EXPERT OPINION

Liposomal quercetin: evaluating drug delivery *in vitro* and biodistribution *in vivo*

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Objective: The drug-loaded PEGylated nanomaterials have shown effective cell-killing *in vitro,* but to the best of authors' knowledge there have been no reports of successful drug delivery *in vitro* and *in vivo* using polyethyleneglycol-2000-distearoyl phosphatidyl ethanolamine (PEG2000-DSPE) nanomaterials loaded with unmodified drug molecules, such as quercetin (QUE). In this study, it remained an open question as to whether such formulations could prove effective in *vitro* and *in vivo,* and to study the distribution and clearance of PEG-DPSE-ylated lipid-based quercetin nanoliposomes (PEG2000-DPSE-QUE-NLs) as delivery vehicles for the anticancer drug *in vitro* and *in vivo.*

Research design and methods: PEG-DPSE layers were attached to QUE-NLs, dispersed in aqueous media and characterized using TEM and HPLC/UV spectroscopy. Tumor cell killing efficacy was assessed *in vitro* using MTT and trypan blue exclusion assays, and the distribution and clearance pathways, as well as repeated administration in rats, were studied by HPLC spectroscopy.

Results: PEG2000-DPSE-QUE-NLs were efficiently dispersed in aqueous media compared with controls, and PEGylated (PEG2000-DPSE) NLs were found to be effective drug delivery vehicles when simply loaded with QUE. The plasma QUE concentration decreased significantly ($p < 0.05$) after repeated administration of PEG2000-DSPE liposomal QUE. There was a slight ABC phenomenon with the PEG2000-DSPE-modified QUE liposomes.

Conclusion: The QUE/PEG2000-DPSE formulation was more effective than QUE *in vitro* on inhibiting the growth of glioma cancer cells. This work demonstrates that nanomaterials (PEG2000-DPSE) are effective drug delivery vehicles *in vivo* as tumor-targeted drug carriers.

Keywords: biodistribution, cancer, drug delivery, nanomaterials, PEG2000-DPSE, quercetin, targeting

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1. Introduction

Many drug candidates have obtained high therapeutic efficacy but have low water solubility, which poses a challenge for *in vivo* delivery [1]. The authors are seeking to develop a safe, modular drug delivery platform that can be loaded with unmodified hydrophobic drugs and thereby enhance their delivery efficiency. In this paper, the authors describe the initial step toward this goal by demonstration of drug delivery systems (DDS) for the hydrophobic drug quercetin (QUE) as potential chemopreventer in considering its involvement in the suppression of many tumor-related processes including oxidative stress, apoptosis, proliferation, metastasis and inhibition of mutant p53 expression [2], and whether it enhances death-receptor-mediated apoptosis in glioma cells [3]. QUE is usually considered an excellent free radical

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scavenging antioxidant owing to the high number of hydroxyl groups and conjugated orbitals by which QUE can donate electrons or hydrogen, and scavenge H_2O_2 and superoxide anion [4]. QUE contains both antioxidant and pro-oxidant properties; thus, in different cellular models low concentrations of QUE induce cell proliferation and increase the antioxidant capacity of the cells, whereas higher concentrations of QUE decrease antioxidant capacity and thiol content, ultimately causing cell death [5]. QUE has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells [6]. However, QUE is low solubility in water, which hinders its anticancer activity. Therefore, development of novel preparations that enhance solubility of QUE to exert its bioactivity in inhibiting tumor is of great significance.

Numerous efforts have been made to find alternative excipients for QUE to increase solubility and drug loading [7,8]. Many of the strategies incorporate QUE into the nanomaterials in order to increase solubility in aqueous solutions [9-13]. Probably, the most well-established approach of this strategy is liposomes, where the drug is sequestered inside the particles [14]. In some cases, this can be a limiting factor, as the drug is never or very poorly released from the liposomes [15]. Owing to its rich formulation history, widespread use, generic availability and continued need for the anticancer drug as drug delivery vehicles, QUE was selected as the initial hydrophobic drug to evaluate for sequestration in this study.

There is a continuing interest in a variant of this strategy of increasing blood circulation time or even control the drug release profile. These are a good choice for this approach as hydrophobic drug molecules are sequestered on the hydrophobic surfaces of polyethyleneglycol (PEG)-distearoyl phosphatidyl ethanolamine (DPSE)-ylated nanomaterials for they are inherently hydrophobic. Several PEG-DPSE-ylated nanomaterials have been shown to successfully sequester hydrophobic compounds [16-19]. PEG–DSPE conjugate polymers have been studied in an attempt to achieve sustained release of drug [20-22]. The mechanisms of drug release were found to be either directly from the micelles by diffusion or due to the dissociation of the micelles into free polymeric chains and hydrolysis of the liable bonds [23-25].

These drug-loaded PEG-DPSE-ylated nanomaterials have shown effective cell killing *in vitro*, but to the best of authors' knowledge there have been no reports of successful drug delivery *in vitro* and *in vivo* using PEG-DPSE-ylated nanomaterials loaded with unmodified drug molecules such as QUE. Until this study, it remained an open question as to whether such formulations could prove effective in *vitro* and *in vivo*.

In general, some nano-DDS increase dispersibility or solubility of drugs and lower toxicity [26-28]. Block copolymers [29], such as poly(L-amino acid), poly(ester) and pluronics, have been used in DDS loaded with doxorubicin, methotrexate, cisplatin and paclitaxel [30-33]. One of the most promising coating materials for such purposes is PEG because of its ability to provide a steric barrier for protein adsorption, which results in reduced uptake by macrophages of the RES (reticuloendothelial system) and ultimately increasing serum half-life [34,35]. In addition, nanoparticles attached with PEG had longer blood circulation times than nanomaterials without surface-attached PEG [36]. PEGylation of nanomaterials not only makes the hydrophobic nanoparticles more soluble, but it also increases their hydrodynamic size and decreases immunogenicity [37].

What remains less clear is whether such a configuration affects the surface-binding capacity of QUE nanoparticles for chemotherapeutic drugs. For DDS to be translated to cancer therapy, low toxicity to normal tissues combined with high potency against cancer is needed [38]. The authors previously demonstrated that quercetin nanoliposomes (QUE-NLs) enhanced ROS (reactive oxygen species) accumulation, which was accompanied by increased cytochrome c and caspase-3 protein levels and killed C6 glioma cancer cells *in vitro* [39]. In this article, the authors investigate the influence of attached PEG2000-DPSE on the performance of QUE DDS. Specifically, investigations of *in vivo* distribution, clearance of PEG-DPSE-QUE-NPs and efficacy for targeted tumor therapy *in vivo* are reported.

In this article, the authors explore the application of PEG-DPSE to prepare QUE-loaded liposomes, investigate their physicochemical properties and the *in vitro* release profiles of desired nanoparticles, and clearance of the biodistribution and antitumor efficiency of desired nanoparticles interacting with cancer cells as drug delivery vehicles for the anticancer drug *in vitro* and *in vivo*.

2. Materials and methods

2.1 Materials and reagents

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG2000-DSPE) was obtained from Japan Oil & Fat Co. Ltd. (Tokyo, Japan); Poloxamer 188 from BASF Aktiengesellschaft, Germany; cholesterol and soy lecithin from Shanghai Youngsun Foods Co., Ltd. (Shanghai, China); Tween 80 from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); glyceryl behenate (ATO) were from Gattefosse S.A. (Cedex, France); QUE from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China); 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 3,3′dihexylocarbocyanine iodide (DiOC6(3)) and Indo-1 AM (acetoxymethyl ester derivative of Indo 1) were purchased from BestBio Biotechnologies Co., Ltd. (Shanghai, China); Roswell Park Memorial Institute 1640 medium (RPMI 1640), penicillin-streptomycin, trypsin-EDTA and fetal bovine serum (FBS) were obtained from GIBCO BRL (Invitrogen Corp, Carlsbad, CA, US). C6 glioma cells were purchased from American Tissue Culture Collection (Rockville, MD, US), cells were grown as recommended by ATCC at 37°C, 5% CO_2.

2.2 Preparation of QUE-NLs

QUE-NLs were prepared according to an established method through emulsification–evaporation and low temperature curing preparation. Aqueous phase was made by dissolving Poloxamer 188 and Tween 80 (1:1, w/v) in pure water and maintained in water bath at 75°C and oil phase was mainly made of glyceryl behenate (ATO), soy lecithin and cholesterol (1:2:1, w/w/w). Glyceryl behenate and cholesterol were melted in water bath at 80°C. QUE 1% (w/v) and soy lecithin were dissolved in the ethanol–acetone mixed solvent (1:1, v/v) to obtain a weight ratio of soy lecithin to QUE of 10:1. QUE and soy lecithin were then dissolved in the oil phase, and then oil phase of the aforementioned mixed solvent was injected into aqueous phase through a plastic needle tubing (internal diameter 0.45 μm, administration rate 2 ml/min) under mechanical agitation at 1000 rpm. After stirring for 2 h, the liposomal suspension was cured at low temperature of 0 ~ 4°C under mechanical agitation at 600 rpm. Then, the suspension was filtered through a dialysis tubing to remove the non-incorporated drug. Briefly, QUE-NLs were injected into dialysis tubing and the dialysis tubing was swished in the mannitol solution for dialyzing three times to remove free QUE, 2 h in the first and the second, 12 h in the third time at 0 ~ 4°C, respectively. Finally, the purification of QUE-NLs were obtained and stored at 0 ~ 4°C. The control liposomes were also prepared by the same method without adding QUE at any stage of the preparation.

