suspended in N, N-DMAC: PEG400: water at 2:4:1 v/v/v) was very low; only about 15% of the drug dissolved in 120 min. On the contrary, the SEOA 4121 NS either in suspension form or in lyophilized powder form both showed a great increase in the dissolution of OA over the coarse suspension; 100% of OA were dissolved in 120 min. According to Noyes–Whitney equation, $\frac{dW}{dt} = \frac{DA(C_s - C)}{L}$ (35), where $\frac{dW}{dt}$ is the rate of dissolution, A is the surface area of the solid, C is the concentration of the solid in the bulk dissolution medium, C_s is the concentration of the solid in the diffusion layer surrounding the solid, D is the diffusion coefficient, and L is the diffusion layer thickness.

An increase in saturation solubility (C_s) and decrease in particle size which led to increased surface area (A) can both enhance dissolution rate ($\frac{dW}{dt}$). Thus, formulating the poorly water-soluble drug as nanosize particles had a dramatic effect both on its saturation solubility and dissolution rate, and the bioavailability could be consequently increased.

The fast-dissolved OA from SEOA 4121 NS may exist as free molecular form or in NS form. Therefore, the dialysis bag, through which only free OA can pass, was needed to identify the different forms. From Fig. 3, by the dialysis method, no free OA was detected within 120 min in the dissolution medium. The findings indicated that most of the dissolved OA existed in the NS droplets and not as the free form.

Cytotoxicity of SEOA NS

In NS form, the saturation solubility of OA was increased from 3.43 μg/mL (free OA) to 660 μg/mL (SEOA91101 NS) and 1890 μg/mL (SEOA4121 NS), respectively. Owing to the increase in OA saturation solubility, the *in vitro* cytotoxicity to A549 cell lines as measured by MTT assay also increased. As shown in Fig. 4, formulation of SE-stabilized OA NS significantly increased the cytotoxicity of OA in both time- and dose-dependent manner (see data in Table VI). The 72 h IC_{50} dropped from 120 μM of free OA to 26 μM of SEOA4121 NS and 18 μM of SEOA91101 NS.

This enhanced cytotoxic effect was most likely to be due to the increased saturation solubility of OA rather than the surface-active effects of the sucrose-ester molecules. This contention was supported by the findings that treatment of A549 cells with drug-free NS resulted in a much less cytotoxic effect than when the cells were treated with drug-loaded SEOA NS.

Since the encapsulation efficiencies of SEOA 4121 NS and SEOA 91101 NS were 45.38% and 79.29%, respectively (Table II), without considering the loss of SE during preparation, the maximal yielding SE:OA ratios in SEOA 4121 NS and SEOA 91101 NS were 4.41: 1 ($\frac{2\times100\ \%}{1\times45.38\%}$) and 12.61: 1 ($\frac{10\times100\ \%}{1\times79.29\%}$), respectively.

At 72 h after the treatment, the IC_{50} of NS prepared with SEL: SEP weight ratio at 4:1 was 17.57 times that of SEOA4121 NS (more than 4.41 times). Similarly, the IC_{50} of NS prepared with SEL: SEP weight ratio at 9:1 was 19.02 times that of SEOA91101 NS at 24 h and 25.61 times at 72 h after treatment (more than 12.61 times).These observations indicated that the cellular toxic effect of SEOA NS was mainly derived from the nanoparticulate drug rather than from the surfactants that formed and stabilized the NS (see Table VI).

Table VI IC_{50} Comparison of SEOA NS and Free OA

IC_{50} values were calculated by nonlinear regression (curve fit) of cytotoxicity data in Fig. 4a-d using sigmoidal dose response (variable slope) equation, Graphpad Prism software.

