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RESEARCH PAPER

Membrane Loaded Copper Oleate PEGylated Liposome Combined with Disulfiram for Improving Synergistic Antitumor Effect *In Vivo*

Lingli Zhou[1] • Liu Yang[1] • Chulei Yang[1] • Yi Liu[1] • Qiuyue Chen[2] • Wenli Pan[3] • Qing Cai[1] • Lifeng Luo[1] • Lu Liu[1] • Shan Jiang[1] • Haibing He[1] • Yu Zhang[1] • Tian Yin[4] • Xing Tang[1]

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ABSTRACT

Purpose This work aims to create a novel Cu^{2+} liposome with excellent loading stability and develop synergistic effect with disulfiram (DSF) for the treatment of tumor.

Methods Copper oleate was incorporated into the liposome membrane via alcohol injection method in this work. *In vitro* release test was applied to evaluate the release profile of the liposomes. Pharmacokinetic studies were performed in rats and the antitumor efficacy was assessed in mice bearing hepatoma xenografts.

Results The copper oleate liposome ($Cu(OI)_2$-L) was formulated and the loading efficiency were more than 85%. TEM images confirmed that the $Cu(OI)_2$-L had a spherical morphology with an average diameter of 100 nm. $Cu(OI)_2$-L displayed a biphasic release profile, with >70% retained drug over 8 h incubation in PBS at pH 7.4. Pharmacokinetic studies demonstrated that $Cu(OI)_2$-L had a prolonged circulation time and increased AUC when compared to the injection of copper oleate solution. The antitumor efficacy test demonstrated an enhanced tumor inhibition rate with the treatment of $Cu(OI)_2$-L and DSF nanoparticles, indicating an improved synergistic antitumor effect.

Conclusions The $Cu(OI)_2$-L was suitable to be employed in combination with disulfiram for tumor treatment and can also open up opportunities for targeted delivery of copper.

✉ Xing Tang
tanglab@126.com

[1] Department of Pharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University Shenyang 110016, China

[2] Wuya College of Innovation, Shenyang Pharmaceutical University Shenyang 110016, China

[3] School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University Shenyang 110016, China

[4] School of Functional food and Wine, Shenyang Pharmaceutical University Shenyang 110016, China

KEY WORDS antitumor efficacy · copper · disulfiram · liposome · synergistic effect

ABBREVIATIONS

5-Fu	5-fluorouracil
AUC	Area under concentration time curve
CTR1	Copper transporter 1
$Cu(DTC)_2$	bis(Dithyldithiocarbamate)-copper
$Cu(OI)_2$-L	Copper oleate liposome
$Cu(OI)_2$-S	Copper oleate solution
DDTC-Na	Sodium diethyldithiocarbamate
DSF	Disulfiram
EPR	Enhanced permeability and retention
IR	Infrared
MPS	Mononuclear phagocyte system
MRT	Mean residence time
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
NS	Normal saline solution
PBS	Phosphate buffer saline
PDI	Polydispersity index
PK	Pharmacokinetics
TEM	Transmission electron microscope
TIR	Tumor inhibition rate
UV	Ultraviolet

INTRODUCTION

Nanoparticle (NP)-based drug delivery systems have long been demonstrated to enhance the therapeutic efficacy and reduce systemic toxicity of antitumor drugs through targeted delivery to tumor sites (1). Prolonged pharmacokinetics can be achieved via surface modification of the nano-carrier with polyethylene glycol (PEG) chains, providing a steric effect that can avoid recognition and phagocytosis by the mononuclear

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phagocyte system (MPS) (2–4). Furthermore, nano-carriers with appropriate sizes can achieve increased accumulation in many types of solid tumors via the enhanced permeability and retention (EPR) effect (5,6). Among those nano-particles investigated in the clinic, the liposome is widely used due to its excellent biocompatibility, and the ability to incorporate both hydrophobic and hydrophilic drugs into its internal cores or lipid membrane, depending on the properties of the drug (7).

Disulfiram (DSF), a conventional anti-alcohol substance, manifests potent effects in cancer treatment in recent studies (8). Moreover, increasing evidence suggests that the anticancer mechanism proceeds in an Cu^{2+} dependent manner (9–11). DSF, as a bivalent metal ion chelator, can form complexes with Cu^{2+} and enhance the intracellular transport of Cu^{2+}. As Cu^{2+} is involved in a wide range of cellular physiological processes, this is speculated to be the main cause of the excess reactive oxygen species (ROS) generation in several solid tumors (12–14). In comparison to DSF and Cu^{2+} alone, DSF/Cu^{2+} complexes exhibit a much stronger ROS induction activity, causing a strong but transient killing effect on tumor cells. As well, the final product $Cu(DTC)_2$ exhibits a delayed but long-lasting cytotoxic effect.

The molecular target of $Cu(DTC)_2$ has not been elucidated clearly until a recent study reveals that it binds and immobilizes NPL4, which is an essential component of p97-NPL4-UFD segregase complex, thereby inhibiting the p97 pathway. (15) As a upstream pathway of proteasome-ubiquitin system, the p97 pathway inhibition triggered a series of cellular reactions, including blocking of protein degradation, accumulation of Poly-Ub protein, inhibition of NF-κB, consequently leading to the apoptosis and necrosis of the cancer cell.

However, due to the strict regulation of copper systems *in vivo*, traditional oral copper preparations are ineffective at enhancing cellular copper content (16,17). Dietary copper absorbed from the intestinal epithelial cells is reduced to Cu^{+} and imported into the liver by copper transporter 1 [CTR1]. Once the cellular copper content rises above the normal value, the genetic expression of CTR1 will be down-regulated, and only limited amounts of exogenous copper will be transferred intracellularly. At the same time, ATP7A and ATP7B, two Cu-transporting P-type ATPases, which are known to take charge of cellular copper efflux in mammals, translocate cellular copper to the membrane to excrete it out of the cell.

Since conventional oral copper formulations are not effective at raising cellular copper content, the approach of formulating copper into liposomes has been considered. Several approaches have previously been proposed to load copper into liposomes, including entrapping soluble copper salts inside the aqueous liposomal core (18), chelating copper with other anticancer drugs to form stable copper-drug complexes in the internal core of the vesicles (19) and attaching copper onto the surface of liposomes via a chelation method (20,21). However, when the highly water soluble cupric salts are employed for a liposome formulation, heavy leakage is prone to occur. To solve this problem, a new drug loading method for copper loading was reported in Jai Woong Seo's study (21), in which a ^{64}Cu-loaded liposome was prepared by incorporating a copper chelator-lipid conjugate in the lipid membrane, and the resultant formulation was stable in serum and exhibited long-circulating PK *in vivo*. This implies that loading a lipophilic form of copper into the membrane may provide more possibilities for *in vivo* copper delivery.