2.3 PEG2000-DPSE-coated loading with QUE-NLs

PEG2000-DPSE dissolved in methanol (5 mg in 1 ml) was added dropwise into QUE-NLs system with a rapidly stirring (1000 rpm/min). The mixture was stirred for 30 min and using a bath sonicator for 30 min, at which time, the solution was translucent and brown/black. To remove most of the methanol, the solution was concentrated on a rotary evaporator at 70°C to 3 ml. After concentration, the PEG2000-DPSE-coated loading with quercetin nanoliposomes (PEG2000-DPSE-QUE-NLs) solution remained translucent. Finally, the solution was diluted back to the original volume (5 ml) with deionized water to give a final concentration of 1 mg/ml of QUE. Before applying in cell experiment, the prepared QUE-NLs were well distributed in RPMI-1640 medium containing 10% (v/v) heating activated FBS by using ultrasound treatment in order to obtain a PEG2000-DPSE-QUE-NL suspension. Control formulations were prepared using the same procedure but without PEG2000-DPSE.

2.4 Characterization

The polydispersity index (PI) and size distribution were determined by photon correlation spectroscopy using laser particle analyzer. (Rise 2208; Shandong Rise Optics Co., Ltd., Shandong, China). The zeta potential was analyzed using a microscopic electrophoresis system (DXD-II; Jiangsu Optics Co., Ltd., Jiangsu, China) at 25°C. The morphology of the QUE-loaded NLs was performed under transmission electron microscopy (TEM-1200EX; JEOL, Tokyo, Japan). UV data were collected from a Purkinje General T90 UV-Vis Spectrophotometer and samples were contained in 1 ml quartz cuvettes. Samples were dissolved in deionized water. The PEG2000-DPSE-QUE-NL core concentration of each sample was determined by the absorbance at 365 nm using the experimentally determined extinction coefficient of 0.0101 l/mg.

2.5 Entrapment efficiency and drug loading

The free drugs were separated from QUE liposomal formulations using a centrifugation technique for measurement of entrapment efficiency (EE). Briefly, PEG2000-DPSE-QUE-NLs or QUE-NLs were dissolved in 1.0 ml purified water and were centrifuged at 16000 rpm for 15 min, and supernatant containing free drugs were collected and adjusted to a volume of 10 ml by ethanol for further analysis. The 1.0 ml of suspension adjusted to a volume of 10 ml by ethanol was added to 10% Triton X-100–ethanol solution 0.5 ml for 5 min to break down the liposomal formulations and dissolve the QUE. The suspension was centrifuged at 16,000 rpm for 10 min. The loading content and EE of PEG2000-DPSE-QUE-NLs were performed using DIONEX Utimate-3000 high performance liquid chromatographic (HPLC) system with a dual λ absorbance detector, the reading obtained at 254 nm was used. A Diamond C18 column was used. A flow of 1.0 ml/min was used for the eluent, which was 30% methanol/20% acetonitrile/50% (5%) phosphoric acid from 0 to 15 min. The loading content and EE measured by HPLC were calculated using the following equations:

Loading content (%) = (weight of drug in the nanoparticles/weight of nanoparticles) × 100%

EE (%) = (weight of drug in the nanoparticles/weight of the feeding drug) × 100%.

2.6 *In vitro* release

QUE liposomal formulations (10 mg) were dispersed into 2 ml of 10% (v/v) human plasma in phosphate buffered saline (PBS). Each suspension was incubated with gentle shaking at 37°C throughout the experiment. At designated intervals, an aliquot (500 μl) was withdrawn and the supernatant was separated from the liposomal formulations by centrifugation at 5000 × *g* for 5 min at 4°C. After collecting the supernatant, the pellet of nano/microspheres was resuspended in the same volume of fresh release medium (500 μl), and then returned to the sample suspension. The amounts of QUE in the supernatant were determined with HPLC, as described above.

2.7 *In vitro* effect on the growth of glioma cancer cells

2.7.1 *MTT assay*

Cell viability was evaluated by the MTT assay. C6 glioma cells were seeded into a 96-well microplate at a density of 1×10^5 cells/well. After growth to 60 – 70% confluence, cells were treated with varying concentrations of PEG2000-DPSE-QUE-NLs. In addition, C6 glioma cells were exposed to QUE-NLs, free QUE and for 12 h, and control liposomes

further incubated for 24 h in drug-free medium before cytotoxicity determination.

Kinetics of C6 glioma cells killing were further detected at 4, 12, 24 and 48 h after treatment with 50, 100, 200 and 400 μM of PEG2000-DPSE-QUE-NLs, QUE-NLs and QUE. Cells were then washed once with PBS, pH 7.4 and incubated with fresh complete DMEM (Dulbecco's Modified Eagle Medium) medium (100 μl/well), MTT solution (25 μl of 2 mg/ml) was added in each well. After incubation at 37°C for 2 h, the media was removed by careful aspiration to avoid suctioning of the cells. Then, blank liposomes (100 μl) were added to the well as the control group. Finally, the plates were analyzed using an ELISA (enzyme-linked immunosorbent assay) plate reader at a wavelength of 570 nm to measure optical density (OD) of each well, which proportionate to number of viable cells in each well. The percent of viable cells relative to control was obtained by dividing the average OD for the treated wells by the OD for the control wells.

Cell surviving rate (%) = OD value of treated group/OD value of control group × 100%.

Cell inhibition rate (%) = (1 – OD value of treated group/OD value of control group) × 100%.

When the cell-surviving rate with the doses was plotted, the 50% inhibitory concentration (IC_{50}) of azurin was calculated by modified Kaber method as follows:

$$IC_{50} = 1_g^{-1}[Xm - I(\sum P - 0.5)]$$

where Xm = the logarithm of maximal dose, P = cell inhibition rate (expressed as a decimal), ΣP = the sum of every group on the inhibition, I = the logarithm of dose ratio between each group of two consecutive numbers.

2.7.2 *Cell viability*

Cell viability was also assessed using the trypan blue exclusion assay. After C6 glioma cells were grown to 70 – 80% confluence and treated with 50 – 400 μM PEG2000-DPSE-QUE-NLs for 12, 24, 36, 48 h, 100 μl of a 0.4% solution of trypan blue in PBS were added to 100 μl of cell suspension. Then, blank liposomes (100 μl) were added to the well as the control group. The aliquots were loaded onto a hemocytometer for the live- and dead-cell counts. Percent of cell survival was calculated by dividing the value obtained for the treated sample by the value obtained for the untreated sample.

2.8 Biodistribution and pharmacokinetic studies

Pharmacokinetic and biodistribution studies of free QUE and drug formulated in the liposomes were compared in Sprague-Dawley (SD) rats (200 ~ 220 g, 10 weeks old, Experimental Animal Center, Hubei Medical University, Hubei, China).

2.8.1 *Biodistribution in rats*

For each material, SD rats were randomized in groups of six to be used over the following ten time points (0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96). Each animal was administered intragastrically with drug being studied in that group at 25 mg/kg body weight, either PEG2000-DPSE-QUE-NLs or QUE-NLs (1.0 mg concentration of QUE, a concentration equivalent to that of the QUE in the other samples). QUE was treated as the control group. After administration, each animal was housed in cages that allowed for freedom of movement and natural postural position. All animals were given access to food and water. For each group and time point, three animals were euthanized and terminal blood samples were collected and various organs were harvested (brain, liver, heart, kidneys, spleen and lungs) and placed into pre-weighed scintillation vials. The organ samples were made homogenate and QUE in tissue homogenate was subsequently extracted by methanol, 10 mol/l HCl and analyzed by HPLC method. During the analysis, each 400 μl organ sample was mixed with 1.0 ml methanol containing 400 μl 10 mol/l HCl. After mixing well, the sample was incubated at 90°C for 5 h and 1.0 ml ethyl acetate was added. After centrifugation at 5000 *g* and 4°C for 10 min, supernatant of organ sample was dried and reconstituted in 500 μl methanol for HPLC analysis.

2.8.2 *Pharmacokinetic studies*

Blood samples were collected in heparin-containing tubes at various time points as mentioned above (0.5, 1, 2, 4, 8, 12, 24, 48). Plasma was isolated by centrifugation at 3000 × *g* for 10 min and stored at -20°C. QUE in plasma was subsequently extracted by methanol, 10 mol/l HCl and analyzed by HPLC method. During the analysis, each 400 μl plasma sample was mixed with 1.0 ml methanol containing 400 μl 10 mol/l HCl. After mixing well, the sample was incubated at 90°C for 5 h and 1.0 ml ethyl acetate was added. After centrifugation at 5000 × *g* and 4°C for 10 min, supernatant was dried and reconstituted in 500 μl methanol for HPLC analysis. Version 3p97 (Quantitative Pharmacology Society of Chinese Pharmacological Society) was used to determine pharmacokinetic parameters, including area under the curve (AUC), total body clearance (CL) and plasma half-life.

