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Lipid nanoemulsions loaded with doxorubicin-oleic acid ionic complex: characterization, *in vitro* and *in vivo* studies

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This study aimed at developing a novel lipid nanoemulsion formulation of doxorubicin (DOX) which is feasible for scale-up production, exhibits good parenteral acceptability, and further improves the therapeutic index of the drug. Oleic acid was used to form ionic complex with DOX in order to enhance its lipophilicity. The lipid nanoemulsions loaded with doxorubicin-oleic acid complex (DOX-OA-LNs) were prepared using a simple high-pressure homogenization method and fully characterized from physicochemical and *in vitro* release standpoint. Afterwards, the DOX-OA-LNs and free DOX were compared with respect to their *in vitro* cellular uptake and cytotoxicity, and their *in vivo* pharmacokinetics and biodistribution behavior in mice were also investigated. The obtained DOX-OA-LNs could achieve high encapsulation efficiency of $93.7 \pm 1.2\%$ under optimal conditions. The *in vitro* release behavior displayed biphasic drug release pattern with rapid release at the initial stage and prolonged release afterwards. The DOX-OA-LNs exhibited higher growth inhibitory effect than free DOX by MTT assay. Flow cytometry and confocal microscopy studies showed that the cellular uptake of free DOX and DOX-OA-LNs were comparable. Pharmacokinetics and *in vivo* distribution studies in mice showed that DOX-OA-LNs demonstrated significantly higher DOX level in blood and longer circulation time than free DOX. Moreover, DOX-OA-LNs significantly decreased DOX concentration in heart, lung and kidney. These results suggested that DOX-OA-LNs could be a promising formulation for the delivery of DOX in tumor chemotherapy.

1. Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is commonly used in the treatment of malignancy, including leukemias, lymphomas, bone sarcoma, and so on. However, its therapeutic use is limited by its cumulative dose-related and irreversible cardiotoxicity and myelosuppression (Young et al. 1981; Singal et al. 2000). Until now, various colloidal particle delivery systems, such as liposomes (Working et al. 1994), polymeric nanoparticles (Park et al. 2009; Zhang et al. 2007; Petri et al. 2007) and solid lipid nanoparticles (SLNs) (Cavalli et al. 1993; Ma et al. 2009; Subedi et al. 2009) have been developed to minimize the toxic side effect of DOX and enhance its therapeutic efficacy. However, all of them had their own drawbacks which limited their clinical use and only the DOX liposomes could be used clinically. Although the liposomal DOX formulation has appeared on the pharmaceutical market in the 1990s, complexity associated with the manufacturing process and enormous cost of the liposomal formulation were the major barriers in the successful commercialization of liposomes (Joshi and Müller 2009; Wissing et al. 2004). The primary two drawbacks of the polymeric nanoparticles which held back their clinical use were the cytotoxicity of polymers and the lack of a suitable large scale production method (Müller et al. 2000). SLNs could be produced easily by the high pressure homogenization method which was feasible for scale-up production. But the parenteral acceptability of the lipids (e.g. stearic acid, hexadecanoic acid) was the major hurdle for the successful commercialization of SLNs for parenteral administration and there is no product of SLNs commercially available for *i.v.* injection on the market so far (Joshi and Müller 2009). In conclusion, despite vast researches in the field of colloidal particle delivery systems of DOX, there has been no colloidal particle delivery system of DOX which could both satisfy the regulatory acceptance of the excipients for *i.v.* administration and the demand on low cost and convenience of large-scale production. Thus, a need for simple, safe and cheap colloidal formulations of DOX persists in the clinical trials.

Recently, lipid nanoemulsions (LNs) have emerged as an interesting carrier system for optimized delivery of drugs. LNs, also frequently known as miniemulsions, ultrafine emulsions, submicron emulsions and so forth (Solans et al. 2005; Gutierrez et al. 2008), were nanometric-scale oil-in-water dispersions, mainly covering a size range of 20–200 nm and showing narrow size distributions (Solans et al. 2005; Huynh et al. 2009; Subedi et al. 2009). The basic structure of a nanoemulsion droplet is a neutral lipid core (e.g. liquid triglyceride) stabilized by amphipathic lipids (e.g. phospholipids). The LNs are appealing to many administration routes: parenteral (Santos-Magalhaes et al. 2000; Zhao et al. 2008), transdermal (Sonneville-Auburn et al. 2004; Yilmaz and Borchert 2005; Mou et al. 2008), oral (Singh et al. 2008; Vyas et al. 2008; Bali et al. 2010) and ocular (Sznitowska et al. 1999).

Intravenously administered LNs are excellent carriers for drug delivery, particularly for lipophilic drugs. They combine the advantages of colloidal drug carrier systems like polymeric nanoparticles and liposomes but at the same time avoid or minimize the drawbacks associated with them. Most importantly, the components of LNs, such as lecithin and soybean oil (injectable grade), are clinically available for several decades and are used in current parenteral formulations.

LNs are useful for the incorporation of lipophilic drugs. Due to limited solubility of the drug in the lipid matrix and drug distribution into the aqueous phase during the production process, conventionally prepared LNs of hydrophilic drugs will probably have a poor drug loading and drug encapsulation efficiency. Thus, the difficulty in preparing hydrophilic drug loaded lipid nanoemulsions formulations combining high drug entrapment efficiency with controlled drug release represents a real challenge.

The aim of this study was to prepare DOX loaded LNs using biocompatible lipid compounds which are used in current parenteral formulations. In the present study, we selected oleic acid (OA) as the lipophilic complexing agent to prepare doxorubicin-oleic acid ionic complex (DOX-OA) to enhance the lipophilicity of DOX. Then we prepared doxorubicin-oleic acid complex loaded lipid nanoemulsions (DOX-OA-LNs) with high encapsulation efficiency by a simple high pressure homogenization method and evaluated the physicochemical and *in vitro* release properties of DOX-OA-LNs. Afterwards, the DOX-OA-LNs and free DOX were compared with respect to their *in vitro* cellular uptake and cytotoxicity, and their *in vivo* pharmacokinetics and biodistribution behaviour in mice were also investigated.

2. Investigations, results and discussion

2.1. Preparation of DOX-OA

Ion pair formation has been used as a method of modifying the lipophilicity of ionizable drugs by shielding their charge with an oppositely charged ion without chemical modifications (Higashiyama et al. 2007). Because DOX has the amino sugar moiety, daunosamine, which bears the positive electrostatic charge localized at the protonated amino nitrogen, lipophilic counterions have been employed to facilitate the entrapment of DOX in lipid vehicles. The Gasco's group (Cavalli et al. 1993) showed that both decyl phosphate and hexadecyl phosphate could form ion-pairs with DOX, and the resulting ion-pair complexes increased the lipophilicity of DOX, which facilitated the incorporation of DOX into the SLNs. Ma et al. (2009) used anionic ion-pairing agents, sodium taurodeoxycholate and sodium tetradecyl sulfate, to neutralize the charges of the cationic DOX and enhance entrapment of DOX in the SLNs. However, the disadvantage of the ion-pair method used by the forementioned two research groups is that the ion-pairing agents they used are potentially toxic and could not be utilized for *i.v.* injection. In our study, OA was used as the complexing agent due to the fact that OA is a kind of biodegradable and physiological long chain fatty acid which shows low toxicity. Moreover, it is clinically available for parenteral usage (Pontes-Arruda 2009).