Group	24 h (μg/mL)	72 h (μg/mL)
Free OA	59. 67 ± 1.01(130.00 μM)	56.75 ± 1.02 (120.00 μM)
SEOA91101 NS	13.10 ± 1.08 (28.00 μM)	8.30 ± 1.05 (18.00 μM)
SEOA4121 NS		12.10 ± 1.09 (26.00 μM)
SE(4:1) NS	211.40 ± 1.03	217.10 ± 1.03
SE (9:1) NS	249.10 ± 1.06	212.60 ± 1.00

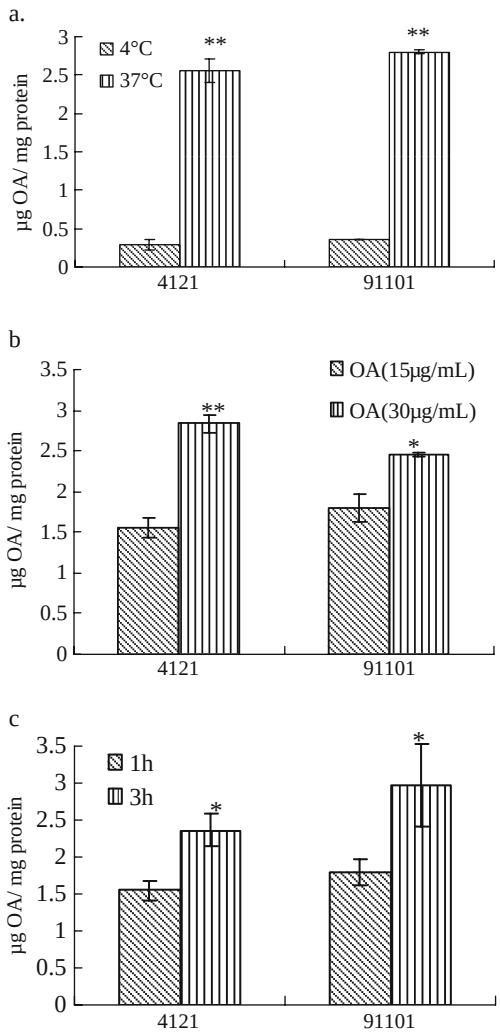

Fig. 5 Cellular uptake of SEOA NS. The cellular uptake of SEOA NS is (**a**) temperature-dependent, (**b**) concentration-dependent, and (**c**) time-dependent. Data are presented as mean (μg OA/mg protein) ± std from three independent experiments repeated triplicate. *, $p < 0.05$; **, $p < 0.01$.

Cellular Uptake of SEOA NS

LC-ESI-MS/MS was used to quantify the cellular OA concentration. In the settings used, the GA (internal standard) and OA peaks emerged at around 2.20 min and 4.29 min, respectively.

The optimal temperature, around 37°C, is crucial for intracellular metabolism (36). When A549 cells were incubated at 4°C, cellular uptake of SEOA NS was significantly lower than that at 37°C (Fig. 5a). The results suggested that uptake of SEOA NS into the A549 cells required a higher temperature. The effects of NS concentration and incubation time on uptake of NS by the A549 cells were studied by treating the cells with different OA concentrations (15 μg/mL or 30 μg/mL) for the same incubation time (1 h), or the same OA concentration (15 μg/mL) for different incubation times (1 h or 3 h). As shown in Fig. 5b and c, significantly higher uptake of SEOA NS compared to control was observed when the cells were treated with a higher concentration of OA or longer incubation time. This may explain that SEOA 4121 NS and SEOA 91101 NS had better cytotoxicity effect than free OA and they yielded better effect after 72 h incubation.

From the *in vitro* dissolution study, it was demonstrated that most of the dissolved OA existed as NS droplets and not as the free molecular form. This may imply that the uptake of OA was mainly in NS form, and by endocytosis. However, more experiments are needed to verify the hypothesis and to explore the underlying mechanism and study the *in vivo* bioeffect of SEOA NS.