2.8.3 *Effect of repeated administration on pharmacokinetics of PEGylated liposomes*

To assess the effect of different liposomes on the pharmacokinetic and tissue distribution of QUE, SD rats were used in the following formulations: In the group of PEGylated liposomes, the first administration was PEG2000-DPSE-QUE-NLs and the second administration was also of PEG2000-DPSE-QUE-NLs at the dose of 25 mg/kg bodyweight. The group contained common QUE-NLs with the first administration using QUE-NLs and the second administration also containing

QUE-NLs at the dose of 25 mg/kg body weight in the group of common liposomes.

2.9 Statistical analysis

Data were represented as mean ± standard deviations (SD) and were analyzed by two-tailed Student's t-tests using the Statistical Program for Social Sciences 13.0 software (SPSS, Co., Shanghai, China); $p < 0.05$ was used as the cutoff for statistically significant differences.

3. Results

3.1 Preparation and characterization of PEG2000-DPSE-QUE-NLs

PEG2000-DPSE-coated loading with PEG2000-DPSE-QUE-NLs size distribution (**Figure 1A**) of 30 – 100 nm and QUE-NLs size distribution (**Figure 1B**) of 50 – 300 nm were observed by laser particle analyzer. Indeed, in the TEM analysis, PEG2000-DPSE-QUE-NLs formed spherical particles with a small diameter and a narrow size distribution (**Figure 2A**) and QUE-NLs formed a wide size distribution (**Figure 2B**) and the control liposomes (**Figure 2C**). Schematic illustration of possible packing of PEG2000-DPSE-coated QUE nanoparticle is shown in **Figure 2D**.

HPLC and UV-Vis spectroscopy are very similar for the PEG2000-DPSE-QUE-NLs and QUE-NLs, indicating that, as expected, the QUE-NLs core remains unchanged by the attachment of the PEG2000-DPSE (**Figures 3A, B**), compared with the control liposomes (**Figures 3C**). HPLC and UV-Vis provide the simplest technique for measuring the concentration of PEG2000-DPSE-QUE-NLs in a sample, although what is measured is the absorbance of the QUE-NLs core.

3.2 Entrapment efficiency and drug loading

The loading content and EE of QUE-NLs and PEG2000-DPSE-QUE-NLs were calculated (**Table 1**). By contrast, EE significantly varied among these formulations, with a range of 69.42 – 85.72%. The addition of PEG-DSPE increased EE of QUE-NLs to 85.72%. Liposomes composed of PEG-DSPE had the higher drug loading content.

3.3 *In vitro* release profiles

The release profile of PEG2000-DPSE-QUE-NLs dispersion shows that the percentage of the drug released from the PEG2000-DPSE-coated QUE-NLs were sustained for 2, 4, 8, 12 and 24 h at 37°C, respectively (**Figure 4**). When the *in vitro* release of QUE was studied from the PEG2000-DPSE-coated QUE-NLs, it was found that about 85% release of drug was obtained from the PEG2000-DPSE-coated nanoliposomal formulation in 12 h, whereas about 66% drug release was achieved in case of the nanoliposomal formulation (QUE-NLs) in 12 h. About 95% release was monitored from PEG2000-DPSE-QUE-NLs after 24 h.

3.4 *In vitro* effect on the growth of glioma and on efficacy of tumor cell killing

For C6 cell lines studied, the assay was performed by MTT assay and the cell-killing efficacy of PEG2000-DPSE-QUE-NLs treatment. MTT assay demonstrated a significantly higher targeted tumor cell killing effect by 24 h treatment of PEG2000-DPSE-QUE-NLs compared with controls. While drug-free QUE-NLs did not show any noticeable cytotoxicity toward C6 glioma cells, and QUE caused only limited tumor cell killing, PEG2000-DPSE-QUE-NLs provoked a significant cell death at the same QUE concentrations. Thus, PEG2000-DPSE-QUE-NLs induced an approximately 2.5-fold increase in C6 glioma cell death compared with QUE concentration of 50 – 200 μM. Stronger time-dependent and faster C6 glioma cell killing by PEG2000-DPSE-QUE-NLs was also evident (**Figure 5A**). Results were observed in a delayed viability test, in which cytotoxicity was performed after 12 h drug treatment and additional 48 h incubation in drug-free medium (**Figure 5B–D**). IC_{50} of PEG2000-DPSE-QUE-NLs is approximately 146.2 μM at 12 h, 103.8 μM at 24 h and 98.6 μM at 48 h, which is much lower than the reported IC_{50} of QUE (above 200 μM) [40].

3.5 Inhibiting effect on tumor cell

For C6 cell lines studied, the assay was performed by trypan blue exclusion assay, and the cell inhibiting effect of PEG2000-DPSE-QUE-NLs or QUE-NLs treatment and the results were further confirmed by trypan blue exclusion assay when C6 glioma cells were treated by 50 – 400 μM PEG2000-DPSE-QUE-NLs for 12, 24, 36, 48 h (**Figure 6**). Indeed, in all cases, the PEG2000-DPSE-coated QUE- NLs formulation was more effective than the QUE-NLs and free QUE.

3.6 Biodistribution of QUE-NLs and PEG2000-DPSE-QUE-NLs

Having demonstrated *in vitro* efficacy, the authors sought to determine the biodistribution of PEG2000-DPSE-QUE-NLs and QUE-NLs in healthy rats, the free QUE was used for control comparison. Data for the brain, liver, heart, kidneys, spleen and lungs are presented in **Figure 7**.

PEG2000-DPSE-QUE-NLs accumulate in the brain, liver and heart in comparison with the QUE-NLs and free QUE control that accumulates in the heart and kidneys. It is interesting to note that the PEG2000-DPSE-QUE-NLs are highly distributed in the brain relative to Tween 80 and the PEGylated NLs [41,42]. High distribution to the brain may be related to that the QUE is interacting with the PEG2000-DPSE, Tween 80 and altering the distribution. Data for the lungs and kidneys are presented in the supporting information as there was little distribution to these organs (**Figure 7**). There was no obvious accumulation of any of the samples in the spleen.

3.7 Pharmacokinetic study

Pharmacokinetics of PEG2000-DPSE-coated loading with QUE-NLs were determined in rats. As shown by the plasma

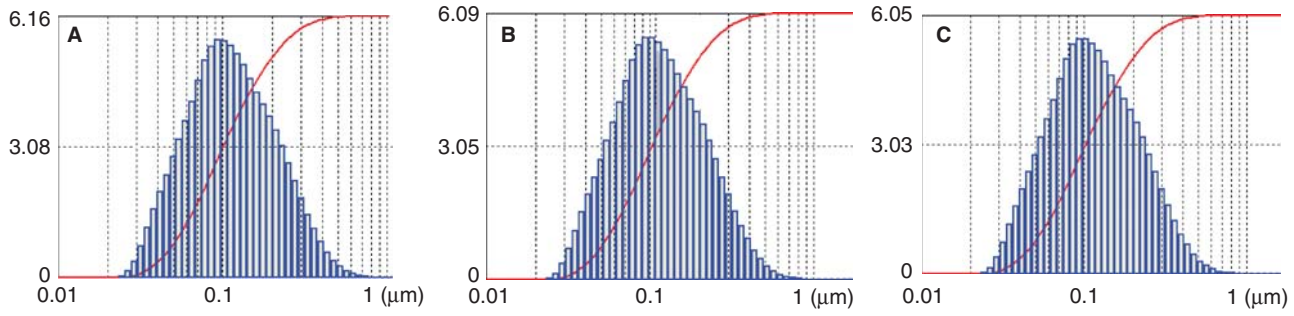

Figure 1. The size distribution of nanolipid formulations. A. The size distribution of PEG2000-DPSE-QUE-NL, and the average diameter of the liposome was 91.3 ± 34.1 nm. **B.** The size distribution of QUE-NL nanoparticles, and the average diameter of the liposome was 134.5 ± 42.2 nm. **C.** The size distribution of control nanoliposomes, and the average diameter of the liposome was 106.8 ± 37.6 nm.