During the preparation process of DOX-OA, bicarbonate solution was used to neutralize the hydrochloric acid of doxorubicin hydrochloride instead of other bases such as sodium hydroxide, because DOX is instable and decomposes easily in strong alkalinous environment. In the preparation process, OA was dissolved in ethanol and subsequently the solution was added to the aqueous solution of doxorubicin base. In this way, OA could disperse in aqueous solution more easily than adding OA directly into the DOX solution, so that it could react with DOX more adequately.

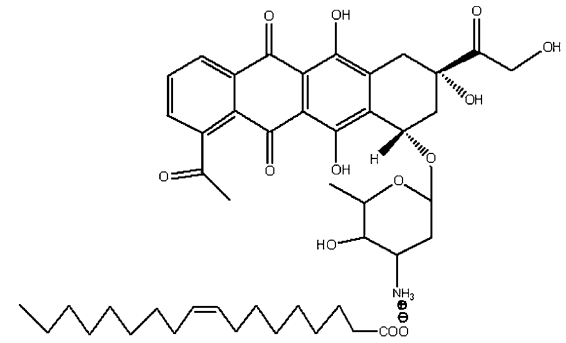

Fig. 1: Structure of the doxorubicin-oleic acid ionic complex

DOX-OA also can be prepared by the codissolve-evaporation method (Olbrich et al. 2002, 2004). Briefly, DOX-OA was prepared by dissolving 50 mg of doxorubicin base and 100 mg of OA in dichloromethane and consequent evaporation of solvent at 30 °C under reduced pressure. Compared with the codissolve-evaporation method, the stirring method could avoid the use of toxic organic solvent. Moreover, the process of this method was more convenient.

The formation of an ionic complex is generally driven by overall forces such as electrostatic attraction, hydrophobic interaction and hydrogen bonding (Higashiyama et al. 2007). Although solvents with low dielectric constant are favored for ion pair formation, the contribution of hydrophobic interaction is relatively large as well as electrostatic force once the ion pair is formed in aqueous solution (Quintanar-Guerrero et al. 1997). What mentioned above explained why the DOX-OA could be prepared both in water and dichloromethane. According to the study performed by Kalinkova (2007), the ionic complexes obtained by interactions between aliphatic amines and carboxylic acids have a structure type of the ion pair and complex composition of 1:1. Similarly, it was presumed that DOX-OA was formed through electrostatic forces derived from the charge neutralization between the basic amino group of DOX and the acidic carboxyl group of OA, and the structure of DOX-OA is shown in Fig. 1. OA was an effective counter-ion for DOX in our LNs formulation. The adding of OA to aqueous solution of doxorubicin base resulted in immediate formation of ion-pair complex as red precipitates. More than 99.7% of DOX could ion-pair with OA during this complexation process, which was almost complete. Investigations showed that the formed DOX-OA was very soluble in organic solvents, especially in ethanol, ethyl acetate, and acetone.

2.2. Determination of octanol/water partition coefficient

The octanol/water partition coefficient (log $P_{O/W}$) of doxorubicin hydrochloride, doxorubicin base and DOX-OA was −1.79, −1.32 and 1.81, respectively. It was shown that the lipophilicity of doxorubicin base was a few stronger than that of doxorubicin hydrochloride. However, the lipophilicity of DOX-OA was much stronger than that of doxorubicin hydrochloride. These results indicated that the ion pair formed between DOX and OA could mask the positive charge of DOX, and that the long alkyl chain of OA increased the lipophilicity of DOX-OA thereby facilitating the distribution of DOX in the organic phase. The enhanced lipophilicity of DOX-OA could contribute to the increased solubility of DOX in lipid matrix and therefore improved the entrapment efficacy of DOX in LNs.

2.3. Preparation of DOX-OA-LNs

In this study, we developed an economical, simple and reproducible method, and it was free of toxic organic solvent during

preparation of LNs. Most importantly, the preparation process of DOX-OA-LNs was feasible for industrial scale-up production. High pressure homogenization (HPH) has emerged as a reliable and powerful technique for the preparation of LNs, which is a technique well established on the large scale since the 1950s and is already available in the pharmaceutical industry.

The parenteral acceptability of the lipids (e.g. stearic acid, hexadecanoic acid) is the major hurdle for the successful commercialization of SLNs. It is more feasible to use glycerides of the fatty acids which are present in the lipid phase of parenteral fat emulsions (e.g. soybean oil, medium-chain triglyceride) to fabricate LNs with high biocompatibility (Joshi and Müller 2009; Wolfram et al. 1989). In our study, the components of LNs were lecithin, OA and soybean oil (injectable grade). They are biodegradable and physiological lipids which show low toxicity. Moreover, these materials are accepted by the regulatory authorities (e.g. FDA) and are clinically available for *i.v.* injection (Pontes-Arruda 2009).

A serious problem related to LNs is the need of large amounts of surfactants. In most cases, surfactants are added to the formulation of LNs to stabilize the highly increased particle surface and hinder the coalescence of droplets. Type and amount of surfactants involved in the fabrication procedure obviously have an important effect on physical and pharmaceutical properties of LNs, such as particle size and zeta potential values being important for physicochemical stability as well as biopharmaceutical properties of the preparation. However, many of these stabilizers such as Cremophor EL and Tween 80 have a low biocompatibility in parenteral administration. The safety and efficacy of the excipients play an important role in case of parenteral application. It should be kept in mind that GRAS (Generally Recognized as Safe) surfactants used to prepare LNs for *i.v* injection should obtain approval and have a good *in vivo* tolerability. In our formulation of DOX-OA-LNs, the only surfactant used was lecithin, which is native and well tolerated in humans. Moreover, it has been widely used in parenteral nutrition emulsions. Therefore, there was no problem related to toxicity of surfactants in DOX-OA-LNs.

In order to optimize the formulation of DOX-OA-LNs, the influence of drug/lipid ratio on the particle size distribution and the polydispersity index (PDI) was investigated. The size and morphology of LNs were significantly influenced by the content of phospholipid. When the drug/lipid ratios were more than 1/4 (w/w), LNs showed large particle size and broad size distribution (data not shown), which could be attributed to insufficient amount of phospholipid as surfactant. As the drug/lipid ratio decreased to 1/6 (w/w), the obtained LNs showed the optimal particle size and polydispersity.

In order to study the conditions of HPH, the influence of parameters such as applied pressure, homogenization cycles and temperature on particle size distribution was investigated. The temperature affected the size distribution of DOX-OA-LNs slightly due to the fact that the melting point of the lipids used in DOX-OA-LNs was relatively low, so the homogenization process could be performed at room temperature to avoid the degradation of DOX which is sensitive to high temperature. In our trials, 7 homogenization cycles at an operating pressure of 100 MPa were sufficient to prepare DOX-OA-LNs with optimal particle size and PDI. Increasing the homogenization pressure or the number of cycles resulted in an increase of the particle size due to droplets coalescence which occurred as a result of the high kinetic energy of the nanoemulsion droplets.