SEOA NS Pharmacokinetics Profile

Recovery, Precision and Accuracy in Analysis of Plasma Samples

The LC-ESI-MS/MS system gave a well-defined separation between the drug, internal standard and endogenous components. The absolute recoveries of OA from the plasma were more than 84.30%, indicating that most of OA in the plasma sample was extracted. The within-day and between-day precision (R.S.D.%, $n=5$) for the OA-spiked control samples at 40, 100, and 800 ng/mL levels varied between 2.50 and 11.20. The corresponding within-day and between-day accuracy (bias%, $n=5$) ranged between −12.00 and 6.50 (Table VII)

Table VII The Recovery, Precision and Accuracy ($n=5$) of the Assay Method

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	R.S.D (%)	Precision (R.S.D%)	Accuracy (Bias%)	Precision (R.S.D%)	Accuracy (Bias%)
40	86.40	6.50	5.60	-8.30	11.20	-12.00
100	94.70	2.10	4.90	5.90	4.00	3.80
800	84.30	1.30	2.50	6.50	1.90	-5.80

Bias% = [(concentration added-concentration found)/concentration added] × 100

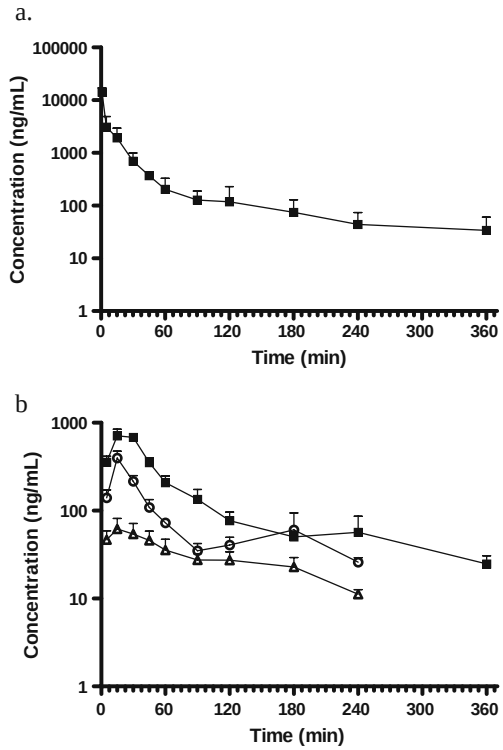

Fig. 6 Mean plasma concentration-time profiles comparison of OA in rats after (**a**) IV injection at 2 mg/kg (■, $n=5$), (**b**) oral administration of OA NS at 10(○, $n=5$),20 (■, $n=5$) mg/kg doses and oral administration of OA coarse suspension (△, $n=5$, control) at 20 mg/kg dose. Vertical bars represent standard deviation.

Pharmacokinetics Results After Intravenous Administration

Figure 6a shows the pharmacokinetics results of OA following single IV bolus dose (2 mg/kg) of NS (Group 1). It demonstrated that the plasma concentration of OA declined rapidly over the first hour and was followed by a slow drop from 2 h onwards. The maximum plasma concentration (C_{max}) was high (21.98 ± 5.79 μg/mL), and plasma elimination half-life ($T_{1/2}$) was found to be 88.41 ± 16.15 min. AUC and Cl values were calculated as 121.49 ± 27.37 μg.min/mL and 17.11 ± 3.67 mL/min/kg, respectively.

Pharmacokinetics Results After Oral Administration

The plasma pharmacokinetic profiles and the pharmacokinetic parameters following single oral dose of SEOA NS (10 and 20 mg/kg) and OA coarse suspensions (20 mg/kg) are shown in Fig. 6b and Table VIII. In all cases, OA in NS groups resulted in a significantly ($p<0.05$) higher C_{max} than the suspension formulations. However, there were no significant differences ($p>0.05$) in T_{max} and $T_{1/2}$. In all groups, the NS group (Groups 2 and 3) had significantly higher bioavailability (F%) values (6 to 7 folds) than the suspension group (Group 4) ($p<0.05$), while between 10 mg/kg and 20 mg/kg NS groups, there were no statistically significant differences ($p>0.05$). These findings indicated that the dosage form of OA can affect the extent of its oral absorption. Figure 6b indicates that the plasma concentration of OA declined rapidly over the first stage and was followed by a second peak at 2 h, 3 h and 4 h for Group 4, Group 3 and Group 2, respectively, which was probably due to the enterohepatic recirculation.