Based on this, the objective of this work was to develop a novel Cu^{2+}-loaded liposome formulation with great serum stability, for a more effective synergy therapy with DSF nanoparticles. Here, to improve the loading stability and encapsulation efficacy, an oil soluble salt form of copper, copper oleate, was synthesized and formulated in the liposome. Unlike the conventional water soluble form of copper, copper oleate is highly lipophilic, and thus a high encapsulation efficiency of more than 84% could be achieved by incorporating it into the lipid membrane. The liposome exhibits negative surface charge at pH 7 and hence, excellent liposome stability. Additionally, a negative surface charge of the liposome is proposed to exert a "fix effect" of copper ions through electrostatic forces. Drug release kinetics of the final formulation were evaluated *in vitro*, and pharmacokinetic studies were performed to assess the serum stability. Finally, the synergistic effects of the liposome formulation with DSF nanoparticles (NPs) (22) towards tumor treatment was evaluated, and the therapeutic efficiency was compared with the treatment of DSF NPs plus oral copper gluconate and DSF NPs alone (Fig. 1).

MATERIALS AND METHODS

Materials

Lipoid@ E80 and DSPE-PEG2000 were purchased from Lipoid (Ludwigshafen, Germany). The sodium oleate and copper chloride were purchased from Sinopharm Chemical Reagent (Shanghai, China). Chloroform was purchased from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). Cholesterol was purchased from J&K Chemical Technology (Beijing., China). All other reagents were purchased from Concord Technology (Tianjin, China).

Synthesis and Characterization of Copper Oleate

Copper oleate was synthesized by an ion exchange method as reported previously (23) and shown in Scheme 1. Based on the distinct dissolving properties, the final product was separated from the reaction mixture (a mixture of NaCl, $CuCl_2$, sodium oleate, copper oleate) by a liquid-separation operation. The

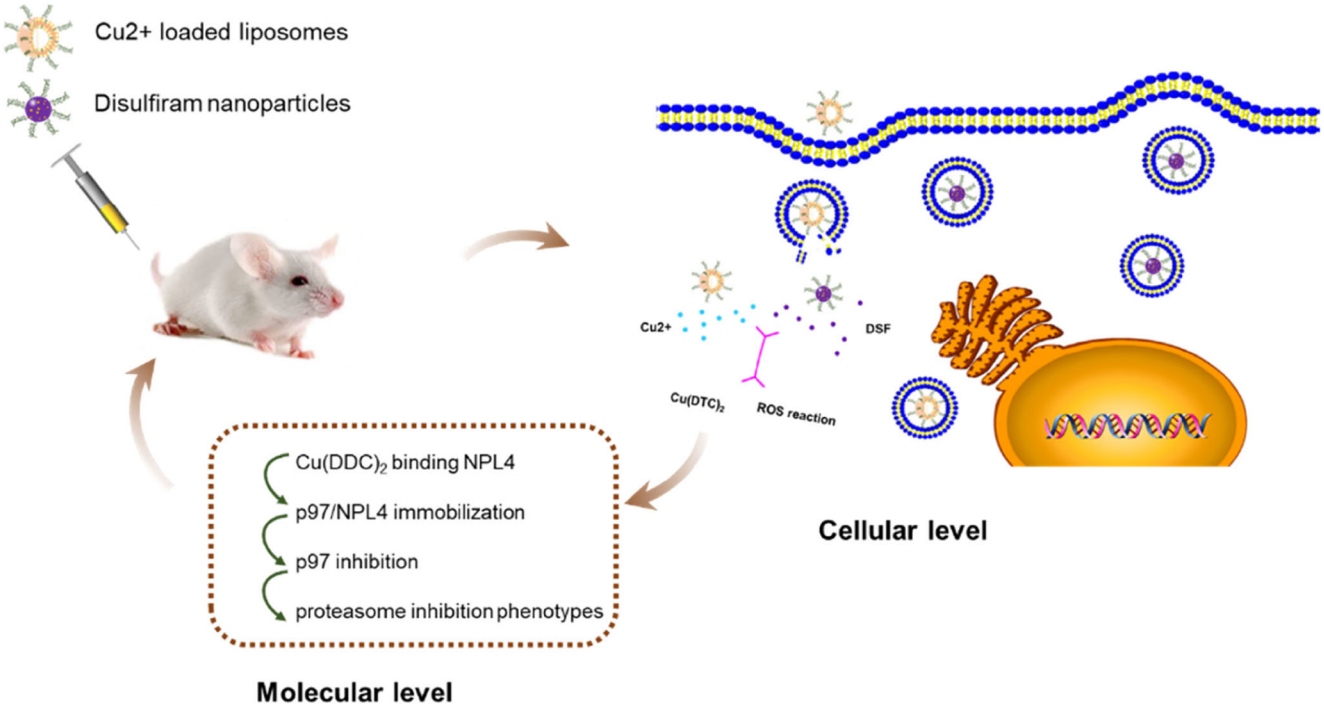


Fig. 1 Anti-tumor mechanisms of combined utilization of Cu^{2+} and disulfiram.

organic phase was washed by sterile water 3 times to purify the copper oleate. The successful synthesis of copper oleate via the ion exchange method was confirmed by IR, ^{1}H-NMR and TGA analysis, and the results are shown in Fig. 2a, b. Solubility tests of copper oleate in different aqueous solutions were carried out, which showed that the solubility of copper oleate was pH-dependent in aqueous solution, as shown in Fig. 2c. The oil-water partition coefficient was evaluated in a n-octanol-water system, and the lgP was approximately 2.7. In order to determine the copper content, sodium diethyldithiocarbamate (DDTC-Na) was utilized to form DDTC-Cu complexes with copper oleate. The ultraviolet absorption of the complexes was measured by UV at 456 nm, and the results were calculated against the absorption of DDTC-Cu formed by a copper standard solution.