2.4. Particle size, Zeta potential and morphology

The size distribution of DOX-OA-LNs measured by photon correlation spectroscopy (PCS) showed one narrow peak indicating that the nanoemulsion droplets population was relatively homogenous in size. The calculated mean size of DOX-OA-LNs, based on three separate measurements, was 208.5 ± 26.7 nm with PDI of 0.136 ± 0.023, which indicated that the DOX-OA-LNs were uniform and mono-disperse. The mean particle size and PDI of DOX-OA-LNs were much desirable due to the optimal formulation and preparation parameters of DOX-OA-LNs. The TEM micrograph of DOX-OA-LNs is shown in Fig. 2, from which it could be found that the shape of nanoemulsion droplets was spherical and the particle size approximately ranged from 100 to 200 nm, corresponding to the PCS results.

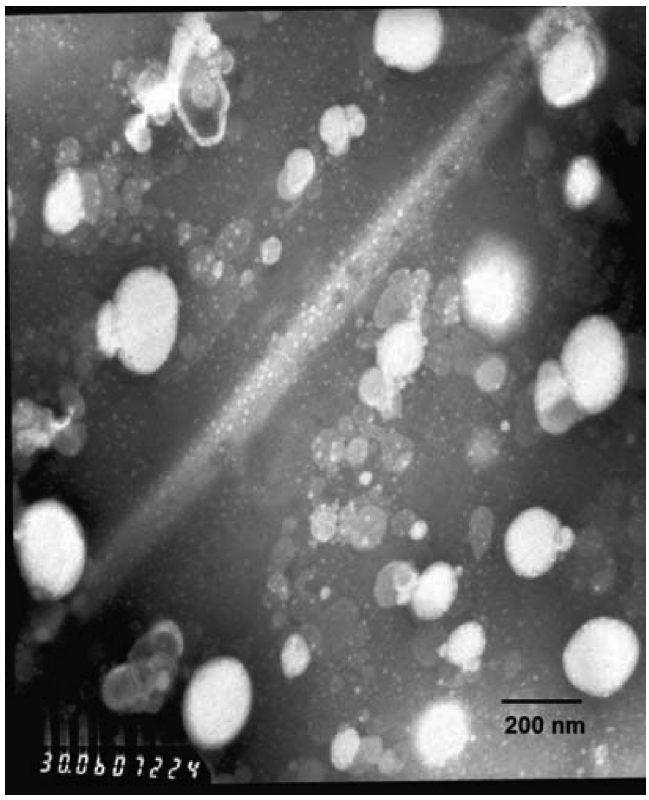

Fig. 2: Transmission electron microscopy micrograph of DOX-OA-LNs aqueous suspensions. Scale bar: 200 nm

The zeta potential was -28.5 ± 3 mV, which was as high as the absolute value of 30 mV to satisfy the stability requirement solely deduced from the electrostatic interaction, showing the good stability of DOX-OA-LNs. Zeta potential is essential to the storage stability of colloidal dispersion, the presence of small amounts of phosphatidylserine and phosphatidylglycerol (2–5%) in Lipoid E80 resulted in a negative surface charge (Zhao et al. 2008; Li et al. 2008). In addition, due to an accumulation of the negatively charged ionized carboxy groups on the interface, OA could also act as co-emulsifier leading to more negative zeta potential, which resulted in a higher resistance to coalescence of the droplets.

2.5. Entrapment efficiency and drug loading

Commonly, it is considered that LNs are mainly applicable to hydrophobic drugs, while they could not achieve high entrapment efficacy for hydrophilic drugs. The incorporation efficiency may be altered by several factors such as the physicochemical properties of drugs and the structure of LNs. In our study, the entrapment efficacy of DOX-OA-LNs based on DOX-OA was $93.7 \pm 1.2\%$. However, it was found in the preliminary experiment that the LNs incorporating DOX without the OA complexation only could reach a low incorporation efficiency

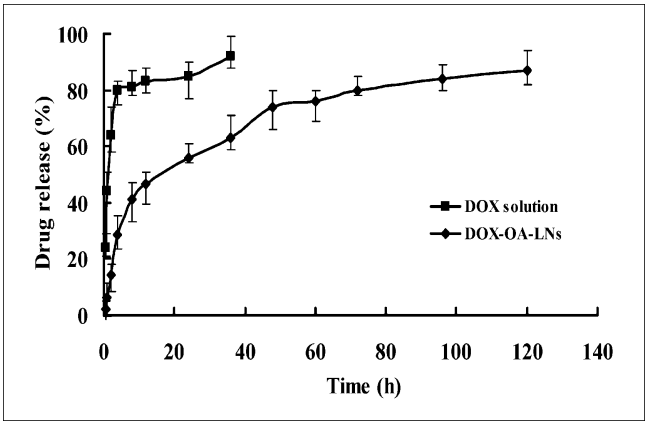

Fig. 3: *In vitro* release curves of DOX from DOX solution and DOX-OA-LNs in PBS (0.01 M, pH 7.4) containing 0.2% w/v of Tween 80 (n = 3)

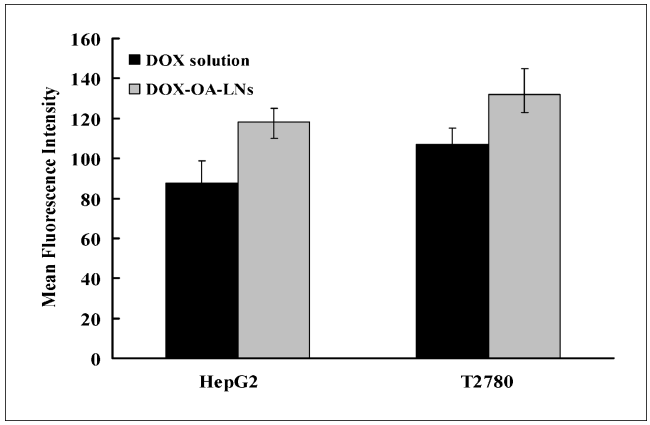

Fig. 4: Intracellular DOX fluorescence intensity in HepG2 and T2780 cells which were incubated with DOX solution or DOX-OA-LN at 10 μM DOX concentration for 3 h at 37 °C. Data represent mean ± SD (n = 3)

of 30%. The high affinity and incorporation of DOX in the lipid matrix of DOX-OA-LNs with high incorporation efficiency were achieved owing to the enhanced lipophilicity of DOX-OA compared to free DOX.

The drug loading content of DOX-OA-LNs was 7.4%. In this system, relatively high drug loading efficiency could be achieved by DOX-OA-LNs, avoiding the administration of redundant excipients.

2.6. *In Vitro* release study

The *in vitro* release behaviors of free DOX solution and DOX-OA-LNs are shown in Fig. 3, from which it could be found that the drug release from free DOX solution was much faster than DOX-OA-LNs. The free DOX solution released 27.8% of DOX within 0.5 h. In contrast, the cumulative release amount of DOX-OA-LNs was only 2.74% within 0.5 h, demonstrating that there was no burst effect for DOX-OA-LNs. This phenomenon can be attributed to the fact that the hydrophobicity of DOX has been notably increased by the formation of DOX-OA and a hydrophobic interaction between drug and matrix lipids may retain the drug.