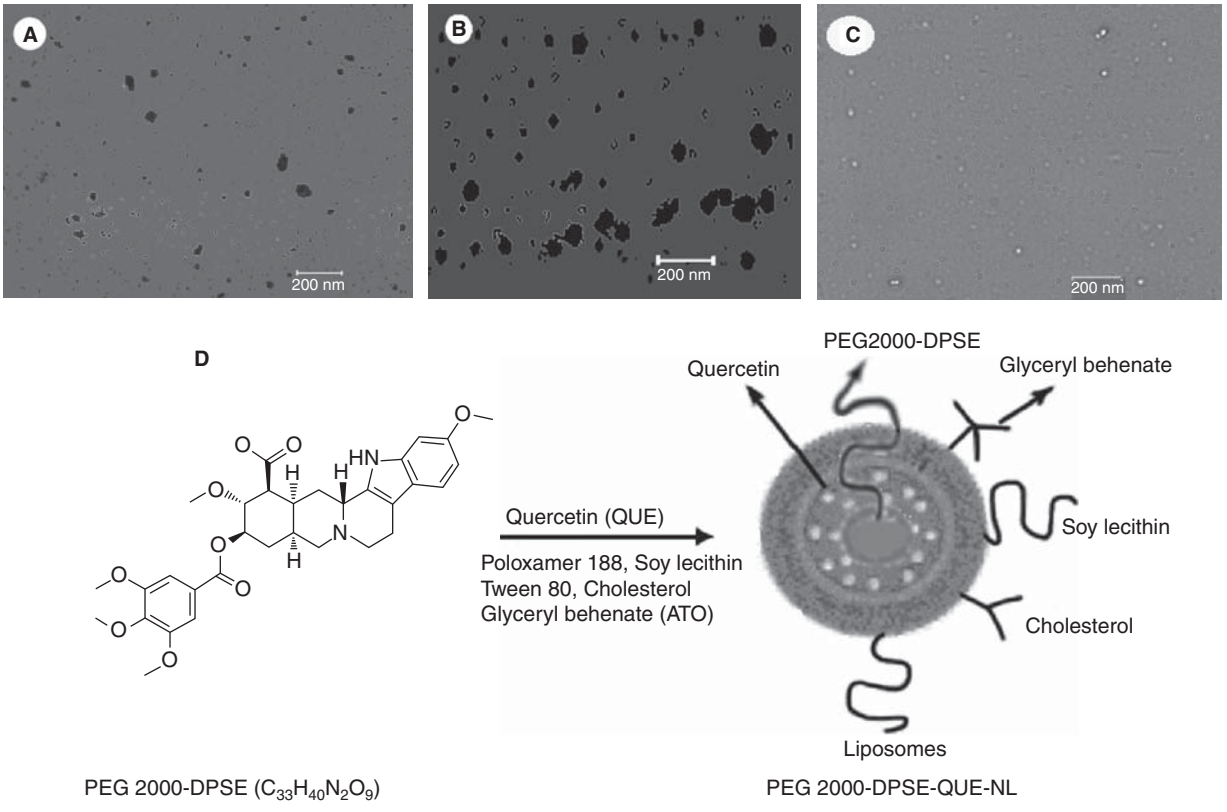

Figure 2. Characterization of PEG2000-DPSE-QUE-NLs. A. Transmission electron microscopic photograph of PEG2000-DPSE-coated quercetin nanoliposomes. **B.** Transmission electron microscopic photograph of quercetin nanoliposomes. **C.** The control nanoliposomes. **D.** Schematic illustration of possible packing of PEG2000-DPSE-coated quercetin nanoliposomes consist of an aqueous core and a lipid bilayer. The hydrophobic components of the nanoliposomes were on the side of the lipid bilayer and quercetin was located in the middle of lipid bilayer.

QUE concentration–time plot (**Figure 8**), the QUE in the liposomes was cleared at a slower rate compared with free QUE. At 8 h after QUE formulation administration, about 10.2% of the PEG2000-DPSE liposomal QUE remained in the plasma as compared with undetectable QUE-NLs or free QUE.

Plasma concentration data were fitted into a two-compartment model and pharmacokinetic parameters were calculated using Version 3p97. **Table 2** shows the pharmacokinetic parameters of PEG2000-DPSE liposomal QUE in comparison with those after QUE-NLs or free drug

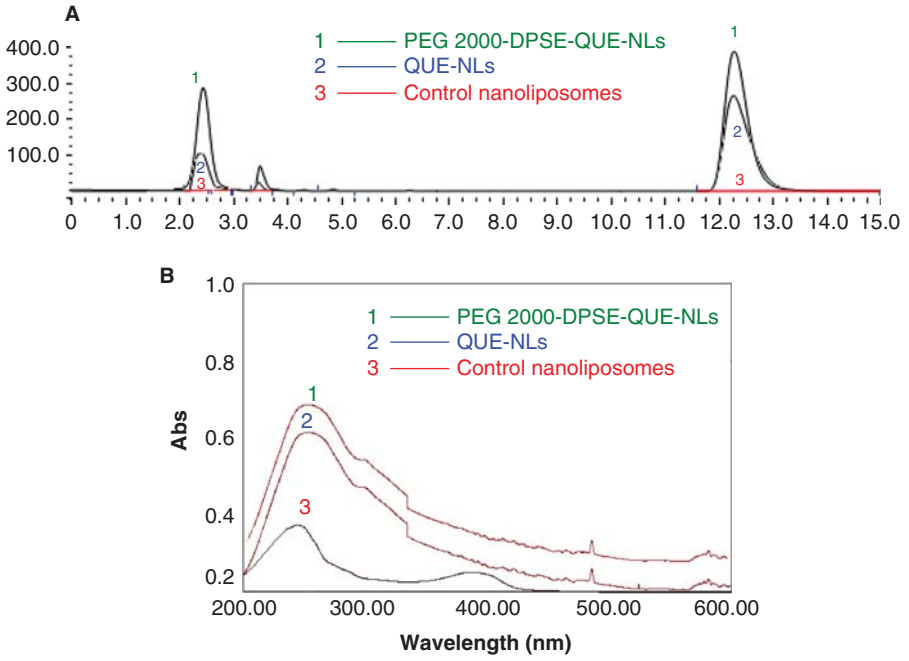

Figure 3. Characterization of QUE-NLs and PEG2000-DPSE-QUE-NLs. A. Stacked HPLC traces of QUE-NLs, PEG2000-DPSE-QUE-NLs and free QUE. **B.** The UV-Vis spectra show similar traces for PEG2000-DPSE-QUE-NLs, QUE-NLs and the control liposomes, indicative that their cores are identical.

Table 1. The loading content, EE, particle size, PI and zeta potential of lipid formulations of QUE.

Lipid formulations	Size (nm)	PI	Zeta potential	Loading content (%)	EE (%)
QUE-NLs	134.6 ± 62.2	0.26 ± 0.06	21.8 ± 5.4	26.23 ± 2.34	69.42 ± 5.92
PEG2000-DPSE-QUE-NLs	91.4 ± 42.2	0.32 ± 0.09	30.5 ± 6.9	43.55 ± 3.56	85.72 ± 7.68
Control liposomes	146.8 ± 47.6	0.17 ± 0.04	16.6 ± 3.8	-	-

Data represent the mean ± SD of three separate experiments (n = 3).
EE: Entrapment efficiency; PEG2000-DPSE-QUE-NLs: PEG-DPSE-ylated lipid-based quercetin nanoliposomes; PI: Polydispersity index; QUE: Quercetin; QUE-NLs: Quercetin nanoliposomes; SD: Standard deviation.

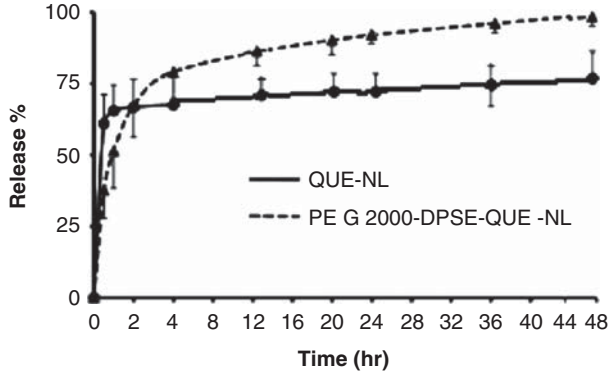

Figure 4. *In vitro* drug release profile from QUE-NLs and PEG2000-DPSE-QUE-NLs. Data shows mean ± SD (n = 3).

administration, with a half-life (T1/2β) of 1.7355 h, apparent volume of distribution (Vd) of 0.0806 l/kg, AUC of 77.72 mg/l and CL of 0.0322 l/h.

Compared with parameters of QUE-NLs and the free drug, half-life of PEG2000-DPSE liposomal QUE was two and six times greater (**Table 2**). Meanwhile, the AUC value of QUE-NLs and free drug in plasma was 5 and 110 (77.72/0.6953) times lower than that of the PEG2000-DPSE coated quercetin liposomal formulation respectively. These data show that the PEG2000-DPSE liposomal QUE had prolonged blood circulation time and decreased clearance.

There was an obvious accelerated blood clearance (ABC) phenomenon with the conventional PEG2000-DSPE modified liposomes [43-46]. There was a slight increase in plasma clearance rate after repeated administration of the PEG2000-DSPE liposomal QUE (**Figure 9**). However, the clearance of