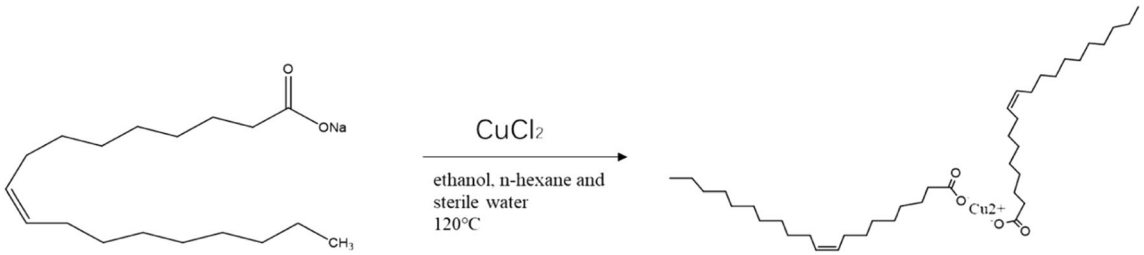
Preparation of $Cu(OI)_2$?-L

$Cu(OI)_2$-L was prepared by an ethanol injection method, and the size was controlled via a membrane extrusion method. Briefly, all lipid materials including lecithin, cholesterol and copper oleate were dissolved in an appropriate amount of ethanol. Next, a constant volume of the ethanol solution was added to sterile water under stirring, and the mixture was heated to remove the ethanol. After further evaporation of the residual ethanol, the liposome suspension was filtered through first 200 nm, then 100 nm polycarbonate filters, to obtain liposomes with the desired size distribution.

In order to improve the stability of the liposomes and prevent oxidation, freeze-drying technology was applied to solidify the liposomal suspension. Influencing factor tests and long-term tests were performed, and the results indicated a high storage stability of the lyo-product (Scheme 2).

Characterization of Copper Oleate Liposomes

The measurement of particle size distribution and zeta potential was carried out by a ZETASIZER NANO-ZSE dynamic light scattering particle size / zeta potential analyzer (Malvern Instruments Ltd). Morphological observations were



Scheme 1 Synthesis of copper oleate.

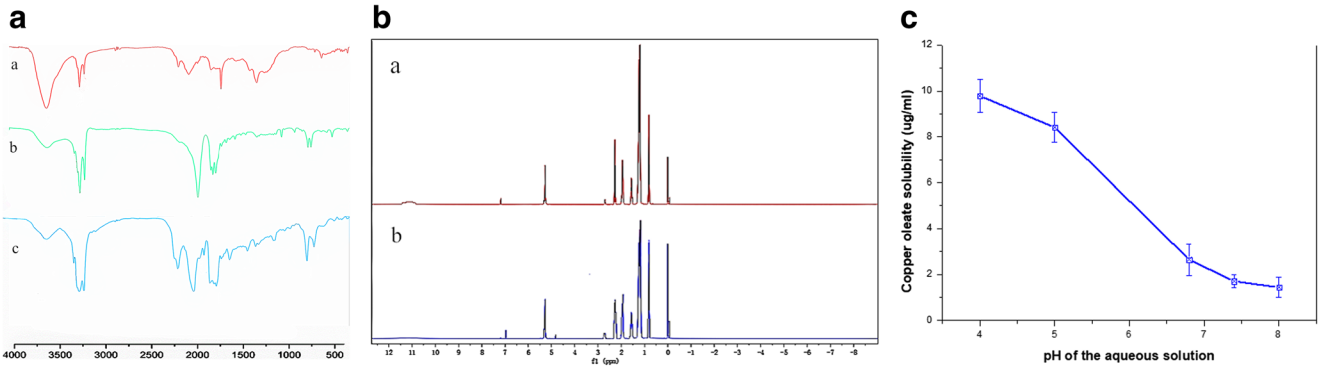


Fig. 2 (**a**) Infrared spectroscopic spectra for oleic acid (a), sodium oleate (b) and copper oleate (c). In comparison to the IR spectra of sodium oleate, a new peak at 1510.1 cm^{-1} appears, due to the larger radius of the copper atom affecting the asymmetric stretching vibration of carboxyl groups, leading to the increased wave number of the asymmetric stretching vibration and reducing the symmetrical stretching vibration frequency. (**b**) The ^{1}H-NMR spectra for Sodium oleate (a) and copper oleate (b). The NMR spectra of oleic acid and copper oleate demonstrated that the oleic acid group retained integrity during the synthesis process. (**c**) The solubility profile of copper oleate in aqueous solutions at different pH values.

performed using a JEM-2100 transmission electron microscope (TEM) (Japan Electron Optics Laboratory, Japan). In order to determine the encapsulation efficiency, free copper oleate was removed by gel filtration on a Sephadex G-50 (Henghui Biotechnology, Beijing, China) column. The eluted liposome fraction was disrupted by isopropyl alcohol for analyzing of the drug content. The copper was quantified by the UV method as described in "Synthesis and Characterization of Copper Oleate" Section. The encapsulation efficiency was calculated as [A_{Cu} (after filtration)]/[A_{Cu} (before filtration)] × 100%.]

In Vitro Release of Copper Oleate Liposomes

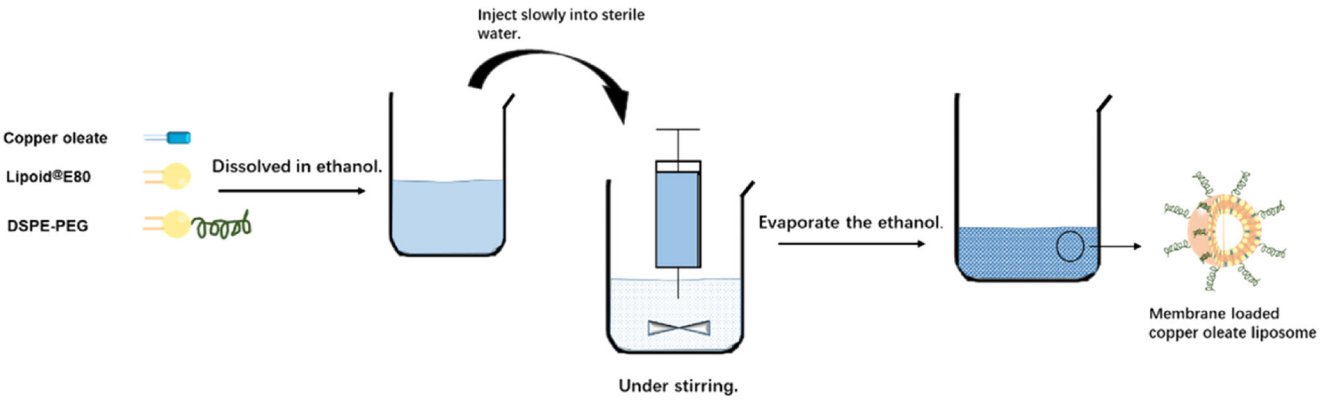
The *in vitro* release performance of the copper oleate liposome was studied via a dialysis method at a physiological temperature of 37°C. Phosphate buffer saline (PBS) of pH 5 and pH 7.4 containing 0.8% SDS (*w*/*v*) was chosen as the release medium. The samples were prepared in quadruplicate for each pH condition. 2 mL of the medium was sampled at 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h, and the same volume of fresh medium was added each time. Samples were filtered through a 0.45 μm microporous membrane and analyzed by the UV method as describes in "Synthesis and Characterization of Copper Oleate" Section to calculate copper(II) release. The cumulative release of each sampling point was calculated according to the following formula.

$$Q\% = \left(C_n V_0 + \sum_{i=1}^{n} C_{n-1} V\right) / M_t \times 100\%$$

Where Q is the quantity of released drug, C_n is the drug concentration measured for the n time, V is the sampling volume, V_0 is the total volume of the release medium, and M_t represents the total drug content.