More than 80% of drug was released from free DOX solution into the medium after 8 h, whereas the cumulative amount of DOX released from DOX-OA-LNs was less than 40% until 12 h, indicating that the dissolution of DOX from the lipid matrix was slowly and DOX-OA-LNs were responsible for the delayed release of DOX.

The drug release profile of DOX-OA-LNs could be best characterized by the exponential model and the following regression equation was given: $F(t) = 15.594 \cdot \ln t + 8.3197$, correlation coefficient $(r) = 0.996$, where $F(t)$ is the accumulative drug release (%), and t is time (h). The release profile of DOX-OA-LNs was biphasic. The initial fast release was observed in the beginning of 24 h, due to the large surface area of the LNs and drug enrichment in the outer shell of the droplets. In the latter stage, drug release was constant and slow. It could be attributed to the assumption that the lipophilic DOX-OA solubilized or dispersed in lipid matrix and therefore DOX released mainly by dissolution and diffusion of drug from the lipid matrix.

In conclusion, the drug release from DOX-OA-LNs was drastically delayed as a result of the increase in the lipophilicity of the complex and the incorporation of the drug into the lipid matrix.

2.7. Flow cytometry analysis

Uptake of DOX-OA-LNs into HepG2 and T2780 cells in comparison to free DOX was quantitatively analyzed by the flow cytometry method. DOX was used directly to measure cellular uptake without additional markers since DOX itself is fluorescent, and its fluorescence intensity is directly proportional to the amount of DOX internalized (Upadhyay et al. 2010). In addition, many researchers have utilized flow cytometry for quantitative determination of DOX uptake in cells (Zheng et al. 2009; Prabaharan et al. 2009; Zhang et al. 2010).

Fig. 4 shows the intracellular DOX fluorescence intensity after HepG2 and T2780 cells were incubated with free DOX or DOX-OA-LNs for 3 h at 37 °C. There was little or no statistical difference in the cell associated fluorescence between free DOX and DOX-OA-LNs within each cell line. For example, the cell-associated fluorescence intensity of free DOX and DOX-OA-LNs was 87.6 ± 11 and 107 ± 8 in HepG2, respectively, and 118 ± 9 and 132 ± 13 in T2780, respectively. The results shown in Fig. 4 indicate that the DOX also could be successfully transported into cells by being loaded in DOX-OA-LNs.

The cellular uptake of DOX might be an integrated result of the nature of drug, the particle size, zeta potential and surface characteristics of the drug carriers, the type and state of cells, and so on. As for the liposomal DOX, the intracellular DOX amount is lower than that of free DOX. Many researchers (Li et al. 2009; Xiong et al. 2005) reported that the highest amount of intracellular DOX was found in cells that had been incubated with free DOX and the intracellular DOX for DOX liposomes in various cells was much lower than that for free DOX. The relative enhanced intracellular DOX of DOX-OA-LNs compared with the liposomal DOX can be ascribed to the presumption that DOX-OA-LNs were more readily internalized than DOX liposomes due to the enhanced lipophilicity of DOX by forming DOX-OA.

2.8. Intracellular distribution of DOX by confocal microscopy analysis

The intracellular distribution of free DOX and DOX-OA-LNs was analyzed by confocal microscopy in HepG2 and T2780 cell lines. Fig. 5 showed the confocal microscopy images of HepG2 and T2780 after incubated with DOX-OA-LNs or free DOX at 37 °C for 3 h.

For both the free DOX and the DOX-OA-LNs, the DOX fluorescence was mainly distributed in the nuclei of the cells. After 3 h of incubation with the DOX solution in HepG2 and T2780 cells, strong fluorescence of DOX was observed in cell nuclei in addition to a very weak fluorescence in the cytoplasm, suggesting rapid intercalation of intracellular DOX to the chromosomal DNA after passive diffusion into the cells without the release

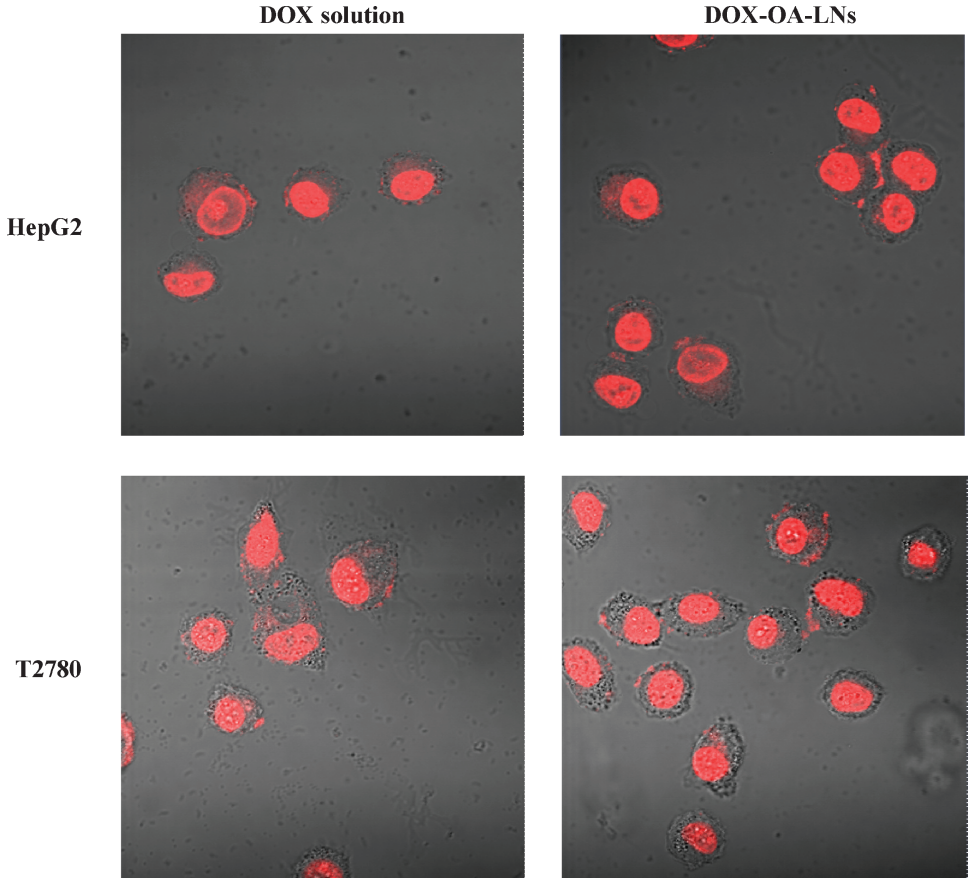

Fig. 5: Confocal microscopy images of HepG2 and T2780 cells incubated with free DOX solution or DOX-OA-LNs at a DOX concentration of 20 μg/mL diluted in culture medium at 37 °C for 3 h. (Magnification 63×)

process. While HepG2 and T2780 cells were incubated with DOX-OA-LNs, as shown in Fig. 5, intense DOX fluorescence at the nucleus was also observed in the two cell lines.
Thus, the results illustrated above evidenced that DOX-OA-LNs nearly did not change the pattern of sub-cellular distribution of DOX and also could deliver DOX to the nuclear compartment of the cells successfully, where the chromosomal DNA was the target site of the DOX.