Oral bioavailability is dependent on various factors including the stability of a drug in the gastrointestinal (GI) tract, its aqueous solubility, its permeability through the intestinal membrane and first-pass elimination (37). A report by D.W. Jeong *et al.* suggested that the low oral bioavailability of OA suspension may be due to its poor solubility, poor gastrointestinal absorption (by Caco-2 cell permeability model), and hepatic first-pass metabolism (28), while our *in vivo* pharmacokinetic study indicated that the bioavailability of OA was highly enhanced by the NS formulation. Among all the NS formulations, the selected SEOA 4121 NS had encapsulated the highest OA concentration, demonstrated to possess relatively good *in vitro* stability in rats' plasma, SGF and SIF for 24 h, and shown increased *in vitro* dissolution rate. The NS formulation not only enhanced the saturation solubility and dissolution rate of OA but also augmented its bioavailability and provided the protection against breakdown in various media.

Table VIII Oral Pharmacokinetics Profiles of SEOA NS and Coarse Suspension

Parameter Formulation	Group 2 NS	Group 3 NS	Group 4 Suspension
Dose (mg/kg)	10.00	20.00	20.00
AUC (μg.min/mL)	21.35 ± 3.89 *, ***	44.06 ± 7.25 *, **	6.74 ± 3.42 **, ***
T_{max} (min)	13.00 ± 4.47	21.00 ± 8.22	13.00 ± 4.47
C_{max} (ng/mL)	397.35 ± 170.19 *, ***	817.19 ± 255.21 *, **	69.95 ± 42.71 **, ***
$T_{1/2}$ (min)	76.38 ± 38.19	78.06 ± 29.21	102.10 ± 16.56
F (%)	3.51 ± 0.64	3.63 ± 0.60	0.56 ± 0.28 **, ***

Data are presented as mean ± std, $N=5$.

*$p<0.05$ between Groups 2 and 3; **$p<0.05$ between 3 and 4; ***$p<0.05$ between 2 and 4

CONCLUSION

NS of OA can be prepared by emulsion/organic solvent evaporation method using SEL and SEP as stabilized surfactants. Diameters of most SEOA NS were around 100 nm. The different weight ratio of SEL to SEP and SE to OA influenced the characteristics of SEOA NS. These particles usually formed clustered agglomerates that were covered by distinct SE coating on the periphery. Preparation of OA as NS increased its saturation solubility considerably. With huge surface area-to-volume ratio, the saturation solubility of resultant SEOA particles ranged from 0.66 mg/mL (SEOA91101 NS) to 1.89 mg/mL (SEOA4121 NS), which were of 191- to 550-fold increase over free OA, respectively. SEOA 4121 NS increased the OA dissolution rate markedly. Most of the dissolved OA exists in the NS droplets but not in the free form. Formulation of OA as NS significantly and substantially increased the cytotoxicity of OA. Both SEOA91101 and SEOA4121 reduced the proliferation rate of A549 cell lines to a much greater extent than free OA. This increased activity was attributed to the nanonized drug and not the SE. Cellular uptake of SEOA NS by A549 cells was shown to be a temperature-, concentration- and time-dependent process. NS of OA not only increased its saturation solubility and dissolution rate to a great extent but also changed the pharmacokinetic profile of OA after oral administration. Oral bioavailability of OA was enhanced by the NS formulation, which showed much higher C_{max} and F (6 to 7 times increase) than those of the coarse suspension group. Dose-independent pharmacokinetics of OA was observed after oral administration at the range of 10 to 20 mg/kg. However, the cellular uptake mechanism and *in vivo* bioeffect of SEOA NS still needed further research.

ACKNOWLEDGMENTS

This project is partially funded from GEA-NUS grant (N-148-000-008-001). The authors would like to thank Associate Professor HO Chi Lui, Paul, Department of Pharmacy, National University of Singapore, Singapore for his support in the DLS measurement. We thank Madam Loy Gek Luan and Mr. Chong Ping Lee, Department of Biological Sciences, National University of Singapore, Singapore for their support in EM experiments. We would also like to thank Compass Foods Pte. Ltd., Singapore, for the generous supply of SE.