Pharmacokinetic Study

A copper oleate solution was prepared by diluting the copper oleate with normal saline solution containing 2% (*w*/*v*) Tween80 and 15% (w/v) PEG400 as the solubilizing compound. The dose of copper was 0.4 $mg{\cdot}kg^{-1}$ in both groups. 10 healthy male SD rats (Experimental Animal Center, Shenyang Pharmaceutical University), weighing approximately 200 g were fasted overnight and randomly divided into



Scheme 2 Preparation of membrane-loaded copper oleate liposome via ethanol injection method.

2 groups ($n = 5$). The rats from each group were administrated with copper oleate injection (Cu (OI) 2-S) or copper oleate liposomes ($Cu(OI)_2$-L) via tail vein injection. At 3 min, 8 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h after administration, 0.5 mL of blood was sampled through the orbital vein and quickly transferred to 1.5 mL EP tubes pretreated with heparin. The plasma was subjected to a wet digestion method to release the copper, and then assayed using an atomic absorption spectrophotometer.

Antitumor Efficacy Study in Combination Therapy with DSF

KunMing mice (5–6 weeks of age) were purchased from the Experimental Animal Center, Shenyang Pharmaceutical University. Mice ascites tumor H-22 cells (Professor Deng Yihui's laboratory, Shenyang Pharmaceutical University) in the logarithmic growth phase were diluted with normal saline solution (NS) to approximately 1.5×10^7cells/ mL. 0.1 mL of the cell suspension was inoculated into the peritoneal cavity of the mice. The ascetic fluid was extracted 2 weeks after inoculation, and was diluted with saline solution to a final cell concentration of 1.5×10^7 mL^{-1}. 0.1 mL of the H-22 cells (1.5×10^7 cells/ mL) suspension were s.c. inoculated into the left axilla of the mice. When the mouse hepatoma model was well established and the tumor volume reached about 100 mm^3, the rats were evenly divided into 5 groups according to the principle of consistency ($n = 6$), receiving normal saline (10 mL· kg^{-1}), 5-FU solution (20 mg· kg^{-1}), DSF NPs (3 mg· mL^{-1}) plus normal saline (10 mL· kg-1), DSF NPs(40 mg· kg-1) plus oral $Cu(OI)_2$-S (copper oleate solution) (0.3 mg·kg^{-1}) or DSF NPs (40 mg· kg^{-1}) plus $Cu(OI)_2$-L(0.3 mg·kg^{-1}) via tail vein injection on days 1, 4, 7, 10, 13. The DSF nanoparticles (NPs) used here were formulated by our group in a previous study (22). The body weight of the mice were monitored, and the morphology of the tumor was recorded to evaluate efficacy and safety of the treatment. The tumor inhibition rate (TIR) used as the evaluation criteria for antitumor efficiency was calculated by the following equation.

$$TIRn = (V_{NS} - Vn)/V_{NS} \cdot 100\%$$

Where TIR_n represents the average tumor inhibition rate in group n, V_{NS} represents the mean tumor volume of the saline group, and V_n represents the mean tumor volume of certain administration group.

Statistical Analysis

All mean values ± SD expressed in the results were compared by two-tailed unpaired t-tests for 2-group comparison or one way ANOVA. P values <0.05 were considered statistically significant.

RESULTS

Preparation and Characterization of the Liposomes

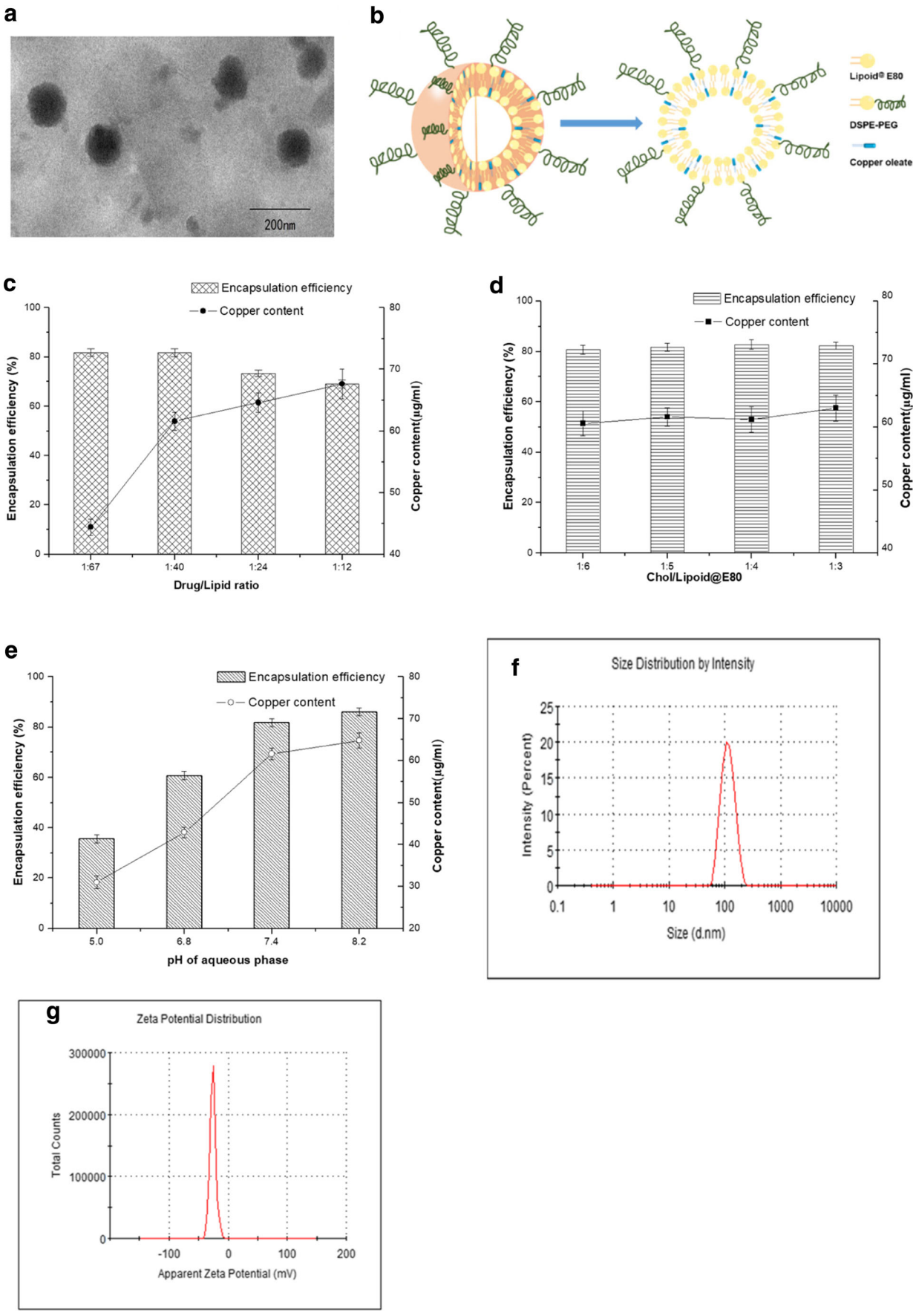
The final liposome formulation was composed of phospholipid E80: cholesterol: DSPE-mPEG2000 with a molar ratio of 20:8:1, and the successful incorporation of copper oleate into the liposomes was confirmed by transmission electron microscopy (TEM). The TEM image in Fig. 3a shows that the $Cu(OI)_2$-L had a spherical morphology, with an average diameter of approximately 100 nm. The dynamic light scattering measurements further validated this result, showing a mean diameter of 109.6 nm. The size distribution of the liposomes, along with the PDI of 0.044, indicates a homogenous formulation. $Cu(OI)_2$-L exhibited a negative zeta potential of −24.91 mV. The optimal encapsulation efficiency of copper was found to be 84% at a drug/lipid ratio of 1:40 (*w*/w), and displayed a pH–dependence, whereby the encapsulation increased by more than 30% when the pH value of the aqueous phase changed from 5.0 to 8.0.