2.9. In Vitro cytotoxicity study

The survival curves of HepG2 and T2780 cells showed a concentration-dependent manner of action, as shown in Fig. 6. It showed that DOX-OA-LNs exhibited higher inhibition rates than those of the free DOX group at the same concentration.
The 50% inhibitory concentration (IC_{50}) of free DOX and DOX-OA-LNs is shown in Table 1. DOX-OA-LNs exhibited significantly higher inhibition rates than free DOX on HepG2 and T2780 cells ($P < 0.05$). Compared with free DOX, the IC_{50} of DOX-OA-LNs decreased from 1.44 ± 0.17 to 0.41 ± 0.33 μg/mL on HepG2, and from 1.11 ± 0.24 to 0.69 ± 0.18 μg/mL on T2780, respectively.

Table 1: IC_{50} values of free DOX solution and DOX-OA-LNs on different cell lines (24 h, $n = 3$)

Cell lines	IC_{50} (μg/mL)	
	Free DOX solution	DOX-OA-LNs
HepG2	1.44 ± 0.17	$0.41 \pm 0.33^*$
T2780	1.11 ± 0.24	$0.69 \pm 0.18^*$

$^*P < 0.05$ versus DOX solution

The cytotoxicity *in vitro* might be influenced by many factors such as the nature of drug, drug release from LNs, the type, characteristics and state of cells, time of contact, and so on (Zhang et al. 2010). The increase cytotoxicity of DOX incorporated in LNs may be related to the successful internalization of DOX-OA-LNs due to the enhanced lipophilicity of DOX by forming DOX-OA and the successive drug release from DOX-OA-LNs inside the cells, enhancing the action of DOX.
In addition, blank LNs exhibited low toxicity or even nontoxicity under various concentrations, as shown in Fig. 6, indicated that the excipients in this formulation were little toxic or even nontoxic under formulation concentrations and the lipid matrix of LNs were well tolerated.

2.10. Pharmacokinetics and biodistribution of DOX-OA-LNs in mice

The plasma concentration-time curves of different DOX formulations are reported in Fig. 7. After injection of DOX-OA-LNs, DOX was still present in the plasma of mice after 6 h, while after 3 h the drug could not be detected in the plasma of the mice injected with the DOX solution. The comparative pharmacokinetic parameters after *i.v.* administration of the DOX formulations are reported in Table 2. The plasma kinetics of DOX-OA-LNs showed a higher AUC, a lower rate of clearance, and a smaller volume of distribution in comparison to those of the DOX solution, as shown in Table 2. This is probably due to the slower release of DOX from DOX-OA-LNs than from the free DOX solution.
However, the circulation time of DOX-OA-LNs was relatively shorter than that of the pegylated DOX liposomes and SLNs

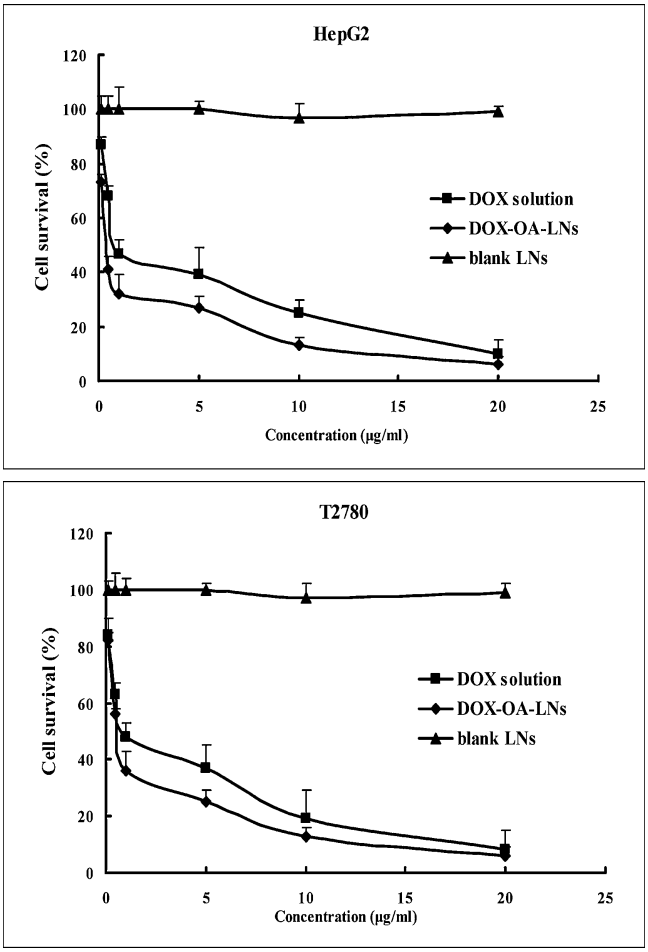

Fig. 6: Survival curves of HepG2 and T2780 cells after 24 h of treatment of free DOX solution, DOX-OA-LNs and blank LNs (n = 3)

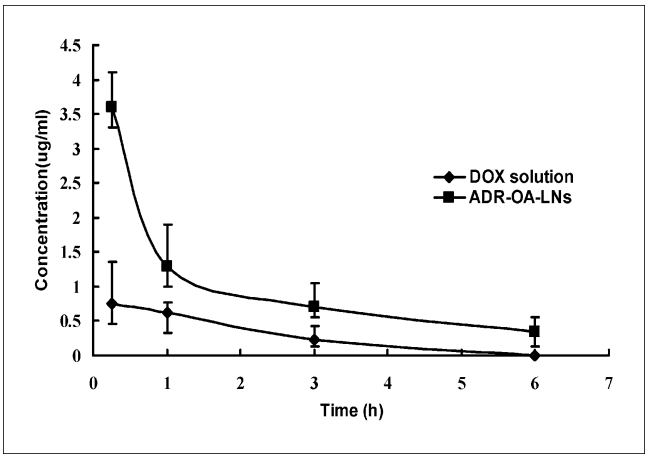

Fig. 7: Plasma concentration-time profiles of DOX following i.v. administration of DOX solution and DOX-OA-LNs (10 mg/kg). Each value represents the mean ± SD (n = 6)

Table 2: Pharmacokinetic parameters of free DOX solution and DOX-OA-LNs

Parameters	DOX solution	DOX-OA-LNs
$T_{1/2}$ (h)	1.554 ± 0.19	2.889 ± 0.48*
AUC (mg/L h)	1.927 ± 0.31	6.923 ± 0.74*
MRT (h)	1.419 ± 0.27	2.323 ± 0.18*
V_d (L/kg)	11.284 ± 1.73	1.483 ± 0.29*
Cl (L/h/kg)	4.057 ± 1.32	1.198 ± 0.48*