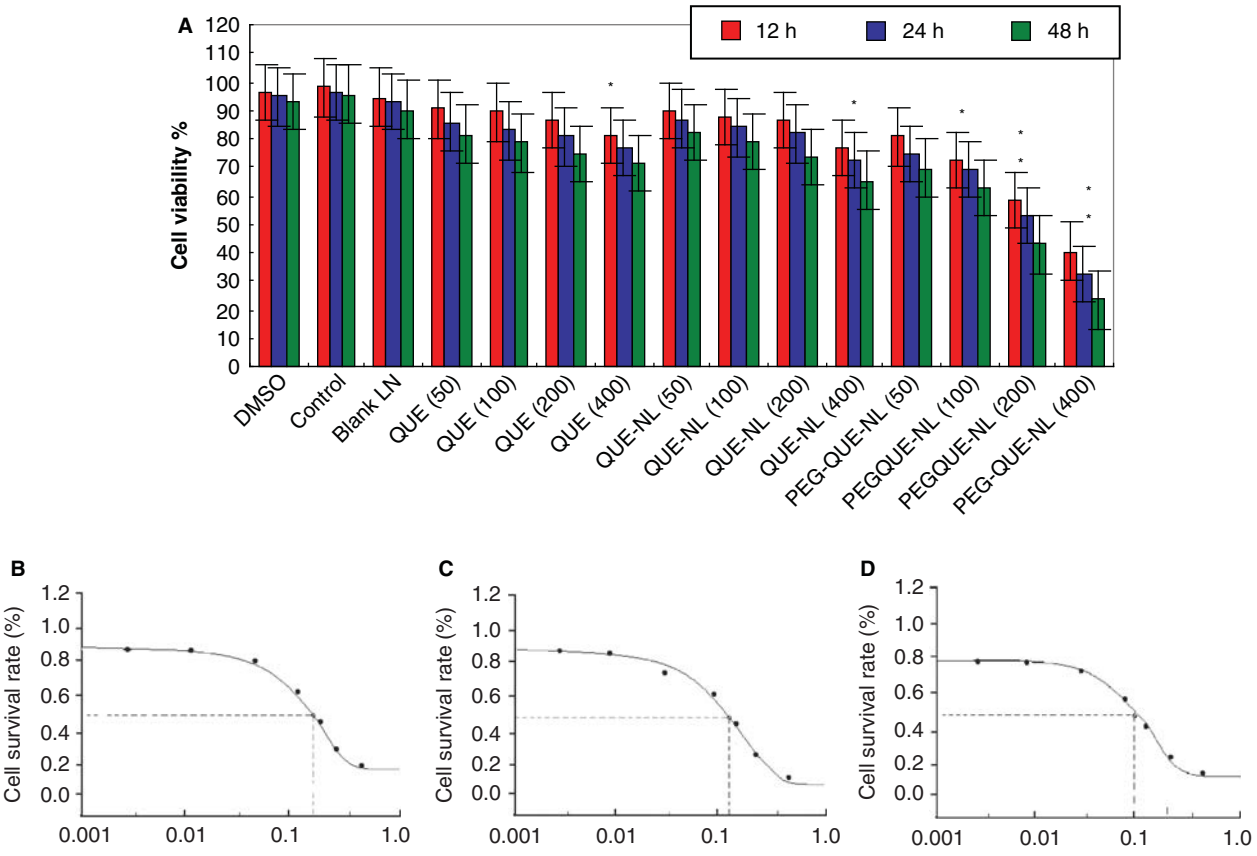

Figure 5. The cell-killing efficacy of PEG2000-DPSE-QUE-NLs treatment by MTT assay. A. *In vitro* efficacy of PEG2000-DPSE-QUE-NLs treatment of C6 glioma cells and cell viability of PEG2000-DPSE-QUE-NLs show the ability of killing cells. **B, C, D.** Presents the IC_{50} data for an average of three times from 12, 24 and 48 h. Each graph represents an individual trial. Error bars are standard errors.

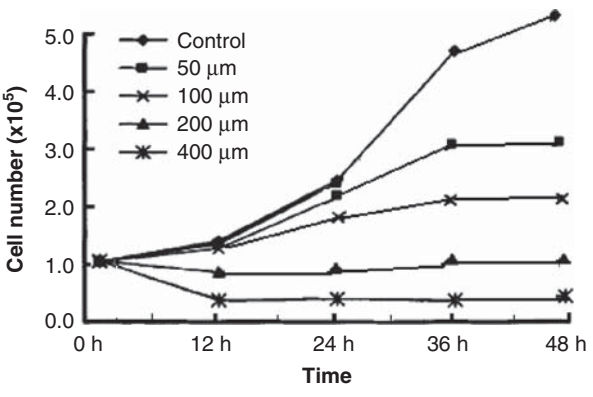

Figure 6. The cell-inhibiting effect of the PEG2000-DPSE-QUE-NLs treatment from 12, 24, 36 and 48 h by trypan blue exclusion assay. Presents the cell-inhibiting effect data for an average of 2.5 ~ 5.0 times from 12, 24 and 48 h. Each graph represents an individual trial. Error bars are standard errors.

PEG2000-DSPE unmodified liposomes (QUE-NLs) showed different pharmacokinetics after the repeated administration compared with the conventional PEG2000-DSPE liposomes. The plasma QUE concentration did not decrease significantly after repeated administration in the QUE-NLs.

With regards to biodistribution of the PEG2000-DSPE liposomes, there was a slight increased uptake in the liver after the second dose, but no significant difference in the spleen. As shown in **Figure 10**, the accumulated amount of QUE in the liver increased after the second dose ($p < 0.05$). However, compared with PEG2000-DSPE modified liposomes, the accumulated amount of QUE in the liver and spleen was not markedly changed in QUE-NLs. As shown in **Figure 10**, there was a marked ABC phenomenon which was induced by PEG2000-DSPE modified liposomes.

4. Discussion

As biodegradable and essentially non-toxic vesicles, liposomes have been used as delivery vehicles for several

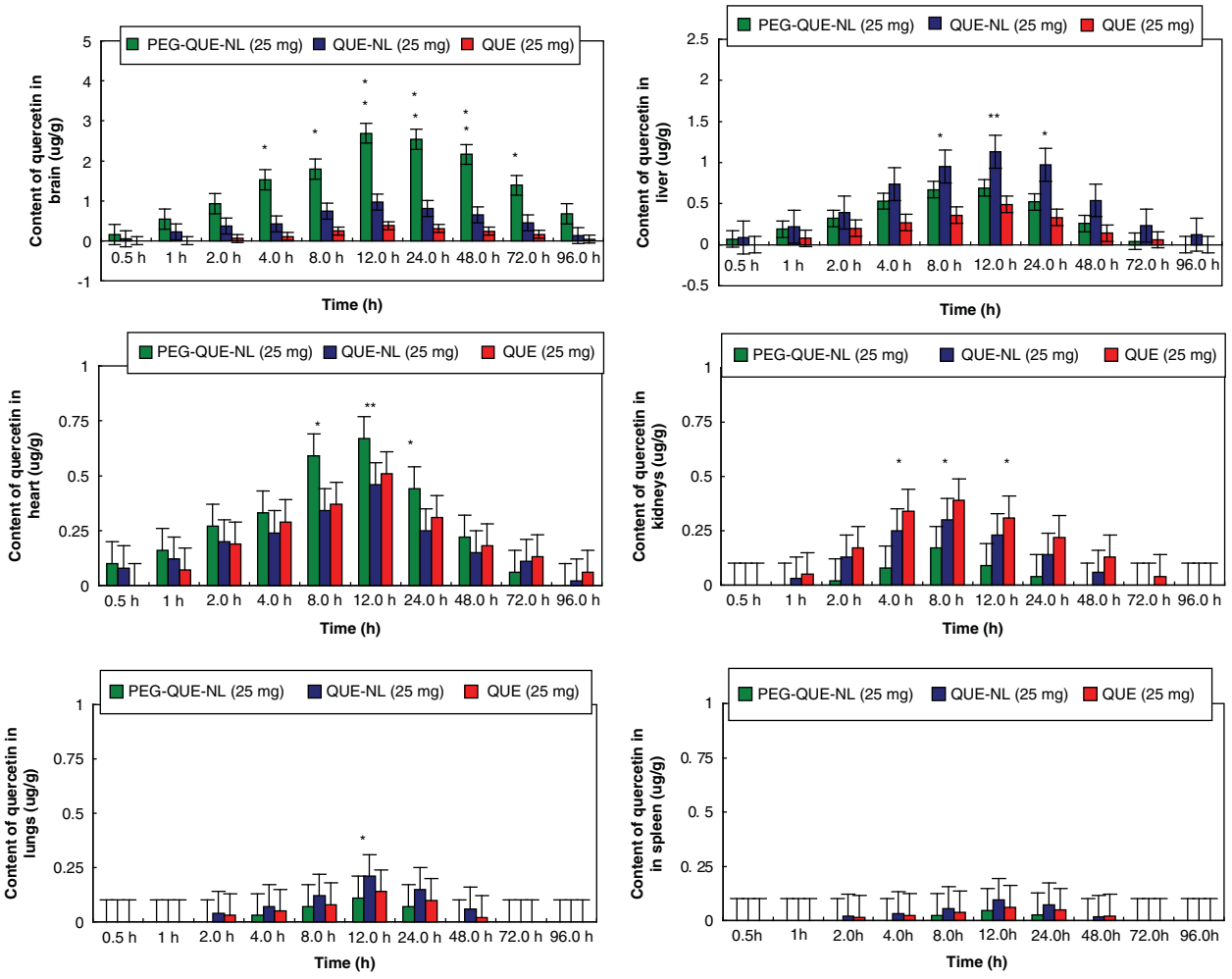

Figure 7. Distribution of quercetin with different formulations in the brain, liver, heart, kidneys, lungs and spleen of healthy rats at different time intervals. Distribution of PEG2000-DPSE- QUE-NLs, QUE-NLs and the free QUE in the brain, liver, heart, kidneys, lungs and spleen of healthy rats. The Y-axis in all figures was normalized to the total QUE content divided by the organ's weight. This value is then divided by the initial QUE content administered in each rat. All plots have the same Y-axis scale to ease comparisons. ANOVA is statistically significant (*$p < 0.05$, **$p < 0.01$), compared with the free quercetin.

anticancer agents [47]. In this study, the authors loaded QUE into a PEGylated liposomal carrier, and characterized its physicochemical and pharmacokinetic properties.