In Vitro Release Profile of $Cu(OI)_2$?-L

To determine the drug retention ability of the carrier in different pH conditions, *in vitro* release assays were performed in pH 5.0 or 7.4 PBS containing 0.8% SDS at 37°C for 48 h. As shown in Fig. 4a**,** the release of $Cu(OI)_2$-L was rapid in the initial 8 h, and gradually slowed down over time in both medium. The release rate was affected by the pH conditions of the release medium; the liposomes incubated in the pH 7.4 medium retained more than 70% copper oleate, while only 50% was retained in the pH 5.0 medium after 8 h of incubation, confirming pH sensitivity of the formulation.

Pharmacokinetic (PK) Study

Pharmacokinetic (PK) studies were performed and the results were calculated using the software DAS 02.1A (Drug and Statistics, China). The data was fitted for a two-compartment model. As shown in Fig. 5, copper oleate solution was cleared rapidly from plasma, likely due to distribution to other tissues or accumulation in the liver for excretion. However, significant differences in the pharmacokinetic profile were observed when a liposome formulation was used to deliver the drug. $Cu(OI)_2$-L exhibited a plasma elimination half-life (t1/2 β) of 2.99 ± 1.78 h, which was nearly 2-fold that of the $Cu(OI)_2$-S, and consistently, the mean residence time (MRT) of the $Cu(OI)_2$-L was ~2 times longer compared with that of the $Cu(OI)_2$-S. Additionally, a much higher bioavailability was achieved by the $Cu(OI)_2$-L, demonstrating an area under concentration time curve (AUC) of 2.94 ± 1.13 mg·L^{-1} ·h, which was approximately 3-fold greater than that of the $Cu(OI)_2$-S.


a
200nm
b
Lipoid® E80
DSPE-PEG
Copper oleate
c
Encapsulation efficiency
Copper content
Encapsulation efficiency (%)
Copper content(μg/ml)
1:67
1:40
1:24
1:12
Drug/Lipid ratio
d
Encapsulation efficiency
Copper content
Encapsulation efficiency (%)
Copper content(μg/ml)
1:6
1:5
1:4
1:3
Chol/Lipoid@E80
e
Encapsulation efficiency
Copper content
Encapsulation efficiency (%)
Copper content(μg/ml)
5.0
6.8
7.4
8.2
pH of aqueous phase
f
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
g
Zeta Potential Distribution
Total Counts
Apparent Zeta Potential (mV)

◀**Fig. 3** Characterization of the liposomes (**a**) TEM image for the Cu(OI)2-L. (**b**) Schematic diagram of $Cu(OI)_2$-L. (**c**) The influence of drug/lipid ratio (w/w) on encapsulation efficiency and copper content in the liposome suspension. (**d**) The influence of mass ratio of cholesterol/Lipoid$^{@}$E80 on encapsulation efficiency and copper content. (**e**) The influence of pH of the aqueous phase on encapsulation efficiency and copper content. (The concentration of lipid material is fixed at 30 mg·mL^{-1}). (**f**) The particle size and (**g**) zeta potential of the Cu(OI)-L.

Anti-Tumor Effect

The antitumor efficacy of $Cu(OI)_2$-L in combination of DSF NPs was evaluated using KunMing mice bearing H-22 cells hepatoma xenografts. The body weight and the tumor volume of each animal was recorded during the experiment, and the data was plotted against time(days) and shown in Fig. 6a, b. The mice were sacrificed 14 days after the treatment and then the tumor was excised and weighted, and the morphology of the tumors is shown in Fig. 6c. It can be seen in Fig. 6b that all the treatments showed a tumor reduction effect compared with the negative control group. The tumor inhibition rate (TIR) was calculated to compare the effects of each treatment, and results showed a tumor reduction effect in the order of

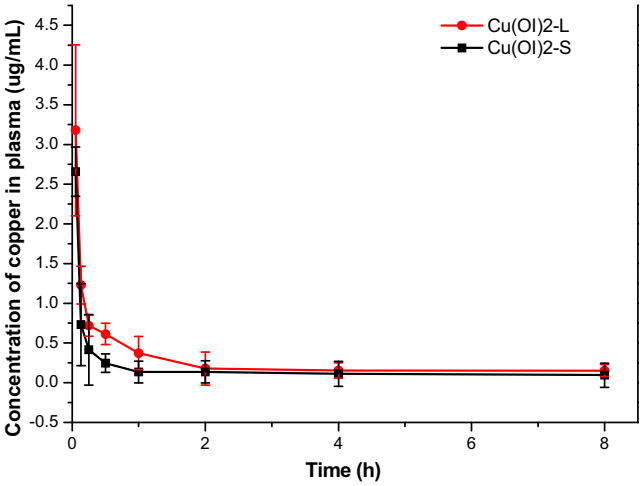


Fig. 5 The average plasma concentration of copper against time after intravenous administration of $Cu(IO)_2$-S and $Cu(OI)_2$-L in rats (n = 5).