Each value represents the mean ± SD ($n = 6$). * $P < 0.05$ versus DOX solution

reported in the literature (Working et al. 1994; Fundarò et al. 2000), and this can be attributed to the following two reasons. Firstly, most of the DOX-OA was located in the oil droplet of the LNs, but some was located at the interfacial lecithin layer between the oil droplet and the aqueous phase and might release rapidly when the DOX-OA-LNs interacted with serum components in the blood stream. Secondly, the formulation of DOX-OA-LNs did not include the stealth agent, such as the pegylated lecithin (e.g. PEG-DSPE). Colloidal drug carriers are rapidly removed from systemic circulation after *i.v.* injection due to their recognition as foreign bodies by the mononuclear phagocyte system (MPS) (Moghimi et al. 2001). The elimination of such colloidal systems is influenced by various parameters such as: the nature of the components, the size, apparent electrical charge and hydrophilicity of the colloidal carriers (Vonarbourg et al. 2006). Coating with hydrophilic polymer chains, such as PEG and its derivatives, to the surface of the colloidal drug carriers provides a highly hydrophilic shield for them from electrostatic and hydrophobic interactions with serum components as well as reduced uptake by MPS. In comparison to conventional nanocarriers without PEG modification, these pegylated colloidal drug delivery systems showed a prolonged retention time in blood, primarily due to the reduced recognition and uptake by phagocytic cells of the MPS located mainly in the liver and spleen. Thus, they are less readily cleared from circulation and showed prolonged circulation time in the blood stream (Mosqueira et al. 2001; Moghimi and Szebeni 2003). Therefore, the DOX loaded pegylated liposomes or SLNs protected by the stealth agent were more stable while circulating in the blood stream than DOX-OA-LNs and exhibited relatively longer circulation time.

Site-specific delivery of drugs and therapeutics can significantly reduce drug toxicity and increase the therapeutic effect. The pharmacokinetic properties of the drug in the nanocarriers, the vesicle size of the carriers and the vascular permeability of individual tissues will determine the extravasation and biodistribution profile of the drug loaded colloidal drug carriers. Biodistribution of DOX in different formulations is shown in Fig. 8. The area under the DOX concentration-time curves (AUC) values calculated for 1–48 h of different formulations in these tissues were listed in Table 3.

In particular, DOX-OA-LNs prevented the accumulation of DOX in the heart. Fig. 8A shows that DOX concentration in the heart (a primary target organ for doxorubicin-related cardiotoxicity) of mice was significantly lower in DOX-OA-LNs group than in free DOX solution group at all time points. The lower DOX heart concentration could determine a lower cardiotoxicity, which was one of the effects limiting the usage of DOX. Fig. 8B and Fig. 8C show that when DOX-OA-LNs were *i.v.* administered, DOX in the lung and kidney was less than that of DOX solution at each time point, respectively. The lower DOX concentration found in heart, lung and kidney in DOX-OA-LNs-treated mice and the concomitant higher plasmatic level, could be related to a slow drug release from DOX-OA-LNs to the blood or to the targeted organs.

Behavior regarding RES tissues differed between DOX-OA-LNs and the DOX solution. Figs. 8D and Fig. 8E showed that when the DOX-OA-LNs were *i.v.* administered, less DOX was taken up by the liver and spleen tissue than that of DOX solution at the initial time (0–3 h), but 6 h later the DOX concentration in the liver and spleen tissue was higher than that of DOX solution. The delayed accumulation of the DOX in the DOX-OA-LNs form in the liver and spleen was in agreement with the slow plasma clearance rates shown above.

Overall, the DOX-OA-LNs showed an improved pharmacokinetic profile, and altered tissue distribution, which could be related to the enhanced lipophilicity of DOX by forming the

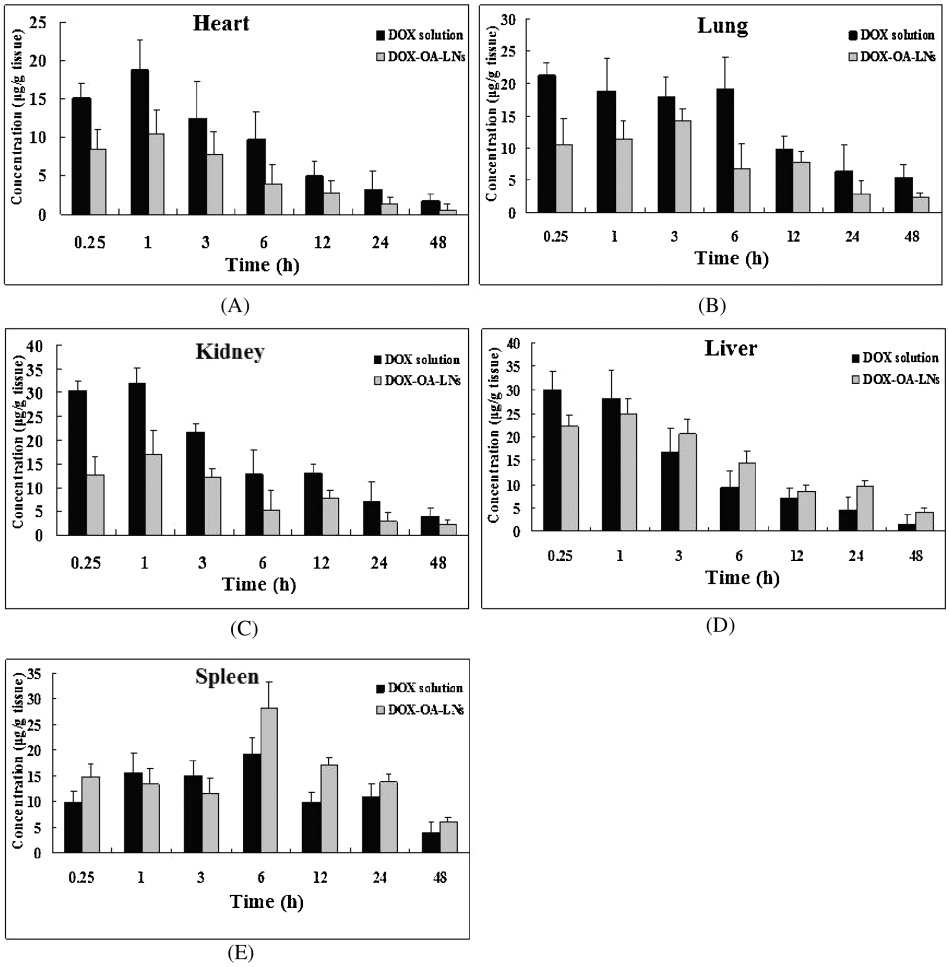

Fig. 8: Tissue distribution of DOX after intravenous administration of DOX solution and DOX-OA-LNs (10 mg/kg). Each value represents the mean ± SD (n = 6)

ion-pair and the slow drug release from the DOX-OA-LNs in the blood or targeted organs.