QUE is a weak water-soluble compound [8]. This study was conducted to prepare PEGylated lipid-based NLs loaded with QUE and to investigate the distribution and clearance of PEG2000-DSPEylated lipid-based PEG2000-DPSE-QUE-NLs as drug delivery vehicles for the anticancer drug *in vitro* and *in vivo*. Entrapment of this compound in ATO/Chol/PEG-DSPE liposomes was achieved by emulsion–evaporation and low temperature curing preparation. Through size measurement, evaluation of encapsulation efficiency and *in vitro* drug release studies, the composition of the lipids and the loading method for the compound were optimized.

Schematic illustration of possible packing of PEG2000-DPSE-coated QUE-NLs consists of an aqueous core and a lipid bilayer. The hydrophobic components of the NLs were on the side of the lipid bilayer and QUE was located in the middle of lipid bilayer. The size distribution of nanoparticles, and the average diameter of PEG2000-DPSE-QUE-NL were 91.3 ± 34.1 nm and PI was 0.32 ± 0.09, while the size distribution of nanoparticles, and the average diameter of QUE-N were 134.5 ± 42.2 nm and PI was 0.26 ± 0.06. The PEGylated layers (PEG2000-DPSE layers) and oil phase (glyceryl behenate and cholesterol) were mainly made of NLs. Cholesterol is known to enhance the rigidity of the soy lecithin bilayer. As a result, incorporation of the PEGylated layers possibly altered the flexibility of the liposomal bilayer, and reduced the average diameter of QUE-NLs. The EE of QUE-NLs was 69.42%.

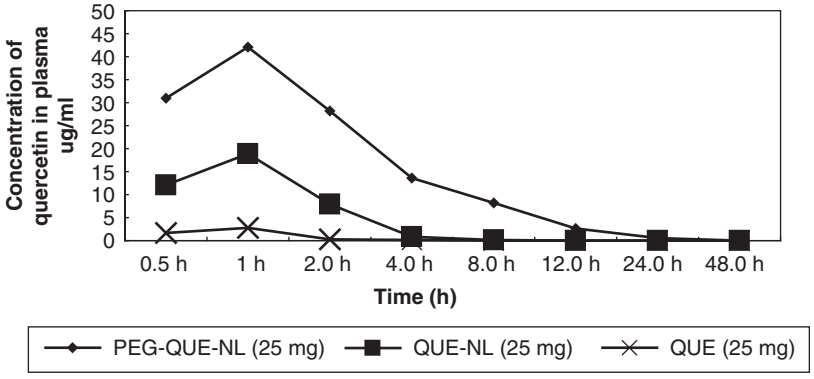

Figure 8. The plasma concentration curve of quercetin with different formulations at different time intervals. Plasma concentration–time curve of PEG2000-DPSE-QUE-NLs, QUE-NLs and the free QUE after administration of different formulations in rats. At indicated time points, rat plasma was collected and quercetin was extracted for concentration analysis by HPLC. Error bars stand for standard deviations (n = 6).

Table 2. Comparison of the relative pharmacokinetic parameters.

Formulations	Ke (h)	Half-life (h)	AUC (mg/l h)	Vd (l/kg)	CL (l/h)
QUE	2.4018	0.2885	0.6953	1.497	3.5955
QUE-NLs	0.8646*	0.8015*	14.0527‡	0.2058*	0.1779*
PEG-QUE-NLs	0.3993‡	1.7355‡	77.72‡	0.0806‡	0.0322‡

Compared with parameters of QUE-NLs and the free drug, half-life of PEG2000-DPSE liposomal QUE was two and six times greater (**Table 2**). Meanwhile, AUC value of QUE-NLs and free drug in plasma was 5 and 110 (77.72/0.6953)times lower than that of the PEG2000-DPSE coated quercetin liposomal formulation respectively. These data show that the PEG2000-DPSE liposomal QUE had prolonged blood circulation time and decreased clearance.
Compared with QUE, *$p < 0.05$; ‡$p < 0.01$; ‡$p < 0.001$.
AUC: Area under the curve; CL: Clearance; Ke: Ke stands for elimination rate constant (CL/Vd); QUE: Quercetin; QUE-NLs: Quercetin nanoliposomes.

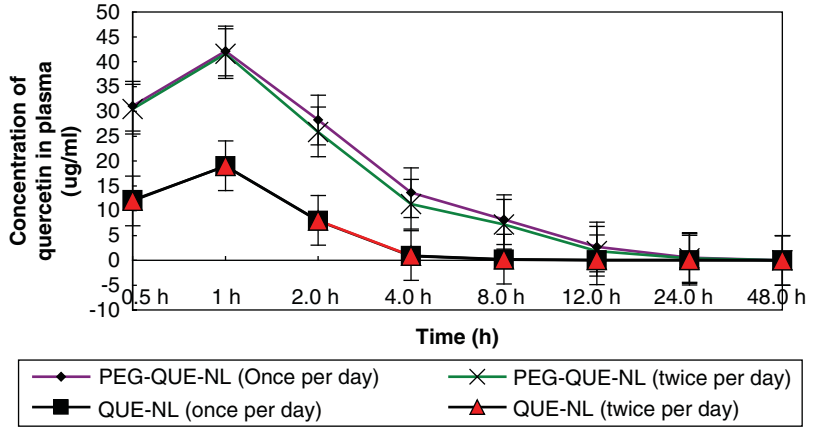

Figure 9. Blood clearance of quercetin in rats after a first and second administration of PEG2000-DSPE QUE liposomes. The plasma QUE concentration decreased significantly ($p < 0.05$) after repeated administration of PEG2000-DSPE liposomal QUE. All plots have the same Y-axis scale to ease comparisons. ANOVA is statistically significant (*$p < 0.05$, **$p < 0.01$), compared with the first administration.

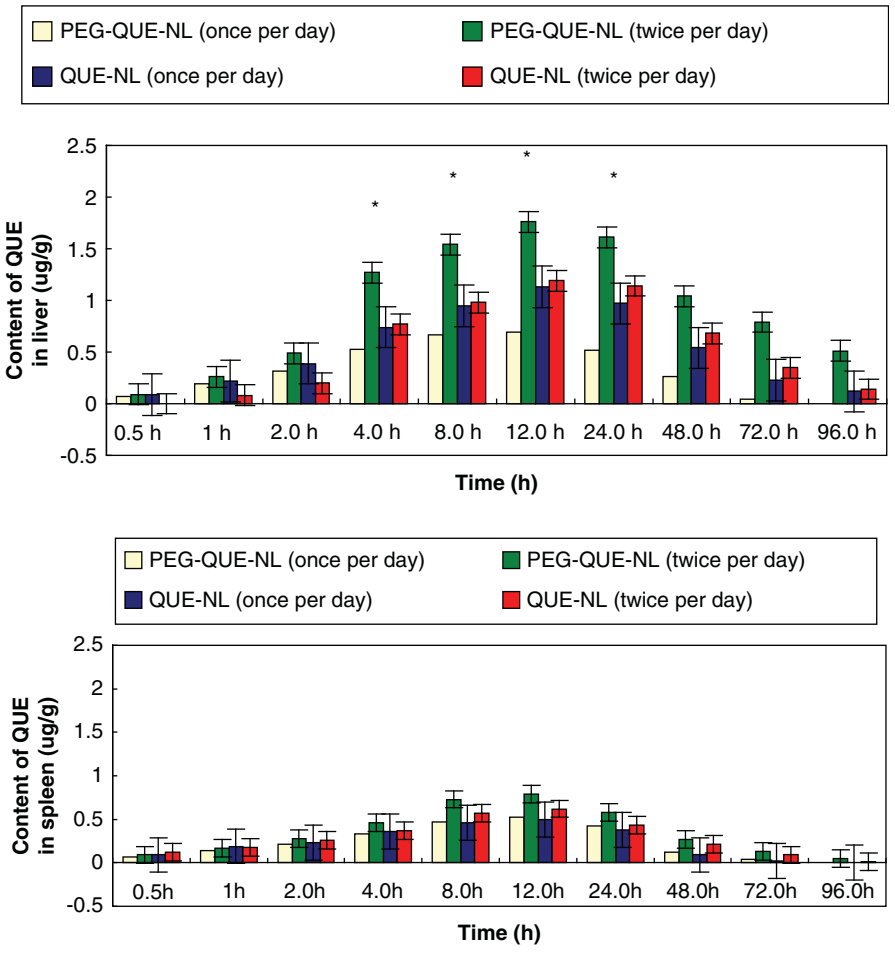

Figure 10. Biodistribution of QUE in liver and spleen in rats after a first and second administration of PEG-DSPE liposomes (n = 6). Distribution of repeated administration of PEG2000-DSPE modified liposomes and QUE-NLs, the accumulated amount of QUE in the liver and spleen of healthy rats. The Y-axis in all figures was normalized to the total QUE content divided by the organ's weight. This value is then divided by the initial QUE content administered in each rat. All plots have the same Y-axis scale to ease comparisons. ANOVA is statistically significant (*$p < 0.05$, **$p < 0.01$), compared with the first administration.