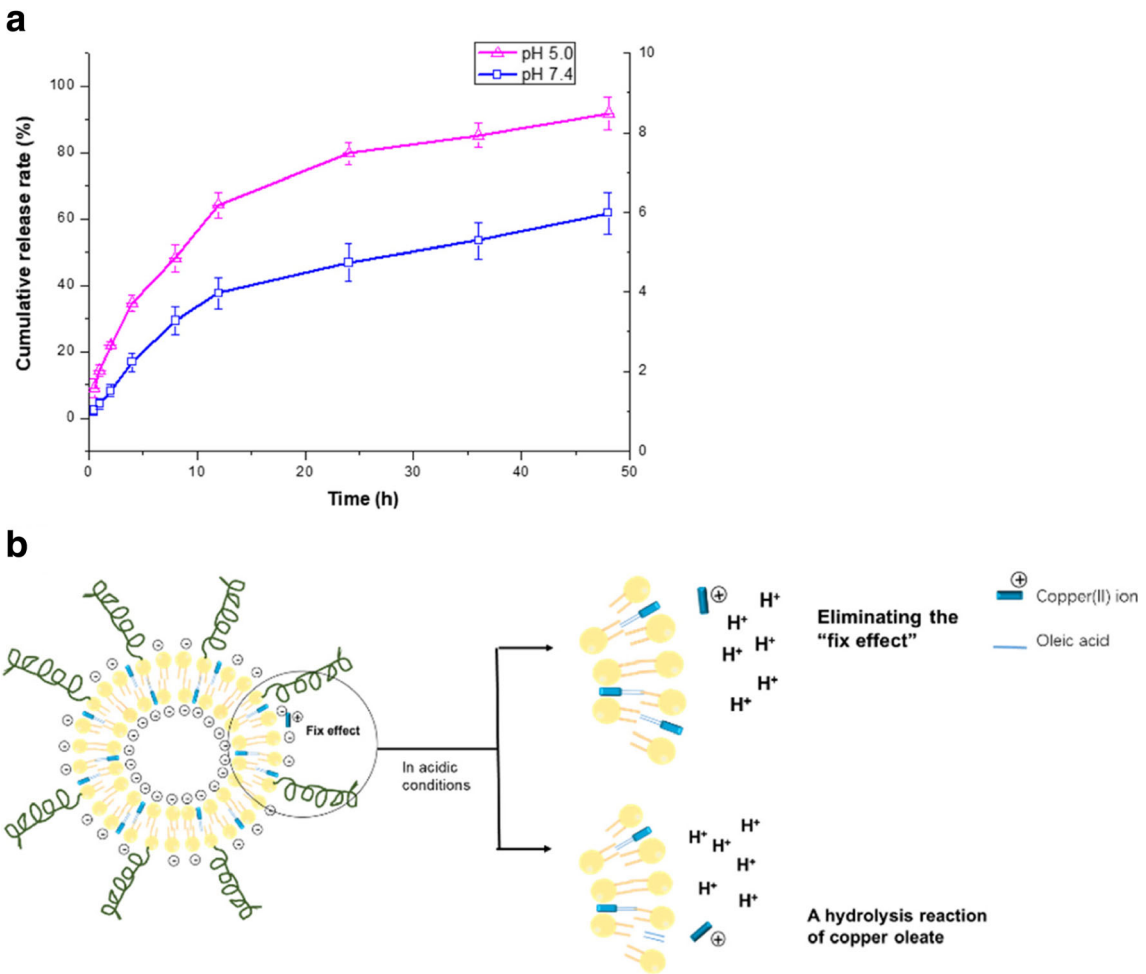


Fig. 4 (**a**) The release profiles of Cu(OI)2-L in PBS of pH 5 and pH 7.4. (**b**) The schematic diagram for the accelerated release profile of Cu^{2+} in low pH conditions.