REFERENCES

1. Rasenack N, Hartenhauer H, Muller BW. Microcrystals for dissolution rate enhancement of poorly water-soluble drugs. Int J Pharm. 2003;254:137–45.
2. Liang L, Tajmir-Riahi HA, Subirade M. Interaction of beta-lactoglobulin with resveratrol and its biological implications. Biomacromolecules. 2008;9:50–6.
3. Betancor-Fernandez A, Perez-Galvez A, Sies H, Stahl W. Screening pharmaceutical preparations containing extracts of turmeric rhizome, artichoke leaf, devil's claw root and garlic or salmon oil for antioxidant capacity. J Pharm Pharmacol. 2003;55:981–6.
4. Eibes G, Moreira MT, Feijoo G, Lema JM. Enzymatic degradation of low soluble compounds in monophasic water: solvent reactors. Kinetics and modeling of anthracene degradation by MnP. Biotechnol Bioeng. 2008;100:619–26.
5. Kinder Jr FR, Versace RW, Bair KW, Bontempo JM, Cesarz D, Chen S, *et al.* Synthesis and antitumor activity of ester-modified analogues of bengamide B. J Med Chem. 2001;44:3692–9.
6. Thiericke R. Drug discovery from nature: automated high-quality sample preparation. J Autom Methods Manag Chem. 2000;22:149–57.
7. Stefaniak AB, Chipera SJ, Day GA, Sabey P, Dickerson RM, Sbarra DC, *et al.* Physicochemical characteristics of aerosol particles generated during the milling of beryllium silicate ores: implications for risk assessment. J Toxicol Environ Health A. 2008;71:1468–81.
8. Lanke SS, Gayakwad SG, Strom JG, and J. D'Souza M. Oral delivery of low molecular weight heparin microspheres prepared using biodegradable polymer matrix system. J Microencapsul. 2008;1–8 .
9. Chingunpitak J, Puttipipatkhachorn S, Chavalitshewinkoon-Petmitr P, Tozuka Y, Moribe K, Yamamoto K. Formation, physical stability and *in vitro* antimalarial activity of dihydroartemisinin nanosuspensions obtained by co-grinding method. Drug Dev Ind Pharm. 2008;34:314–22.
10. Zhang D, Tan T, Gao L, Zhao W, Wang P. Preparation of azithromycin nanosuspensions by high pressure homogenization and its physicochemical characteristics studies. Drug Dev Ind Pharm. 2007;33:569–75.
11. Chen Y, Liu J, Yang X, Zhao X, Xu H. Oleanolic acid nanosuspensions: preparation, *in-vitro* characterization and enhanced hepatoprotective effect. J Pharm Pharmacol. 2005;57:259–64.
12. Pauland BK, Mitra RK. Percolation phenomenon in mixed reverse micelles: the effect of additives. J Colloid Interface Sci. 2006;295:230–42.
13. Glatter O, Orthaber D, Stradner A, Scherf G, Fanun M, Garti N, *et al.* Sugar-Ester nonionic microemulsion: structural characterization. J Colloid Interface Sci. 2001;241:215–25.
14. Honeywell-Nguyen PL, Frederik PM, Bomans PH, Junginger HE, Bouwstra JA. Transdermal delivery of pergolide from surfactant-based elastic and rigid vesicles: characterization and *in vitro* transport studies. Pharm Res. 2002;19:991–7.
15. Grossiord JL, Thevenin MA, Poelman MC. Sucrose esters/cosurfactant microemulsion systems for transdermal delivery: assessment of bicontinuous structures. Int J Pharm. 1996;137:177–86.
16. Kuntsche J, Koch MH, Steiniger F, Bunjes H. Influence of stabilizer systems on the properties and phase behavior of supercooled smectic nanoparticles. J Colloid Interface Sci. 2010;350:229–39.
17. Klang V, Matsko N, Zimmermann AM, Vojnikovic E, Valenta C. Enhancement of stability and skin permeation by sucrose stearate and cyclodextrins in progesterone nanoemulsions. Int J Pharm. 2010;393:152–60.
18. Takegami S, Kitamura K, Kawada H, Matsumoto Y, Kitade T, Ishida H, *et al.* Preparation and characterization of a new lipid nano-emulsion containing two cosurfactants, sodium palmitate for droplet size reduction and sucrose palmitate for stability enhancement. Chem Pharm Bull (Tokyo). 2008;56:1097–102.
19. Ullrich S, Metz H, Mader K. Sucrose ester nanodispersions: microviscosity and viscoelastic properties. Eur J Pharm Biopharm. 2008;70:550–5.