In conclusion, the present study demonstrated that a novel doxorubicin-oleic acid ionic complex was prepared to alter the solubility of DOX in water and organic solvents and entrap DOX into lipid nanoemulsions. The method of preparing the ionic complex with oleic acid opens a new prospective for carrying positively charged drugs or drugs with basic amino groups into lipid-based colloidal drug carriers with high entrapment efficiency and controlled release, which needs further research. In addition, a simple but successful high pressure homogenization method, feasible for scale-up production, was employed to prepare DOX-OA-LNs. This HPH method allowed instantaneous and reproducible formation of DOX-OA-LNs, with a diameter value around 200 nm, small PDI (< 0.2), high entrapment efficiency (> 90%) and improved release properties. Most importantly, all the used materials were approved for *i.v.* injection, so the preparation has a great potential for clinical application. The drug release behavior from the lipid nanoemulsions exhibited a biphasic pattern with rapid release at the initial stage and sustained release afterwards. The *in vitro* cytotoxicity studies revealed that the IC_{50} value of DOX-OA-LNs was lower than that of free DOX. Flow cytometry and confocal microscopy studies showed that the cellular uptake of free DOX and DOX-OA-LNs were comparable. Pharmacokinetics and biodistribution studies in mice showed that DOX-OA-LNs demonstrated higher DOX levels in blood and longer circulation time than free DOX. Moreover, DOX-OA-LNs significantly decreased DOX concentration in heart, lung and kidney. Hence, this novel formulation has a promising potential as an alternative parenteral colloidal delivery system of DOX for cancer treatment.

3. Experimental

3.1. Materials

Doxorubicin hydrochloride was kindly offered by Haizheng Pharmaceutical CO., Ltd (Taizhou, China). Purified yolk lecithin (Lipoid E80) and oleic acid were purchased from Lipoid CO., Ltd (Germany). Vitamin E was purchased from Southwest Synthetic Pharmaceutical Co., Ltd (Chongqing, China). Soybean oil was provided by Beiya Medical oil CO., Ltd (Tieling, China). Trypsin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenltetrazolium bromide (MTT) were purchased from Sigma (USA). All the other chemical solvents and reagents were of analytical grade or better.

Table 3: Tissue AUC_{0-48} (h μg/g) values of DOX solution and DOX-OA-LNs in heart, liver, spleen, lung and kidney

Formulations	Heart	Liver	Spleen	Lung	Kidney
DOX solution	240.84 ± 57.42	337.68 ± 78.58	591.85 ± 87.32	629.19 ± 93.78	504.38 ± 132.17
DOX-OA-LNs	124.68 ± 36.14*	577.57 ± 62.17*	821.69 ± 104.46*	241.47 ± 74.51*	295.21 ± 46.25*

Each value represents the mean ± SD ($n = 6$). * $P < 0.05$ versus DOX solution

3.2. Tumor cell lines and cell culture

HepG2 cell line and T2780 cell line were provided by Shanghai Institutes for Biological Sciences (SIBS; Shanghai, China). HepG2 cells were grown in RPMI 1640 medium and T2780 cells were grown in Dulbecco's modified Eagle's medium (DMEM), respectively. Both cells lines were cultured in medium supplemented with 10% calf serum (Minhai, Gansu, China), 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

3.3. Animals

Healthy male Kunming mice (15–25 g) were obtained from Laboratory Animal Center of Sichuan University (Chengdu, China). Prior to the experiments, the mice were housed in a temperature and humidity controlled room (25 ± 1 °C, 55% air humidity) with free access to water and standard mice chow, and they were acclimated for at least 5 days. All the animal experiment protocols and procedures were approved and supervised by Animal Ethics Committee of Sichuan University.

3.4. Preparation of DOX-OA

To neutralize the charges of cationic doxorubicin hydrochloride salt form and facilitate drug entrapment in the oil phase, DOX-OA was prepared using a co-precipitation method. The amount of DOX used was calculated as the base form throughout the experiments. Aqueous solution of doxorubicin base was prepared by addition of 0.2 mL sodium bicarbonate solution (50 mg/mL) to 10 mL aqueous solution of doxorubicin hydrochloride (5 mg/mL) in a 25 mL round bottom flask. DOX-OA was prepared by adding 2 mL ethanol solution of oleic acid (50 mg/mL) to the obtained doxorubicin base solution under stirring. Under continuous stirring at room temperature, a cloudy solution was spontaneously developed as a result of the complex formation. DOX-OA, a red precipitate, was collected by centrifuging. The supernatant was diluted with 0.12 M HCl in ethanol and subsequently quantified (excitation/emission: 490/580 nm) by fluorescence spectrophotometer (RF-5301 PC, Shimadzu, Kyoto, Japan) to determine the concentration of DOX in the supernatant. The percentage of DOX complexed with OA was calculated as follows:

$$\text{Percentage of DOX ion-paired with OA} = [\,1\text{- (weight of drug in supernatant/weight of the drug added)}] \times 100\% \quad (1)$$

The resultant complex was washed three times with water for injection, and then it was sealed and kept in a desiccator at room temperature.

3.5. Determination of octanol/water partition coefficient

Octanol/water partition experiments were performed by the shaking flask method previously reported (Montero et al. 1997) using the following experimental conditions.

Doxorubicin base was prepared by addition of excess sodium bicarbonate to an aqueous solution of doxorubicin hydrochloride, followed by extraction into chloroform and solvent evaporation. Water and octanol were mutually saturated for 24 h before the experiment.

Firstly, 1 mL aqueous solution of doxorubicin hydrochloride (1 mg/mL) and 1 mL aqueous solution of doxorubicin base (1 mg/mL) were mixed with 3 mL octanol respectively, and 1 mL octanol solution of doxorubicin-oleic acid complex (equivalent to 1 mg/mL doxorubicin base) was mixed with 3 mL water. Consequently, the two phases were vigorously vortexed for 10 min and agitated for 24 h in a thermostatted shaker bath (Shenzhen worldwide industry, Co., Ltd., China) at 25 ± 0.1 °C.

After equilibration, the samples were centrifuged at 18000 × g at 25 °C for 15 min, and then both phases were diluted with 0.12 M HCl in ethanol and subsequently assayed by fluorescence spectrophotometry to determine the concentration. Each experiment was performed at least in triplicate. The partition coefficient was calculated by the following equation:

$$P = (Co \times Vw)/(Cw \times Vo) \quad (2)$$

(Co: concentration of DOX in octanol at equilibrium; Vo: volume of octanol in sample; Cw: concentration of DOX in water at equilibrium; Vw: volume of water in sample)

3.6. Preparation of DOX-OA-LNs

DOX-OA-LNs were prepared by a simple lipid film hydration-high pressure homogenization method. Briefly, 300 mg DOX-OA (DOX/OA, 1/2, w/w), 600 mg lipoid E80, 50 mg vitamin E and 400 mg soybean oil were dissolved into 200 mL ethanol in a 500 mL round bottom flask. The organic phase was subsequently removed by a rotary evaporator (Büchi, Switzerland) under reduced pressure at 45 °C until a thin lipid film formed.

The dried lipid film was rehydrated in 50 mL water, followed by vigorous vortex. Afterwards, this predispersion was passed through a high-pressure homogenizer (EmulsiFlex-C5, AVESTIN, Canada) for seven cycles at an operating pressure of 96.4–115.6 MPa, resulting in the formation of DOX-OA-LNs solution.

3.7. Characterization of DOX-OA-LNs

3.7.1. Measurement of particle size and Zeta potential

The mean particle size and zeta potential of DOX-OA-LNs were determined by photon correlation spectroscopy (PCS) (Malvern Zetasizer Nano ZS90, UK) at 25 °C.

3.7.2. Transmission Electron Microscopy (TEM)

The morphology of DOX-OA-LNs was examined by transmission electron microscopy (H-600, Hitachi, Japan). The sample was stained with 2% (w/v) phosphotungstic acid for 30 s and placed on copper grids with films for viewing.