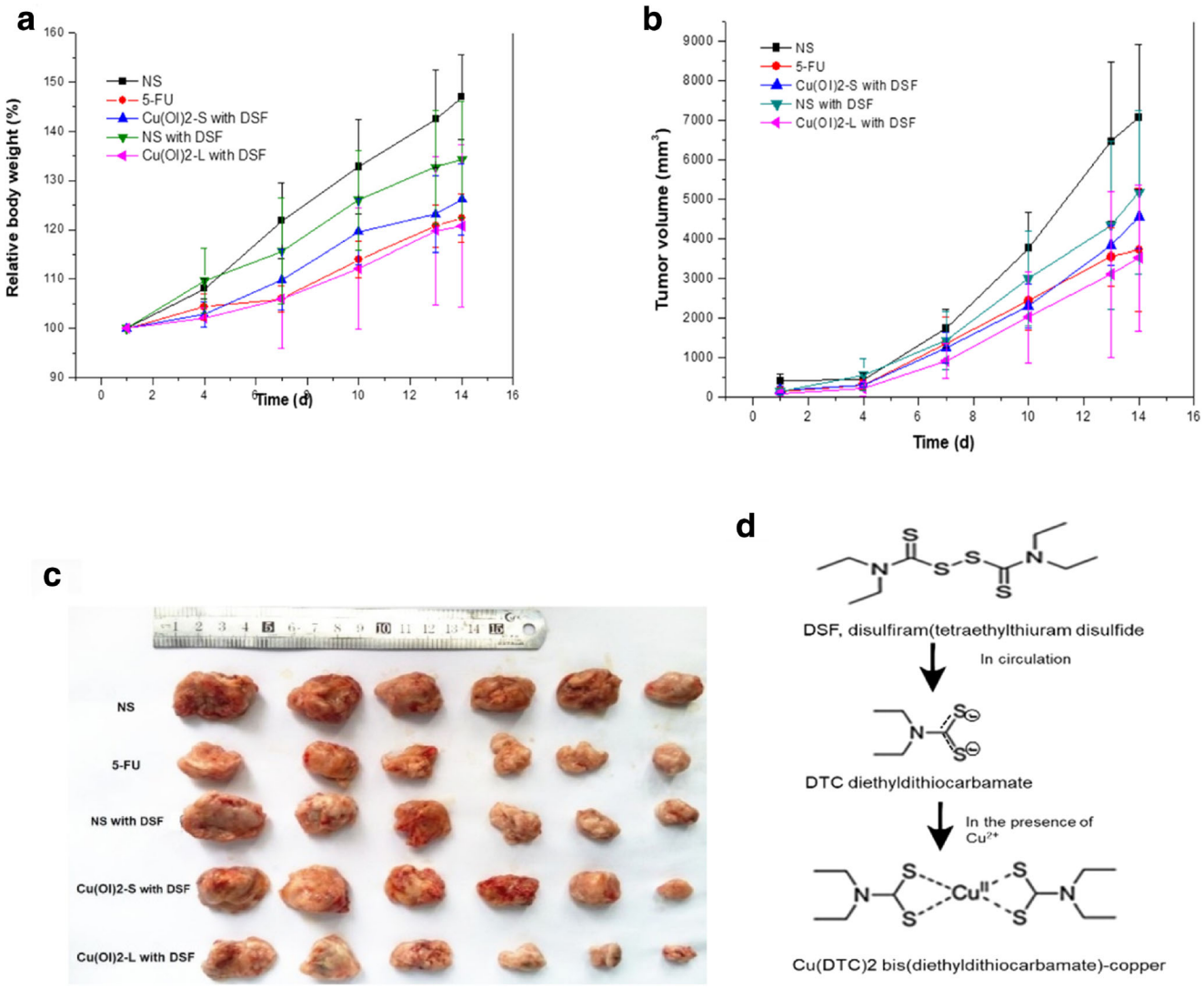


Fig. 6 (**a**) The relative body weight and (**b**) the tumor volume of mice in different groups ($n = 6$). (**c**) Photograph of the tumor in different groups.

DSF NPs alone < DSF NPs plus oral $Cu(OI)_2$-S < 5-Fu < $Cu(OI)_2$-L plus DSF NPs, with TIRs of 26.8%, 35.5%, 47.4% and 50.3%, respectively.

DSF: Disulfiram nanoparticles. NS: Normal saline, as a negative control. 5-Fu: 5-fluorouracil, as a positive control. $Cu(OI)_2$-S: Administration of copper oleate solution per oral. $Cu(OI)_2$-L: Administration of copper oleate liposome via tail vein injection. (D) The reaction of disulfiram with Cu^{2+}.

DISCUSSION

Since Bangham from the United Kingdom first proposed the concept of liposomes, liposomal drug delivery systems have made significant progress in the fields of anti-tumor and anti-infection treatments in the last 5 decades. Liposomes generally present as spherical vesicles, in which aqueous cores are enclosed by one or more concentric bilayers formed by lecithin and cholesterol. By taking advantage of their unique structure, both hydrophilic and hydrophobic substances can be formulated into liposomal preparations. Several anti-tumor drugs such as doxorubicin, and vincristine have been formulated into liposomes and been clinically approved due to their considerable chemotherapeutic effects (24–26).

Unlike hydrophilic agents, which dissolve in the aqueous core, hydrophobic drugs are located within the membrane bilayer. Thus, the maximum solubility of the drug in the membrane may be the main factor that influences the loading efficiency of lipophilic drugs. Theoretically, based on different affinities between the drug and the lipid membrane, the drug/lipid ratio can be raised from 1: 100 to 1: 1, particularly when the drug is similar to the structure of the membrane component. However, excess membrane solubility may lead to slower drug release rate. In this work, copper oleate was synthesized and shown to be lipophilic, with a partition coefficient (lgP) of 2.7, and as a result an optimal loading efficiency of

84% was obtained at a drug/lipid ratio of 1:40, indicating copper oleate is suitable for incorporation in the liposome membrane. If the drug binding capacity of a membrane is limited, excess drugs may damage the stability of the membrane and even lead to disruption. As shown in Fig. 4c, an obvious decline of encapsulation efficiency was observed when the drug/lipid ratio was increased from 1:40 to 1:24. It was also noted that alterations in the lipid composition (increasing the mass ratio of cholesterol) did not affect the encapsulation efficiency significantly, as shown in Fig. 3d, and thus it is assumed that the incorporation of copper oleate was mainly due to hydrophobic interactions, rather than interactions(such as hydrogen-bond interactions, Van der Waals, electrostatic interactions, etc.)between the drug and the lipid materials. This assumption was confirmed by the pH-reliant encapsulation efficiency and the pH-reliant water solubility, as shown in Figs. 2c and 3e, where low pH values led to increased solubility of copper oleate in the aqueous solution, and thus reduced the incorporation in the membrane.

The liposomes prepared in this study exhibited negative zeta potential in physiological pH conditions, and this may be due to the use of the negatively charged lipids Lipoid@ E80 and DSPE-mPEG (27). This inter-particle electrostatic repulsion caused by the negative surface charge likely contributed to stability of the liposomes by avoiding aggregation. The opposite charges between Cu^{2+} and the liposome surface are assumed to exert a "fix effect" on free copper ions, increasing the loading stability, as shown in Fig. 4b. As well, the variable spatial structure of PEG chains should provide a steric hindrance on the surface, furthering protecting the Cu^{2+} located near the external aqueous phase by burying within the PEG shell.

It is well known that only drug released from the carrier produces a therapeutic effect, and the drug-delivery efficacy of a liposomal formulation is determined by how the drug releases and where it is released. The *in vitro* release kinetics were investigated to assess the implications for *in vivo* delivery efficiency. As discussed earlier, hydrophobic drugs are trapped in the lipid membrane through interactions with membrane materials and hydrophobic effects (28), and thus the drug can be released only after these interactions are disrupted or weakened by changes in the external environment. When tested in aqueous medium, as shown in Fig. 4a, more than 70% of the encapsulated copper oleate was retained over 8 h incubation in the simulated physiological medium of pH 7.4, confirming excellent copper retaining ability while in circulation. Additionally, pH-sensitive release kinetics were also observed, and three mechanisms are proposed to explain this pH-sensitivity of the liposomes. (1) Under acidic conditions, copper oleate present in the outer leaflet is exposed to excess hydrogen ions and contacts to form a more stable oleic acid molecule, leading to the release of Cu^{2+}; moreover, one copper oleate molecule breaks down into two oleic acid molecules in the ion exchange reaction, and this results in increments in degrees of freedom in the membrane. (2) In acidic conditions, increased surface charge of the liposome eliminates the "fix effect" on Cu^{2+}, as shown in Fig. 4b, leading to the escape of the copper(II) ions. (3) The Lipoid@ E80 used in this study contains a small amount of acidic lipid. When the acid phospholipids are protonated by excessive hydrogen ions in the acidic medium, the size of polar head group increases and the charge changes, resulting in reduced Van der Waals interactions and increased electrostatic repulsion between acyl chains. All of the above mechanisms were hypothesized to destroy the stability of bilayer structure, and accelerating the release of encapsulated drugs in acidic conditions. In addition, a typical biphasic release profile (29) was also observed, and this phenomenon is particularly obvious in the acidic release medium. The release profile may also be explained by the distribution of copper oleate in the liposome membrane. Any copper oleate incorporated in the periphery of the liposome membrane may undergo the following decomposition reaction when in contact with the aqueous solution, thereby releasing copper(II) ions.