20. Arica Yegin B, Benoit JP, Lamprecht A. Paclitaxel-loaded lipid nanoparticles prepared by solvent injection or ultrasound emulsification. Drug Dev Ind Pharm. 2006;32:1089–94.
21. Lippacher A, Muller RH, Mader K. Semisolid SLN dispersions for topical application: influence of formulation and production parameters on viscoelastic properties. Eur J Pharm Biopharm. 2002;53:155–60.
22. Liu J, Liu Y, Parkinson A, Klaassen CD. Effect of oleanolic acid on hepatic toxicant-activating and detoxifying systems in mice. J Pharmacol Exp Ther. 1995;275:768–74.
23. Ovesna Z, Vachalkova A, Horvathova K, Tothova D. Pentacyclic triterpenoic acids: new chemoprotective compounds. Minireview. Neoplasma. 2004;51:327–33.
24. Konopleva M, Tsao T, Estrov Z, Lee RM, Wang RY, Jackson CE, *et al.* The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. Cancer Res. 2004;64:7927–35.
25. Singh GB, Singh S, Bani S, Gupta BD, Banerjee SK. Anti-inflammatory activity of oleanolic acid in rats and mice. J Pharm Pharmacol. 1992;44:456–8.
26. Gao D, Li Q, Li Y, Liu Z, Liu Z, Fan Y, *et al.* Antidiabetic potential of oleanolic acid from Ligustrum lucidum Ait. Can J Physiol Pharmacol. 2007;85:1076–83.
27. Ji HY, Shin BS, Jeong DW, Park EJ, Park ES, Yoo SD, *et al.* Interspecies scaling of oleanolic acid in mice, rats, rabbits and dogs and prediction of human pharmacokinetics. Arch Pharm Res. 2009;32:251–7.
28. Jeong DW, Kim YH, Kim HH, Ji HY, Yoo SD, Choi WR, *et al.* Dose-linear pharmacokinetics of oleanolic acid after intravenous and oral administration in rats. Biopharm Drug Dispos. 2007;28:51–7.
29. Song M, Hang TJ, Wang Y, Jiang L, Wu XL, Zhang Z, *et al.* Determination of oleanolic acid in human plasma and study of its pharmacokinetics in Chinese healthy male volunteers by HPLC tandem mass spectrometry. J Pharm Biomed Anal. 2006;40:190–6.
30. Gao Z, Maloney DJ, Dedkova LM, Hecht SM. Inhibitors of DNA polymerase beta: activity and mechanism. Bioorg Med Chem. 2008;16:4331–40.
31. Das S, Lin HS, Ho PC, Ng KY. The impact of aqueous solubility and dose on the pharmacokinetic profiles of resveratrol. Pharm Res. 2008;25:2593–600.
32. Patravale VB, Date AA, Kulkarni RM. Nanosuspensions: a promising drug delivery strategy. J Pharm Pharmacol. 2004;56:827–40.
33. Sucrodet - Compound Summary (CID 5360814) http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=5360814&loc=ec_rcs.
34. SUCROSE MONOLAURATE - Compound Summary (CID 4182596) http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4182596&loc=ec_rcs.
35. Noyesand A, Whitney W. The rate of solution of solid substances in their own solutions. J Am Chem Soc. 1897;19:930–4.
36. Carey HV, Andrews MT, Martin SL. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev. 2003;83:1153–81.
37. Chan OH, Stewart BH. Physicochemical and drug-delivery considerations for oral drug bioavailability. Drug Discov Today. 1996;1:12.