The addition of PEG-DSPE increased EE of PEG-DSPE-QUE-NLs to 85.72%. Liposomes composed of PEG-DSPE had the higher drug loading content.

Currently, the limited solubility of QUE in water presents a major problem for its administration as a chemopreventer. Accordingly, many studies analyzed possible complexes able to transport QUE to various tissues [48,49]. Promising studies have been obtained with PEG [50]. In this article, the association of QUE-NLs with PEG2000-DSPE (PEG2000-DSPE-QUE-NLs) has been tested *in vitro* and *in vivo* through administrations. Pharmacokinetic study demonstrated that PEG2000-DSPE liposomal QUE had prolonged blood circulation time and decreased clearance compared with the free drug. The biodistribution and the antitumor activity of QUE have been evaluated *in vitro*. Interestingly, PEG2000-DSPE-QUE-NLs not only have a better solubility in water and prolong the circulation times of QUE in blood, but also enhance its antitumor activity.

Perhaps one of the most extensively studied carriers in drug delivery, NLs are traditionally thought of as spherical vesicles composed of phospholipids. Drug-loading capacity is a key feature of DDS. Among NLs, loading hydrophobic drugs into the lipophilic membrane follows general approaches developed for their classic spherical counterparts [51]. Loading of the hydrophobic drugs into polymersomes reduces their systemic toxicity and enhances antitumor effects in animal models [52-54]. However, to be widely applicable in medicine, DDS should be biodegradable and their degradation products non-toxic, amenable to physiological excretion. Polymeric carriers including spheres and flat disks are less amenable to drug loading into the carrier. Since polymeric particles are not biodegradable, they are not commonly considered as drug carriers for medical use [55].

Among biodegradable polymeric DDS materials, there are two classic examples at opposite ends of the degradation kinetics spectrum. On the fast end are polyanhydrides and on the slow end are polyesters (used for synthesis of many nanocarriers),

with degradation times ranging from weeks to years, dependent on their environment and geometry [56-58]. Phospholipid NLs represent a classical example of fully degradable DDS. In order to enhance circulation properties of carriers in the bloodstream, the typical course of action is to coat a carrier with PEG. For example, PEGylation of liposomes increased their half-life from less than 30 min to approximately 5 h in mice [59]. Other benefits, such as reduced interaction with the immune system, have also been found. For example, due to their dense PEG surface brush, polymersomes and filomicelles are compatible with blood [60], as they: i) remain suspended and flexible in plasma; ii) do not adhere to red blood cells and leukocytes in blood; iii) do not fix opsonins or activate complement [61] and iv) do not cause hemolysis [62].

PEGylation prolonged circulation half-life of 200 nm spherical liposomes from ~ 30 min to nearly 5 h in mice [63]. Spherical polymersomes persist in the circulation slightly longer with half-live of nearly 1 day [64]. This is thought to be due to the much denser PEG brush present on the surface of polymersomes relative to liposomes (and, perhaps, due to their thicker bilayer and resultant structural integrity).

In the majority of cases, the nanomaterials are functionalized with PEG2000-DPSE to provide solubility in aqueous solutions and increase blood circulation times. These polymer conjugates via the hydrophobic interaction between the alkyl tails and the nanomaterials. *In vivo* formulation was more effective than QUE-NLs and showed prolonged retention in the liver and brain.

The ABC phenomenon is important for the development of DDS, especially for nanoparticles in the case of repeated administration of nanomedicines. Researchers have used different animal models to investigate the mechanism of the ABC phenomenon, including rats, mice, rhesus monkeys and rabbits, among others. Dams *et al.* demonstrated that rhesus monkeys and rats could bring out the ABC phenomenon after repeated administration of PEGylated liposomes [65], whereas mice could not. Ishida *et al.* studied the first liposomal characteristics that affect the ABC phenomenon and demonstrated that an intense accelerated clearance can be induced in mice [66]. Although the conclusion from animal experiments might not be fully consistent with the clinical manifestations of liposomes, when there is a need for repeated administration, thorough study of their distribution in the body and their pharmacokinetics is necessary.

The PEG2000-DPSE-QUE-NLs were more effective in killing C6 glioma cell than QUE-NLs, and this may be attributable to the smaller diameter of PEG-DSPE-QUE-NLs, leading to enhance drug uptake in C6 glioma cell and killing tumor cell. Here, the authors showed that PEG2000-DPSE were effective drug delivery vehicles *in vitro* and *in vivo*. Drug loading was accomplished by PEG2000-DPSE-coated loading with a hydrophobic drug NLs; no covalent chemistry is required and the drug-loaded formulation is highly stable.

5. Conclusion

In conclusion, a novel liposomal QUE formulation has been designed and evaluated. The authors have demonstrated that a carbon nanomaterial (PEG2000-DPSE) can be an effective drug delivery platform, both *in vitro* and *in vivo*, when loaded with a drug (QUE). The QUE/PEG2000-DPSE formulation proved to be more effective than QUE *in vitro* efficacy of tumor cell killing on the growth of glioma cancer cells. This work demonstrates that nanomaterials (PEG2000-DPSE) are effective drug delivery vehicles *in vivo* as tumor-targeted drug carriers. Biodistribution studies demonstrated that, similar to other nanoparticles, PEG2000-DPSE-QUE-NLs reach the tissues but primarily accumulate in the brain and liver. The ABC phenomenon was induced by prolonged administration of PEGylated liposomes into the rats and was accompanied by substantially increased uptake into the liver. The present study may have a considerable impact on the future development of PEGylated liposomal formulations for use in multiple drug therapy.

This formulation provides characteristics such as high drug encapsulation ratio, low *in vitro* release rate and slow drug clearance and prolonged circulation time *in vivo*. This provides an alternative solubilization vehicle for administration of QUE. Further preclinical studies are warranted to define the safety and therapeutic efficacy of this novel formulation. While preparing a novel nanovector drug delivery platform that is as effective as a commercial formulation is noteworthy, in the future it will be essential to develop a targeted PEG-HCCs formulation that improves on current clinical formulations. Switching to a liposomal formulation offers the potential to alter the pharmacokinetics of QUE and provide a more favorable efficacy profile for this promising chemotherapeutic agent.

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Declaration of interest

The authors declare no conflict of interest. This work was funded by the project on the Anti-glioma Effects of nano-liposomes of quercetin funded by the Education Department of Hubei province.

Bibliography

1. O'Driscoll CM, Griffin BT. Biopharmaceutical challenges associated with drugs with low aqueous solubility—the potential impact of lipid-based formulations. Adv Drug Deliv Rev 2008;60:617-24
2. Xavier CP, Lima CF, Rohde M, et al. Quercetin enhances 5-fluorouracil - induced apoptosis in MSI colorectal cancer cells through p53 modulation. Cancer Chemother Pharmacol 2011;68:1449-57
3. Siegelin MD, Reuss DE. Quercetin promotes degradation of surviving and thereby enhances death-receptor–mediated apoptosis in glioma cells. Neuro-oncol 2009;6:123-9
4. Heijnen CG, Haenen GR, van Acker FA, et al. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. Toxicology in Vitro 2001;15(1):3-6
5. Robaszkiewicz A, Balcerczyk A, Bartosz G. Antioxidative and prooxidative effects of quercetin on A549 cells. Cell Biology International 2007;31:1245-50
6. Lugli E, Ferraresi R, Roat E, et al. Quercetin inhibits lymphocyte activation and proliferation without inducing apoptosis in peripheral mononuclear cells. Leukemia Research 2009;33:140-50
7. Sun M, Gao Y, Pei Y, et al. Development of nanosuspension formulation for oral delivery of quercetin. J Biomed Nanotechnol 2010;6:325-32
8. Li H, Zhao X, Ma Y, et al. Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. J Control Release 2009;133:238-44
9. Sahoo NG, Kakran M, Shaal LA, et al. Preparation and characterization of quercetin nanocrystals. J Pharm Sci 2011;100:2379-90
10. Wu TH, Yen FL, Lin LT, et al. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. Int J Pharm 2008;346:160-8
11. Smith AJ, Kavuru P, Wojtas L, et al. Cocrystals of quercetin with improved solubility and oral bioavailability. Mol Pharm 2011;8:1867-76
12. Kim MK, Park KS, Lee C, et al. Enhanced stability and intracellular accumulation of quercetin by protection of the chemically or metabolically susceptible hydroxyl groups with a pivaloxymethyl (POM) promoiety. J Med Chem 2010;53:8597-607
13. Chetoni P, Rossi S, Burgalassi S, et al. Comparison of liposome encapsulated acyclovir with acyclovir ointment: ocular pharmacokineticsin rabbits. J Ocul Pharmacol Ther 2004;20:169-77
14. Jesorka A, Orwar O. Liposomes: technologies and analytical applications. Annu Rev Anal Chem 2008;1:801-32
15. Harrington KJ, Lewanski CR, Northcote AD, et al. Phase I-II study of pegylated liposomal cisplatin (SPI-077™) in patients with inoperable head and neck cancer. Ann Oncol 2001;12:493-6
16. Crielaard BJ, van der Wal S, Le HT, et al. Liposomes as carriers for colchicine-derived prodrugs: Vascular disrupting nanomedicines with tailorable drug release kinetics. Eur J Pharm Sci. 2012;45(4):429-35
17. Wu FG, Luo JJ, Yu ZW. Infrared spectroscopy reveals the nonsynchronicity phenomenon in the glassy to fluid micellar transition of DSPE-PEG2000 aqueous dispersions. Langmuir 2010;26:12777-84
18. Sochanik A, Mitrus I, Smolarczyk R, et al. Experimental anticancer therapy with vascular-disruptive peptide and liposome-entrapped chemotherapeutic agent. Arch Immunol Ther Exp (Warsz) 2010;58:235-45
19. Moribe K, Maruyama S, Inoue Y, et al. Ascorbyl dipalmitate/PEG-lipid nanoparticles as a novel carrier for hydrophobic drugs. Int J Pharm 2010;387:236-43
20. Gaber NN, Darwis Y, Peh KK, et al. Characterization of polymeric micelles for pulmonary delivery of beclomethasone dipropionate. J Nanosci Nanotechnol 2006;6:1-7
21. Torchilin VP. Polymer-coated long-circulating microparticulate pharmaceuticals. J Microencapsul 1998;15:11-19
22. Zalipsky S. Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine. FEBS Lett 1994;353:71-4
23. Allen C, Maysinger D, Eisenberg A. Nano-engineering block copolymer aggregates for drug delivery. Colloids Surf B Biointerfaces 1999;16:23-7
24. Jones MC, Leroux JC. Polymeric micelles—a new generation of colloidal drug carriers. Eur J Pharm Biopharm 1999;48:101-11
25. Lukyanov AN, Gao Z, Torchilin VP. Micelles from polyethylene glycol/ phosphatidylethanolamine conjugates for tumor drug delivery. J Control Release 2003;91:97-102
26. Veronese F, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today 2005;10:1451-8
27. Jiang W, Mardyani S, Fischer H, et al. Design and characterization of lysine cross-linked mercapto-acid biocompatible quantum dots. Chem Mater 2006;4:872-8
28. Mulder WJM, Koole R, Brandwijk RJ, et al. Surfactant-assisted synthesis of water-soluble and biocompatible semiconductor quantum dot ratlles. Nano Lett 2006;6:1-6
29. Adams M, Lavasanifar A, Kwon G. Amphiphilic block copolymers for drug delivery. J Pharm Sci 2003;92:1343-55
30. Kwon GS, Suwa S, Yokoyama M, et al. Enhanced tumor accumulation and prolonged circulation times of ratlle-forming poly(ethylene oxide-aspartate) block copolymer–adriamycin conjugates. J Control Release 1994;29:17-23
31. Li Y, Kwon GS. Methotrexate esters of poly(ethylene oxide)-block-poly (2-hydroxyethyl-laspartamide). I. Effects of the level of methotrexate conjugation on the stability of ratlles and on drug release. Pharm Res 2000;17:607-11