$$Cu(OI)_2 \rightleftarrows Cu^{2+} + 2OI^-; OI^- + H^+ \rightleftarrows HOI.$$

Thus, free drug in the outer aqueous phase, the drug physically adsorbed on the liposome surface and the drug wrapped in the outer layer of the phospholipid bilayer will be preferentially released. The Cu^{2+} wrapped in the lipid layer closed to the internal water phase could be released only by diffusion through the lipid matrix or upon rupturing of the liposomes.

In the pharmacokinetics study, the half-life of $Cu(OI)_2$-L and $Cu(OI)_2$-S was found to be 2.99 h and 1.84 h respectively. A much higher bioavailability was also observed from the $Cu(OI)_2$-L, with the concentration time curve (AUC) of 2.94 $mg \cdot L^{-1} \cdot h$, which was approximately 3-fold greater than that of the $Cu(OI)_2$-S. The results confirmed that the nanoparticles modified with PEG possess the ability to avoid recognition by mononuclear phagocyte system (MPS) (30), and provide an improved pharmacokinetic profile compared with the copper oleate solution. A steep slope in the blood concentration curve in the initial 8 min was observed, after administration of both the liposomal formulations and copper oleate solution, as shown in Fig. 5. This indicates an initial sharp decrease of blood copper content after injection, followed by a gentler decline. This phenomenon is consistent with the pharmacokinetics of most endogenous substances *in vivo*. As it is a trace element involved in many physiological and pathologic activities in mammals, copper content *in vivo* is strictly controlled by a regulatory system. Free copper ions in the blood circulation can quickly accumulate in the liver after injection. In a high copper environment, ATP7A and ATP7B, two Cu-transporting P-type ATPase, which are known to take charge of cellular copper efflux in mammals,

translocate cellular Cu^{2+} to the membrane for clearance and promote copper excretion through the biliary duct. As time passes, the proteins involved in copper transportation are gradually saturated, and thus the clearance rate of copper is reduced. In this case, with the liposome formulation, the rapid decline in the plasma concentration curve may be explained by the initial burst release profile of $Cu(OI)_2$-L as discussed earlier. Assuming that PEGylated liposomes loaded Cu^{2+} can entirely escape the capture of the MPS, then only the copper(II) ions released from the vesicle can be cleared. Therefore, those copper oleate molecules that are well protected in the inner leaflet of the bilayer will bear the important task of tumor-targeting delivery and developing the synergistic effect with DSF.

Generally, vesicles with a particle size around 100 nm are more likely to penetrate and accumulate into solid tumors via the enhanced penetration effect (EPR) effect (31). Moreover, the pH sensitivity of liposomes increases the potential for the drug to be released in the tumor. The poorly-developed vasculature in a tumor is often unable to provide enough nutrition to the rapid dividing cells, resulting in lack of oxygen and nutrients in the tumor cells. Lactic acid produced by the anaerobic metabolism activity can lead to an acidic pH in many solid tumors. Although the acidic tumor environment may contribute to tumor invasion in some cases, it also provides an opportunity for tumor-targeted delivery systems (32,33). As mentioned above, the liposome prepared in this work displayed considerable pH sensitivity, which may help prevent drug leakage in the blood circulation, but promote the release of the copper in tumor tissue. As the liposomes loaded with Cu^{2+} reach tumor tissues, the copper ions encapsulated in the liposomes may transport into tumor cells by the following two ways: (1) by colliding with the cell membrane, followed by transference of the copper ions by a concentration gradient or lipid exchange (34), without being limited by the numbers of Ctr1, or (2) the free Cu^{2+} released in the tumor tissue can be chelated by DSF and this process can facilitate the internalization of copper in a CTR1 independent manner, leading to an excessive ROS reaction, and final product $Cu(DTC)_2$ further providing a lasting antitumor effect. The tumor-targeted release of copper ions from the liposome was validated in the tumor therapy results. When tested in mice bearing hepatoma xenografts, the $Cu(OI)_2$-L demonstrated a significant synergistic effect efficacy with the DSF NPs. As shown in Fig. 6c, the tumor growth is effectively inhibited by the $Cu(OI)_2$-L- DSF NPs combination therapy with a TIR of 50.3%, while DSF NPs plus oral copper supplementation exhibited a much weaker antitumor effect with a TIR of 35.5%, which is similar to the therapy of DSF NPs alone. The efficacy test data indicated that $Cu(OI)_2$-L is able to increase the cellular copper level effectively and develop synergism with DSF.

CONCLUSION

In order to improve the antitumor effect of disulfiram, a PEGylated liposome copper preparation was formulated in this study. The encapsulation efficiency of the liposomes was improved by the synthesis of copper oleate, which also overcame the common leakage problems faced when water-soluble copper is used to incorporate in a liposome. The copper ions and disulfiram could accumulate in tumor cells when $Cu(OI)_2$-L was injected combined with disulfiram nanoparticles. The properties of the copper oleate liposomes resulted in drug release in acidic media, made the vesicles more likely to accumulate in tumor tissue, and improved the efficacy and safety while reducing side effects of the preparation. The vesicle size of about 100 nm is more easily able to accumulate in the tumor through the EPR effect. Compared with the traditional oral copper preparations, copper liposomes exhibited excellent *in vitro* and *in vivo* stability, and were able to extend the circulation time and improve the bioavailability, and thus were more suitable to be employed in combination with disulfiram for tumor treatment. The strategy of incorporating copper in a liposome can also be applied to treat other copper-deficiency diseases, and open up opportunities for targeted delivery of copper.

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