3.7.3. Entrapment efficiency and drug loading

The entrapment efficiency of DOX-OA-LNs was determined by the ultrafiltration method previously reported (Ma et al. 2009). To separate the free drug from the LNs suspension, Nanosep® Centrifugal Filtration Devices (Mw cut-off 300 kDa; PALL Life Science, USA) were used. A fixed volume (400 μL) of the freshly prepared DOX-OA-LNs (1 mg/mL) was added to the sample reservoir tube and spun at 14000 × g at 25 °C for 60 min. The collected filtrate in the retentate vial was diluted with 0.12 M HCl in ethanol and analyzed by fluorescence spectrophotometry.

The encapsulation efficiency and drug loading were calculated by the following formulas:

$$\text{Encapsulation efficiency (\%)} = [\,1\text{ - (amount of drug in filtrate/amount of drug added)}] \times 100\% \quad (3)$$

$$\text{Drug loading content (\%)} = \text{(weight of drug added/weight of drug and excipients added)} \times 100\% \quad (4)$$

3.7.4. In Vitro release study

In vitro drug release from DOX-OA-LNs was investigated using a dialysis bag diffusion technique previously reported (Zhang et al. 2007) with some modifications. PBS (400 mL, 0.01 M, pH 7.4) containing 0.2% w/v of Tween 80 was used as the release medium at 37 ± 1 °C under constant shaking at 100 rpm with a thermostatted shaker bath (Shenzhen worldwide industry, Co., Ltd., China). An appropriate amount of DOX-OA-LNs was suspended in the cellulose membrane dialysis bag (Mw cut-off: 8000–12000; Millipore, USA) and immersed into the release medium. During the whole process the device was protected from light in order to avoid DOX photodegradation. At certain time intervals, 1 mL aliquot of the medium was pipetted out, and each withdrawal was followed by replacement with the same volume of fresh medium.

The amount of DOX released in the supernatant was determined by fluorescence spectrophotometry after the sample solution being diluted with 0.12 M HCl in ethanol. Simultaneously, the same amount of free DOX was tested as the control. Each experiment was performed in triplicate.

3.8. Cell experiments

3.8.1. Flow cytometry analysis

HepG2 and T2780 cell lines were used as model cancer cells for the examination of endocytosis. HepG2 and T2780 cell suspension (50×10^5 cells/well) was seeded in a six-well tissue culture plate (Corning, NY, USA) and incubated for 24 h at 37 °C, respectively. Then DOX-OA-LNs or free DOX solution was added to designated wells with a concentration of DOX as 10 μM. After the incubation period of 3 h, cells were trypsinized and pelleted by centrifugation, then washed three times with cold PBS and examined by flow cytometry using the FACScan Aria™ (Becton Dickinson, San Jose, CA, USA). The intracellular DOX was excited with an argon laser (488 nm) and fluorescence was detected at 540 nm. Files were collected of 10000 gated events and analyzed with the FACStation software program.

3.8.2. Confocal microscopy studies

A confocal fluorescent microscopy was used to compare the intracellular distribution of DOX (excitation/emission: 488/534 nm) between the free DOX and DOX-OA-LNs. Followed culture of HepG2 and T2780 cells for 24 h on 14-mm^2 sterile glass coverslips that were pre-soaked in culture dishes, free DOX solution and DOX-OA-LNs diluted in culture medium at a DOX concentration of 20 μg/mL were added to each dish and incubated for another 3 h at 37 °C, respectively. The medium was removed and cells were washed with cold PBS followed by fixing with 4% paraformaldehyde in PBS for 15 min. Fluorescent images of cells were examined by confocal microscopy (Carl Zeiss LSM510, Germany).

3.8.3. In Vitro cytotoxicity study.

In Vitro cytotoxicity of free DOX, DOX-OA-LNs and blank LNs were conducted in HepG2 and T2780 cell lines by MTT assay. Cells were plated at the density of 8000 cells per well in 200 μL medium in 96-well plates and grew for 24 h. Then the cells were exposed to series concentrations of free DOX, DOX-OA-LNs (0.1, 0.5, 1, 5, 10, and 20 μg/mL, respectively) and blank LNs for 24 h. A mixture of 20 μL of MTT solution (5 mg/mL in PBS, pH 7.4) was added to each well, and samples were incubated for 4 h in the absence of light. The medium was then removed and the converted dye was solubilized by adding 200 μL of DMSO. The absorbance was measured on a microplate reader (Bio-Rad Model 550; USA) at 570 nm. The cell viability (%) related to control wells containing cell culture medium was calculated according to the following equation and the 50% inhibitory concentration (IC_{50}) was evaluated, as well.

$$\text{Cell viability percentage (\%)} = \text{(OD of tested sample/OD of negative control)} \times 100 \quad (5)$$

3.9. Pharmacokinetics and biodistribution studies in mice

3.9.1. Drug administration and sample collection

The DOX-OA-LNs and DOX solution were administrated to mice via the tail vein at a dose of 10 mg/kg. Six mice were used at each predetermined time after *i.v.* injection (0.25, 1, 3, 6, 12, 24, and 48 h), and their blood and tissues (heart, lung, liver, kidney, and spleen) were collected. Plasma samples were obtained following centrifugation at 4000 × g for 4 min.

3.9.2. Sample preparation

Every tissue sample was accurately weighed, homogenized, and extracted with three volumes of 0.9% NaCl solution to gain a concentration of 250 mg/mL. Homogenized tissue samples (heart and spleen: 0.3 mL; liver, lung and kidney: 0.5 mL) and plasma samples (0.2 mL) were mixed with 100 μL daunomycin solution (5 μg/mL, dissolved in 0.9% NaCl solution) as the internal standard solution and 100 μL 10% trichloroacetic acid solution was added to the samples to precipitate protein, then 200 μL 3% sodium dodecyl sulfonate solution was added to the samples to ion pair with DOX. After the mixtures were vortexed for 3 min, they were mixed thoroughly with 3 mL ethyl acetate for 10 min on a vortexer and then centrifuged at 8000 × g for 10 min. The organic layer was transferred to a test tube, and the extract was evaporated to dryness under nitrogen gas at 50 °C in a water bath system. Dried samples were dissolved in 200 μL of the mobile phase and centrifuged at 12000 × g for 15 min. Then 50 μL of the clear supernatants were injected into the HPLC system.

3.9.3. HPLC analysis

The HPLC system was composed of a Series 3 Digital HPLC pump (Alltech, USA), a Model LC305 fluorescence detector (Ex, 460 nm, Em, 550 nm, Alltech, USA) and Kromasil ODS-1 column (250 mm × 4.6 mm, 5 μm). The mobile phase was a mixture of 0.05 M acetic acid (adjusted to pH 4.0 with triethylamine) and acetonitrile(55:45, v/v), and the flow rate was 1.0 mL/min.

3.9.4. Pharmacokinetics data analysis

The data was analyzed using non-linear regression analysis by the computer program DAS 2.0 (Drug and Statistics, Anhui, China).

3.10. Statistical analysis

All the experiments were repeated at least three times. The statistical analysis of the samples was performed by using a Student's t-test with P-values < 0.05 as the minimal level of significance.

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