32. Nishiyama N, Kataoka K. Preparation and characterization of size-controlled polymeric ratlle containing cis-dichlorodiammineplatinum (II) in the core. J Control Release 2001;74:83-94

33. Burt HM, Zhang X, Toleikis P, et al. Development of copolymers of poly(d, llactide) and methoxypolyethylene glycol as ratllar carriers of paclitaxel. Colloids Surf B Biointerfaces 1999;16:161-71

34. Kohler N, Fryxell GE, Zhang MQ. A bifunctional poly(Ethylene Glycol) silane immobilized on metallic oxide-based nanoparticles for conjugation with cell targeting agents. J Am Chem Soc 2004;126:7206-11

35. Larsen EK, Nielsen T, Wittenborn T, et al. Size-dependent accumulation of pegylated silane-coated magnetic iron oxide nanoparticles in murine tumors. ACS Nano 2009;3:1947-51

36. Wang A, Gu F, Zhang L, et al. Biofunctionalized targeted nanoparticles for therapeutic applications. Expert Opin Biol Ther 2008;8:1063-70

37. Ryan SM, Mantovani G, Wang X, et al. Advances in PEGylation of important biotech molecules: delivery aspects. Expert Opin Drug Deliv 2008;5:371-83

38. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 2007;7:573-84

39. Wang G, Wang JJ, Du SM, et al. Effects of quercetin nanoliposomes on C6 glioma cells through induction of type III programmed cell death. Int J Nanomedicine 2012;7:271-80

40. Siegelin MD, Reuss DE, Habel A, et al. Quercetin promotes degradation of survivin and thereby enhances death-receptor–mediated apoptosis in glioma cells. Neuro-oncol 2009;3:122-31

41. Xin H, Sha X, Jiang X, et al. The brain targeting mechanism of angiopep-conjugated poly(ethylene glycol)-co-poly(epsilon-caprolactone) nanoparticles. Biomaterials 2012;33:1673-81

42. Tian XH, Lin XN, Wei F, et al. Enhanced brain targeting of temozolomide in polysorbate-80 coated polybutylcyanoacrylate nanoparticles. Int J Nanomed 2011;6:445-52

43. Ishida T, Harada M, Wang XY, et al. Accelerated blood clearance of PEGylated liposomes following preceding liposome administration: effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. J Control Release 2005;105(3):305-17

44. Koide H, Asai T, Hatanaka K, et al. T cell-independent B cell response is responsible for ABC phenomenon induced by repeated administration of PEGylated liposomes. Int J Pharm 2010;392(1-2):218-23

45. Ishida T, Atobe K, Wang X, et al. Accelerated blood clearance of PEGylated liposomes upon repeated injections: effect of doxorubicinencapsulation and high-dose first injection. J Control Release 2006;115(3):251-8

46. Ishihara T, Takeda M, Sakamoto H, et al. Accelerated blood clearance phenomenon upon repeated injection of PEG modified PLA-nanoparticles. Pharm Res 2009;26(10):2270-9

47. Hofheinz RD, Gnad-Vogt SU, Beyer U, et al. Liposomal encapsulated anti-cancer drugs. Anticancer Drugs 2005;16:691-707

48. Ghosh A, Mandal AK, Sarkar S, et al. Nanoencapsulation of quercetin enhances its dietary efficacy in combating arsenic-induced oxidative damage in liver and brain of rats. Life Sci 2009;84:75-80

49. Vicentini FT, Simi TR, Del Ciampo JO, et al. Quercetin in w/o microemulsion: in vitro and in vivo skin penetration and efficacy against UVB-induced skin damages evaluated in vivo. Eur J Pharm Biopharm 2008;69:948-57

50. Kale R, Saraf M, Juvekar A, et al. Decreased B16F10 melanoma growth and impaired tumour vascularization in BDF1 mice with quercetin-cyclodextrin binary system. J Pharm Pharmacol 2006;58:1351-8

51. Zarif L. Drug delivery by lipid cochleates. Methods Enzymol 2005;391:314-29

52. Ahmed F, Discher DE. Self-porating polymersomes of PEG–PLA and PEG–PCL: hydrolysis triggered controlled release vesicles. J Control Release 2004;96(1):37-53

53. Cai S, Vijayan K, Cheng D, et al. Micelles of different morphologies – advantages of worm-like filomicelles of PEO–PCL in paclitaxel delivery. Pharm Res 2007;24(6):2099-109

54. Ahmed F, Pakunlu RI, Srinivas G, et al. Shrinkage of a rapidly growing tumor by drug-loaded polymersomes: pH-triggered release through copolymer degradation. Mol Pharm 2006;3(3):340-50

55. Tokiwa Y, Jarerat A. Biodegradation of poly(L-lactide). Biotechnol Lett 2004;26(10):771-7

56. Kumar N, Ravikumar MN, Domb AJ. Biodegradable block copolymers. Adv Drug Deliv Rev 2001;53(1):23-44

57. Shive MS, Anderson JM. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev 1997;28(1):5-24

58. Siepmann J, Gopferich A. Mathematical modeling of bioerodible, polymeric drug delivery systems. Adv Drug Deliv Rev 2001;48(2–3):229-47

59. Klibanov AL, Maruyama K, Torchilin VP, et al. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 1990;268(1):235-7

60. Lee JC, Wong DT, Discher DE. Direct measures of large, anisotropic strains in deformation of the erythrocyte cytoskeleton. Biophys J 1999;77(2):853-64

61. Photos PJ, Bacakova L, Discher B, et al. Polymer vesicles in vivo: correlations with PEG molecular weight. J Control Release 2003;90(3):323-34

62. Bermudez H, Brannan AK, Hammer DA, et al. Molecular weight dependence of polymersome membrane structure, elasticity, and stability. Macromolecules 2002;35(21):8203-8

63. Dalhaimer P, Bates FS, Discher DE. Single molecule visualization of stable, stiffness-tunable, flow-conforming worm micelles. Macromolecules 2003;36(18):6873-7

64. Geng Y, Dalhaimer P, Cai S, et al. Shape effects of filaments versus spherical particles in flow and drug delivery. Nat Nano 2007;2(4):249-55

65. Dams ET, Laverman P, Oyen WJ, et al. Accelerated blood clearance and altered biodistribution of repeated

administration of sterically stabilized liposomes. J Pharmacol Exp Ther 2000;292(3):1071-9

66. Ishida T, Ichikawa T, Ichihara M, et al. Effect of the physicochemical properties of initially administrate liposomes on the clearance of subsequently administrate PEGylated liposomes in mice. J Control Release 2004;95(3):403-12

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